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Manganese Binding and Oxidation by Spores of a Marine Bacillus

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Mature, dormant spores of a marine bacillus, SG-1, bound and oxidized (precipitated) manganese on their surfaces. The binding and oxidation occurred under dormant conditions, with mature spores suspended in natural seawater. These heat-stable spores were formed in the absence of added manganese in the growth medium. The rate and amount of manganese bound by SG-1 spores was a function of spore concentration. Temperatures greater than 45°C, pH values below 6.5, or the addition of EDTA or the metabolic inhibitors sodium azide, potassium cyanide, and mercuric chloride inhibited manganese binding and oxidation. However, SG-1 spores bound and oxidized manganese after treatment with glutaraldehyde, formaldehyde, ethylene oxide gas, or UV light, all of which killed the spores. Manganese oxidation never occurred in the absence of manganese binding to spores. The data suggest that Mn^{2+} was complexed by a spore component, perhaps an exosporium or a spore coat protein; once bound, the manganese was rapidly oxidized.

In many environments, manganese-oxidizing bacteria are thought to be geochemically important because they initiate or accelerate the deposition of minerals and ores (27). Both gram-positive (3, 11, 13, 22, 34) and gram-negative (13, 16, 33, 39, 40, 43) bacteria can catalyze manganese deposition, and for some of these, their activities have been shown to be environmentally significant (8, 32, 38–40). Although no mechanisms have been elucidated, several are probably involved, including direct (enzymatic or organic matrix-mediated) and indirect (e.g., localized changes in pH or Eh) oxidations. The precipitated manganese is generally, but not always, found outside the cell (35), often associated with cell surface acidic polysaccharides (16) or sheaths, which may contain acidic polysaccharides (31, 36, 41). Oxidized manganese has also been observed to accumulate on spores of fungi (40), algae (5, 25, 37), and bacteria (40).

All bacteria require low, submicromolar, levels of manganese for growth and metabolism (2). Bacilli in general require more manganese for sporulation than during vegetative growth (7, 14), and *Bacillus fastidiosus* (1) and *Bacillus megaterium* (20) require Mn^{2+} concentrations greater than 10^{-5} M in some media to produce maximally heat-resistant spores. We report here the binding and oxidation (deposition) of manganese by mature, dormant spores of a marine bacillus, SG-1.

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MATERIALS AND METHODS

Organisms and their cultivation. Three bacillus strains were used: SG-1, a salt-requiring strain isolated from a near-shore sediment enrichment culture (34); SG-1W, a spontaneous mutant of SG-1 that no longer oxidizes manganese; and *Bacillus subtilis* CU1037, obtained from J. A. Hoch. SG-1 and SG-1W were grown on K medium: 5 g of peptone (Difco Laboratories), 3 g of yeast extract (Difco), 0.2 g of $MnCl_2 \cdot 4H_2O$ (filter sterilized and added to cooled medium), and 1 liter of 75% natural seawater or artificial seawater (ASW; 0.3 M NaCl, 0.01 M KCl, 0.05 M $MgSO_4$, 0.01 M $CaCl_2$, pH 7.0). *B. subtilis* was grown in a medium containing the same nutrients, but with 25% natural seawater or ASW. Usually, cultures were greater than 95% sporulated in 3 to 4 days when shaken at 24°C.

Isolation and purification of spores. Completely sporulated cultures, as judged by phase-contrast microscopy, were harvested and cleaned by the method of Goldman and Tipper (17). Spores were harvested and washed by centrifugation at 10,000 × *g* and 4°C as follows: cells were washed with deionized water, suspended in 10 mM Tris buffer (pH 7.5), and treated with lysozyme (50 μg/ml) for 30 min at 37°C to lyse any remaining vegetative cells, washed one time each with 1 M NaCl, 0.15 M NaCl, and 0.1% sodium dodecyl sulfate, and washed five times with deionized water. With the exception of the final sodium dodecyl sulfate and deionized water washes, all treatments were supplemented with 10 mM EDTA and 5% (wt/vol) phenylmethylsulfonyl fluoride (stock solution, 6 mg/ml in ethanol), pH 7.5, to inhibit protease activities (17). Purified spores were stored in deionized water at 4°C.

Vegetative cell and spore suspensions. The number of vegetative cells or spores in suspensions was determined by direct counts with a Petroff-Hausser count-

ing chamber. Numbers of viable cells were determined by dilution of suspensions into sterile seawater followed by plating onto K agar medium. Stock spore suspensions typically contained 10^9 spores (about 900 μg of protein) per ml.

Manganese binding assay. Mn^{2+} binding to cells or spores was determined by measuring the partitioning of a radioactive tracer, ^{54}Mn (New England Nuclear Corp., Boston, Mass.), between soluble and insoluble phases by filtration. Soluble manganese (Mn^{2+}) passed freely through the filters, whereas manganese bound to cells or spores was retained. Since oxidized manganese, MnO_2, is insoluble and forms aggregates that are trapped on filters (30), controls without cells or spores were included with all experiments. This abiological Mn^{2+} oxidation was largely alleviated by buffering the assay mixture at a pH of 7.5, which resulted in extremely slow autocatalytic oxidation of manganese (see Fig. 4A, control; 29).

Cells or spores suspended in 75% natural seawater (pH 7.5) were supplemented with 1 to 1,000 μM $MnCl_2$ and 2 μCi of carrier-free $^{54}MnCl_2$ per ml and gently shaken at 24°C (unless otherwise noted). At specified times, duplicate 0.2-ml portions of the suspensions were filtered through 0.2-μm cellulose triacetate membrane filters (Gelman Sciences, Ann Arbor, Mich.). Particulate material trapped on the filters was rinsed with 5 ml of filtered seawater, and both the filters and filtrates were counted on a Beckman Biogamma II Counter (Beckman Inst. Inc., Irvine, Calif.).

Not all manganese trapped on the filters is necessarily oxidized (4, 6, 28, 32). Therefore, in some experiments the particulates on the filters were treated to remove any adsorbed, but not oxidized manganese. The particulates were incubated in 2.0 ml of 0.01 M $CuSO_4$ (pH 7.8) for 18 to 24 h at room temperature, filtered again, and rinsed with an additional 2.0 ml of 0.01 M $CuSO_4$. Since Cu^{2+} ions can displace adsorbed Mn^{2+} (6, 28), the amount of oxidized manganese could be estimated by counting filters and filtrates after this treatment.

Spore treatments. Spores (10^6 to 10^7/ml) were suspended in deionized water. Subsequently these suspensions were treated in one of the following ways: (i) heated for 15 min at various temperatures and quickly chilled to 4°C; (ii) treated with ethylene oxide (12% ethylene oxide–88% dichlorodifluoromethane) by bubbling spore suspensions in loosely capped tubes for 5 min to displace the air, and the tubes then sealed and incubated for 2 h at room temperature; (iii) incubated with 2% glutaraldehyde or 3.7% formaldehyde for 1 h; (iv) irradiated for 2.0 h with a General Electric germicidal lamp (G8T5) positioned 20 cm above the suspension (10 ml in an open standard petri dish); or (v) incubated in 75% seawater (pH 7.5) with 100 μg of pronase, trypsin, or lysozyme per ml for 60 min at 30°C. Treated spores were harvested by centrifugation, washed, and suspended in deionized water.

Chemical assays. Protein was determined by the method of Lowry et al. (26). Since manganese interferes with this method, spores were washed with an ice-cold trichloracetic acid-oxalic acid solution before protein analysis to remove manganese, as described by Hajj and Makemson (19). The presence of precipitated (oxidized) manganese was confirmed by colorimetric spot tests with either benzidinium reagent (15) or leukoberbelin blue (25); a positive test with either reagent resulted in a blue color. For quantitative analysis of oxidized manganese, a small volume (0.05 to 0.25 ml) of a spore suspension containing oxidized manganese was added to 0.5 ml of 0.04% (wt/vol) leukoberbelin blue in 0.25% acetic acid (pH 4.0). Leukoberbelin blue is oxidized by manganese of oxidation states of +3 or higher, producing a blue color; Mn^{2+} does not interfere (25). The optical density (620 nm) was measured after 15 to 24 h at 24°C. Potassium permanganate was used as a standard.

Transmission electron microscopy. Samples for transmission electron microscopy were fixed in 1% glutaraldehyde in ASW for 3 days, washed three times (15 min each) in ASW, and postfixed in 1% osmium tetroxide in ASW for 1 to 2 h. Samples were then dehydrated in ethanol and propylene oxide and embedded and polymerized in Spurr's medium as previously described (35).

RESULTS

Binding of manganese by spores. Transmission electron microscopy of thin sections of precipitates formed during growth of SG-1 in K medium showed that mature spores invariably had oxidized manganese associated with them (Fig. 1; 36). As manganese was bound, the spores aggregated, forming flocculent dark brown precipitates. Spot tests with either benzidinium-hydrochloride (15) or leukoberbelin blue (25) confirmed that oxidized manganese was accumulating. Whether manganese was oxidized by the spores themselves or by the vegetative cells and later deposited around the spores was not determined.

SG-1 spores efficiently removed Mn^{2+} from solution when suspended in manganese-supplemented seawater, whereas neither vegetative cells nor spores of SG-1W, a mutant that does not oxidize manganese, bound manganese (Fig. 2A). Neither vegetative cells nor spores of *B. subtilis* CU1037 bound manganese in ASW, seawater, or low-salt media (data not shown). Both the initial rate and final amount of manganese bound was dependent on the concentration of SG-1 spores (Fig. 2B, inset), and the binding exhibited saturation kinetics (Fig. 2B).

Spores from cells grown in K medium without added manganese oxidized manganese to the same extent as spores formed by cells in media supplemented with 1 mM $MnCl_2$ (Fig. 2C).

In all experiments, there was no significant manganese oxidation in the absence of spores (Fig. 2B, inset). Thus, under the conditions tested, the spores themselves bound Mn^{2+} and deposited oxidized manganese.

Bound versus oxidized manganese. Spore-associated manganese was not always oxidized (Fig. 3). Washing with $CuSO_4$, which should remove bound, but not oxidized, Mn^{2+} (7), removed 20 to 30% of the total manganese that was bound. With very low levels of added manganese, 40 to 50% of the Mn^{2+} was bound by the spores as

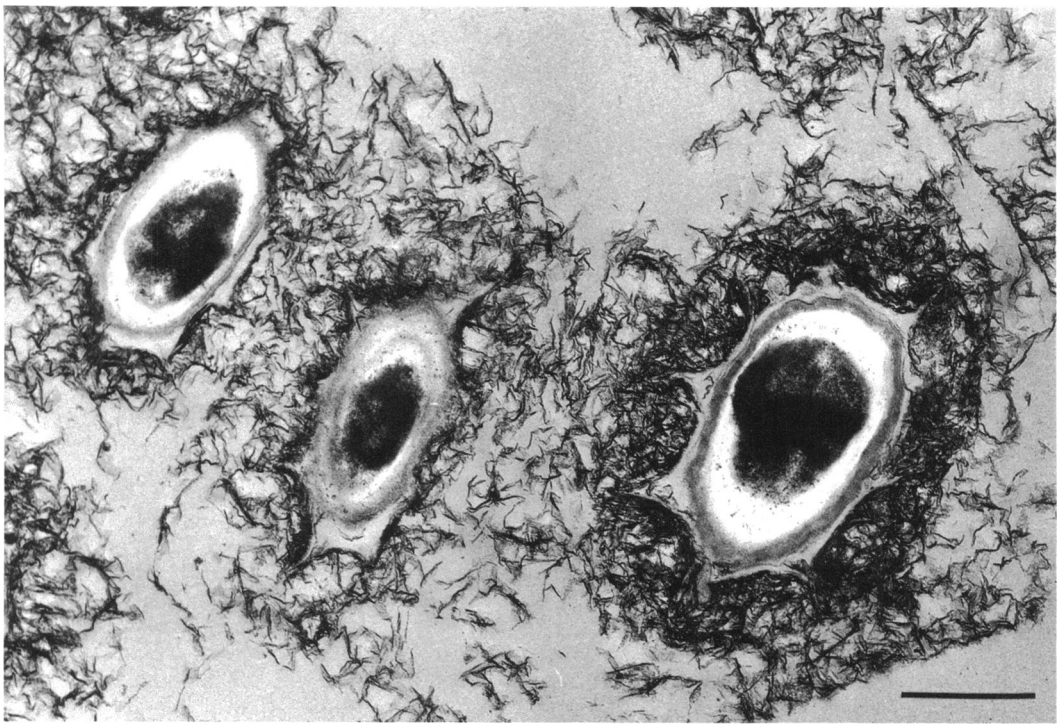

FIG. 1. Transmission electron micrograph of SG-1 spores coated with MnO_2. Bar, 0.5 μm.

quickly as samples were taken (about 15 s), yet only 20% of this bound manganese was removed by $CuSO_4$ washing. Thus, manganese bound to spores was either not readily exchangeable with $CuSO_4$ or rapidly oxidized upon binding. Experiments to compare the kinetics of these events by simultaneously measuring both binding and oxidation rates are in progress.

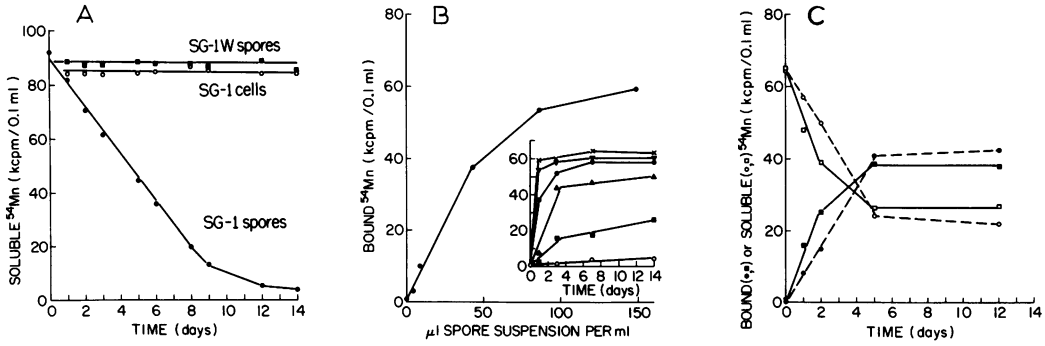

FIG. 2. Removal of dissolved manganese (Mn^{2+}) from solution by SG-1 spores suspended in seawater. Washed spores or cells were added to 75% natural seawater supplemented with 1 mM $MnCl_2$ and tracer $^{54}Mn^{2+}$. Portions (0.1 ml) of a mixture were removed at various times and filtered (0.2-μm membrane filters), and radioactivity in supernatants (soluble) or on filters (bound) was measured. Control indicates an assay without spores or cells. The assay procedure was used in this and all subsequent figures. (A) Manganese remaining soluble as a function of time with (●) SG-1 spores (4×10^6 spores per ml), (○) SG-1 vegetative cells (6.5×10^7 cells per ml), or (■) SG-1W spores (5×10^6 spores per ml). *B. subtilis* CU1037 cells and spores also did not bind and oxidize manganese, similar to SG-1W cells and spores (data not shown). (B) Manganese bound at 24 h by SG-1 spores as a function of spore concentration (volume of stock spore suspension per ml of assay mixture). Inset graph shows the kinetics of manganese binding with the following SG-1 spore concentrations (microliters of stock spore suspension per milliliter of assay mixture): ○, no spore control; ■, 4.3; ▲, 8.6; ●, 43; ▼, 86; and ×, 149. The stock spore suspension contained 10^8 spores per ml. (C) Manganese bound (●, ■) and soluble (○, □) with SG-1 spores prepared from cultures grown in K-medium either with (●, ○) or without (■, □) added 1 mM $MnCl_2$. The spore concentration was 2×10^6 spores per ml.

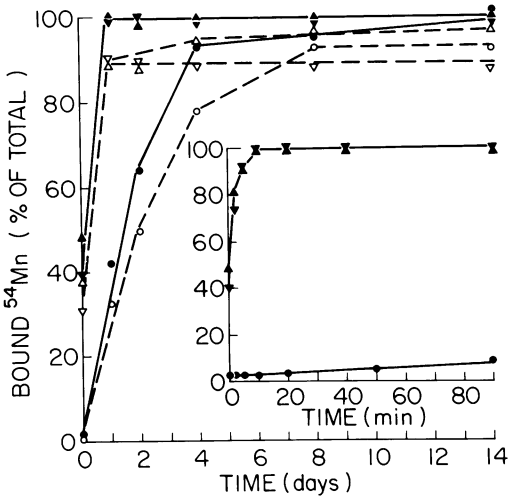

FIG. 3. Manganese bound by SG-1 spores suspended in seawater supplemented with various initial manganese concentrations: (▲, △), ~10 nM (only carrier-free tracer $^{54}Mn^{2+}$ added); (▼, ▽), 1 μM; (●, ○), 1 mM. Open symbols are samples washed with $CuSO_4$ to remove bound (Mn^{2+}) but not oxidized (Mn^{4+}) manganese from spores (see text). The initial soluble radioactivity was 130,000 cpm per 0.1 ml. Radioactivity bound by spore suspensions (percent of initial) was 1 nM and 1 μM manganese (100%), and 1 mM manganese (70%). The spore concentration was 8 × 10^5 spores per ml.

Conditions affecting Mn^{2+} binding by spores. Spores removed Mn^{2+} from solution over a wide range (nanomolar to millimolar) of initial manganese concentrations (Fig. 3). No solid manganese dioxide was added in these experiments; however, as Mn^{2+} was oxidized and deposited, further binding may have been associated with both spores and freshly produced MnO_2 (6, 28, 32).

The rate of manganese binding by spores increased between pH 6.5 and 8.0. The maximal amount of manganese bound by spores was typically equal to 100% of the initial soluble manganese for pH 7.0 to 8.0. Above pH 8.0, the rate of inorganic oxidation, as judged by the simultaneous appearance of brown precipitates and by radioactivity on control filters, was significant. However, the initial rate at which Mn^{2+} was trapped on filters was greater in the presence of spores, indicating that the rate of Mn^{2+} binding by spores was faster than the autocatalytic oxidation rate at pH 8.5 (Fig. 4A).

The temperature of incubation affected manganese binding by spores (Fig. 4B). The initial rate of binding at 45°C was 2.5-fold greater than that at 4°C. The 45°C rate decreased by 90% after 1 day. The total manganese bound at all temperatures up to 45°C was nearly equal, again suggesting a saturation-type system. At 60 and 80°C, only 50% of the 45°C amount was bound.

Qualitatively, oxidation of bound manganese was apparent and equivalent at pHs between 7.0 and 8.5 and at incubation temperatures between 4 and 45°C. Quantitation of oxidized manganese showed that after 14 days, between 65 and 69% of the bound manganese was oxidized at temperatures between 4 and 45°C, and less than 40% was oxidized at 60 and 80°C (Table 1).

The binding and oxidation of manganese by

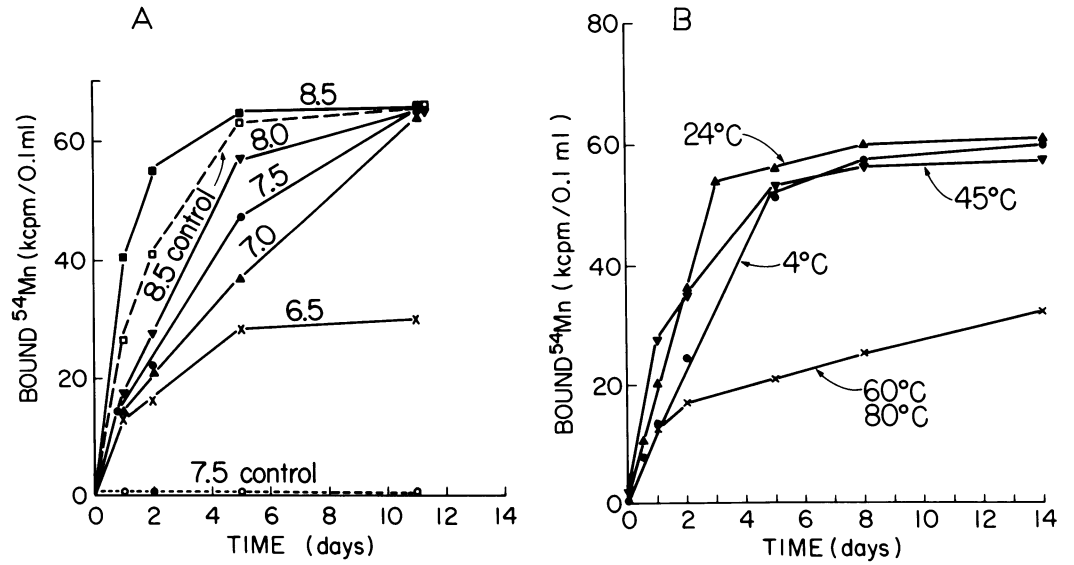

FIG. 4. Effect of (A) pH and (B) temperature of incubation on manganese bound by SG-1 spores. The spore concentration was 8 × 10^6 spores per ml.

TABLE 1. Manganese bound and oxidized by SG-1 spores incubated at different temperatures[a]

Incubation temp (°C)	Manganese (nmol/ml)		Oxidized manganese (% of bound)
	Bound	Oxidized	
4	800	550	69
24	870	565	65
45	770	520	68
60	470	180	38
80	470	120	26

[a] Manganese bound or oxidized at 14 days by SG-1 spores (8×10^6 per ml) suspended in 75% seawater initially supplemented with 1 mM $^{54}MnCl_2$ (2.0 μCi/ml).

SG-1 spores occurred at significant rates only in natural seawater suspensions. There was no binding or oxidation in 3% NaCl solution (data not shown), ASW, or deionized water suspensions (Table 2).

Mechanisms(s) of manganese binding and oxidation. The temperature optimum observed in the manganese removal experiments is suggestive of protein and perhaps enzyme involvement. If a protein were involved, it would be possible to thermally denature it and abolish the ability of the spores to oxidize manganese. Spores were pretreated for 15 min at 80, 100, or 121°C and then incubated for 12 days at 24°C to test for the ability to bind and oxidize manganese (Fig. 5A). Pretreatment at 80°C decreased both the initial rate and the final amount of manganese removed by about 30 to 35%, whereas pretreatment at either 100 or 121°C completely blocked manganese binding. These data indicate that a heat-labile spore component was involved in the binding. However, spore germinability was reduced to about 30% of control levels by the 80°C treatment and to about 3% at 100°C. Treatment at 121°C rendered the spores completely nongerminable (Table 3). It could be argued, therefore, that the adverse effects of Mn^{2+} binding related more to effects on spore germinability than to denaturation of a Mn^{2+} binding protein.

Pretreatment of spores with glutaraldehyde, formaldehyde, ethylene oxide gas, or UV light also rendered SG-1 spores nongerminable (Table 3). However, these treatments had no significant effect on Mn^{2+} removal from solution or Mn^{2+} binding by spores (Fig. 5B and C). Therefore, the potential for metabolic activity and germinability was apparently unnecessary for the binding and oxidation of manganese by spores.

Mature bacterial spores are ametabolic and thus insensitive to most respiratory inhibitors (23), as are newly germinating spores (10). It was thus somewhat surprising to find that manganese removal from solution by SG-1 spores was severely inhibited by potassium cyanide and sodium azide (Fig. 6A). This effect may have been due to complexation of dissolved manganese by these inhibitors; indeed, EDTA, a chelator of divalent cations, blocked the binding of manganese by spores (Fig. 6B). In separate experiments, however, this was shown not to be the case. Whereas potassium cyanide interfered significantly with manganese chemistry, neither sodium azide nor mercuric chloride had measurable Mn^{2+} complexing activity (Rosson et al., manuscript in preparation).

Mercuric chloride completely blocked both removal of Mn^{2+} from solution and manganese oxidation, further suggesting protein involvement (Fig. 6A). An attempt to non-specifically degrade spore proteins with trypsin or pronase, and hence inhibit manganese binding, actually resulted in slightly enhanced levels of binding (Table 4). Lysozyme, which hydrolyzes peptidoglycan, had no measurable effect on manganese binding (Table 4). If a protein is involved with the binding of manganese, it, like many other proteins of the spore, is apparently insensitive to hydrolytic attack by the enzymes tested (18, 24, 38).

DISCUSSION

The data presented show that mature, dormant *Bacillus* sp. strain SG-1 spores bind and oxidize Mn^{2+}. Is this manganese oxidation coupled to spore metabolism or viability? Azide and cyanide inhibited manganese binding, suggesting a metabolic involvement, but all other data and observations did not support this idea. Some treatments that rendered the spores nongerminable did not decrease manganese binding or oxidation, and oxygen consumption was not measurable in spore preparations (data not shown). Thus, the azide and cyanide effects can not be explained simply by the inhibition of metabolism. Furthermore, spores coated with oxidized manganese showed no evidence of germination (Fig. 1) and retained their heat resistance and dormancy until placed in a rich medium, which

TABLE 2. Manganese binding by SG-1 spores suspended in various solutions

Solution	% of initial manganese radioactivity bound[a]	
	1 μM $MnCl_2$	1 mM $MnCl_2$
Natural seawater	100.0	57.0
ASW	0.5	2.0
Deionized water	0.7	3.1

[a] Percentage of initial manganese radioactivity bound at 4 days. No additional manganese was bound up to day 14. The initial $^{54}MnCl_2$ contained 90,000 cpm/ml. The spore concentration was 2×10^6/ml.

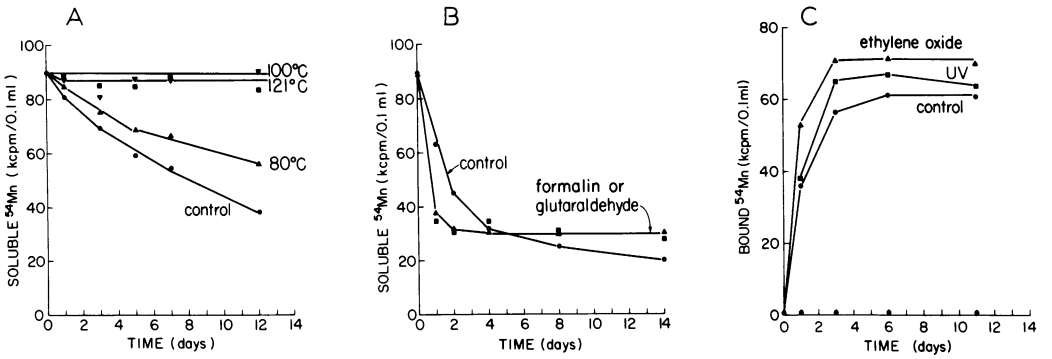

FIG. 5. Effect of various pretreatments on manganese removal or binding by SG-1 spores. For details of pretreatments, see the text. The spore concentrations were (A) 2×10^5 spores per ml, (B) 8×10^6 spores per ml, and (C) 7×10^6 spores per ml.

stimulated germination. Apparently the SG-1 spores, like other bacillus spores (23), are not metabolically active, and manganese precipitation is not related to metabolism of spore preparations.

SG-1 spores not only bound Mn^{2+}, but also rapidly catalyzed its oxidation to MnO_2. This activity is distinct from the effect of inorganic particles, such as clays, carbonates, and silicates, that bind Mn^{2+} but do not catalyze its oxidation. So far, the identity or even the nature of the catalyst for the reaction is unknown. It probably is not an enzyme, since dormant spore enzymes are generally inactive (23). One suspects that a manganese-binding component is involved, perhaps a polysaccharide similar to that reported by Ghiorse and Hirsch (16), or a protein, as reported by Jung and Schweisfurth (21). The component is sensitive to pH, temperature, and mercuric chloride, which suggests that it might be a sulfur-containing protein, perhaps a spore coat protein, but does not eliminate other possibilities. However, its activity is unaffected by treatment with glutaraldehyde, formaldehyde, or proteases.

TABLE 3. Spore germinability after a variety of treatments[a]

Pretreatment	% Survivors	
	SG-1	*B. subtilis*
70°C	72.0	131.0
80°C	26.0	38.0
90°C	7.0	4.0
100°C	2.0	0.1
Ethylene oxide	<0.1	<0.1
UV light	0.2	<0.1

[a] Spores suspended in deionized water (10^9 spores per ml) were treated as follows (see text): (i) heated at the indicated temperature for 15 min, (ii) treated with ethylene oxide gas for 15 min, or (iii) treated with UV light. Germinability was determined by plating on K medium. The ratio of colony-forming units from treated and untreated spore suspensions was determined and reported as percent survivors.

The actual mechanism of oxidation is not known, but if a binding protein is involved, one need only hypothesize that complexation lowers the activation energy of the reaction, allowing it to proceed spontaneously (27). With regard to the mechanism, it should be noted that in our experiments at both low and high manganese concentrations, SG-1 spores removed Mn^{2+} from solution without the addition of solid MnO_2, which has been reported to be necessary for manganese oxidation by some other marine bacteria (11, 12). Whatever the mechanism is, it apparently does not involve preformed MnO_2.

It is difficult to assess whether there is any connection between the manganese oxidation by SG-1 spores and previously reported effects of manganese on sporulation of other bacilli. Manganese is required in trace levels for vegetative growth (2), micromolar levels for sporulation (2, 7, 14), and, in some instances, high levels (50 μM or more) for the production of maximally heat-resistant spores (1, 20). In none of these studies was the specific relationship between mature spores and manganese investigated, so it is difficult to directly compare our results. Rather these studies focused on the effect of manganese on sporulation or germination or on the properties of the spores once formed. Since our experiments used spores formed in a complex medium, it is difficult to rule out a manganese requirement for either growth or sporulation. In fact, in less rich media, and in some batches of K medium, it is necessary to add up to 1 μM Mn^{2+} to achieve good sporulation (R. Rosson, unpublished results). However, strain SG-1W, a spontaneous mutant that does not oxidize manganese, exhibits the same Mn^{2+} requirements for sporulation, so a manganese requirement per se can not be linked to the ability of the mature

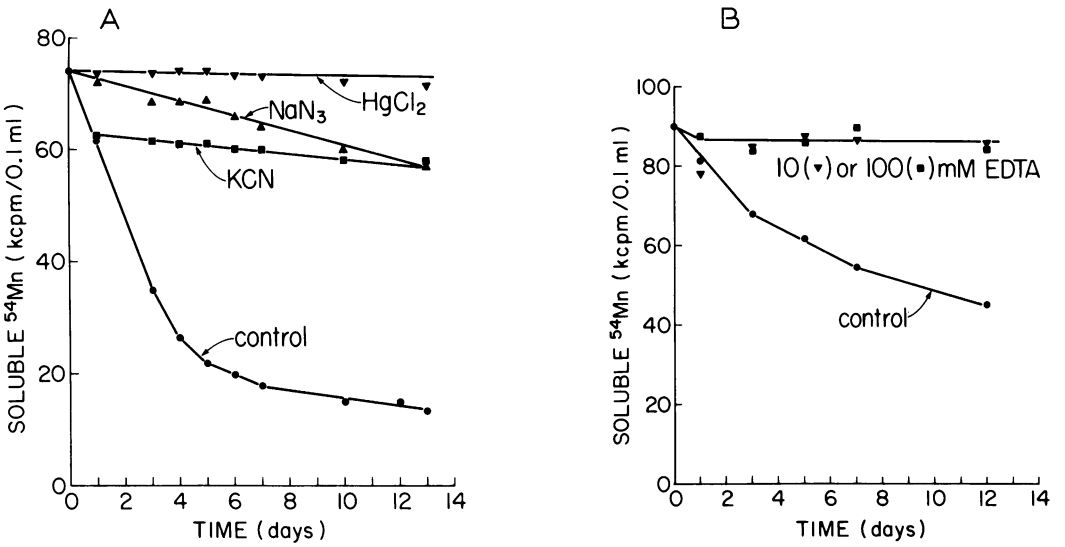

FIG. 6. Removal of manganese from solution as a function of time by SG-1 spores in the presence of (A) added inhibitors (4×10^6 spores per ml) or (B) EDTA (2×10^5 spores per ml). Inhibitor concentrations were as follows: 0.01% $HgCl_2$, 50 mM KCN, and 0.05% NaN_3.

spores to oxidize manganese. When grown and sporulated in the same medium, strains SG-1 and SG-1W formed spores that were identical in appearance and heat sensitivity, but SG-1W spores failed to bind manganese. These spores are being analyzed biochemically to try to identify a spore-specific component involved in manganese binding.

A final consideration is whether the organisms or activities reported here might be significant in natural environments. There are two parts to this question: (i) are the properties of spores consistent with activities in nature, and (ii) are the numbers of spores ever high enough to be significant? In the binding assay, SG-1 spores were seen to bind and oxidize manganese over a wide range of initial concentrations (10^{-9} to 2.5×10^{-2} M). In marine environments, soluble manganese varies between 10^{-9} M in seawater to 10^{-4} M or higher in the pore waters of some sediments (9, 42). Thus, the Mn^{2+} binding affinities of the spores are sufficient for them to be active in virtually all marine environments, and it is clear that if spores reached high concentrations they could have significant effects on manganese chemistry of natural systems. Initial rates of Mn^{2+} binding, calculated from the data of Fig. 3, range from 0.15 to 6.5 nM Mn^{2+} min^{-1} per mg of protein, depending on the initial Mn^{2+} concentration in the experiment. Since the data also show the bound Mn^{2+} is rapidly oxidized (Fig. 3, Table 1), these values may also represent maximum oxidation (deposition) rates for SG-1 spores once the spores are formed.

In nature, these or similar spores may initiate the deposition of manganese oxides that can catalyze further oxidation of Mn^{2+}. It is well established that manganese oxides complex Mn^{2+} and increase the rate of its oxidation (28–30, 32). Thus, any process that results in the formation of fresh MnO_2, even in small amounts, could have important geochemical implications.

TABLE 4. Manganese binding by SG-1 spores pretreated with various enzymes

Enzyme	% of initial manganese radioactivity bound[a]
None (untreated)	53
Lysozyme	53
Pronase	65
Trypsin	64

[a] Percentage of initial manganese radioactivity bound at 4 days. The initial 1 mM $^{54}MnCl_2$ contained 90,000 cpm per ml. The spore concentration, was 8×10^5 per ml.

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