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# Cobalt(II) Oxidation by the Marine Manganese(II)-Oxidizing *Bacillus* sp. Strain SG-1

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**The geochemical cycling of cobalt (Co) has often been considered to be controlled by the scavenging and oxidation of Co(II) on the surface of manganese [Mn(III,IV)] oxides or manganates. Because Mn(II) oxidation in the environment is often catalyzed by bacteria, we have investigated the ability of Mn(II)-oxidizing bacteria to bind and oxidize Co(II) in the absence of Mn(II) to determine whether some Mn(II)-oxidizing bacteria also oxidize Co(II) independently of Mn oxidation. We used the marine *Bacillus* sp. strain SG-1, which produces mature spores that oxidize Mn(II), apparently due to a protein in their spore coats (R.A. Rosson and K. H. Nealson, J. Bacteriol. 151:1027–1034, 1982; J. P. M. de Vrind et al., Appl. Environ. Microbiol. 52:1096–1100, 1986). A method to measure Co(II) oxidation using radioactive $^{57}Co$ as a tracer and treatments with nonradioactive (cold) Co(II) and ascorbate to discriminate bound Co from oxidized Co was developed. SG-1 spores were found to oxidize Co(II) over a wide range of pH, temperature, and Co(II) concentration. Leucoberbelin blue, a reagent that reacts with Mn(III,IV) oxides forming a blue color, was found to also react with Co(III) oxides and was used to verify the presence of oxidized Co in the absence of added Mn(II). Co(II) oxidation occurred optimally around pH 8 and between 55 and 65°C. SG-1 spores oxidized Co(II) at all Co(II) concentrations tested from the trace levels found in seawater to 100 mM. Co(II) oxidation was found to follow Michaelis-Menten kinetics. An Eadie-Hofstee plot of the data suggests that SG-1 spores have two oxidation systems, a high-affinity–low-rate system ($K_m$, $3.3 \times 10^{-8}$ M; $V_{max}$, $1.7 \times 10^{-15}$ M · spore$^{-1}$ · h$^{-1}$) and a low-affinity–high-rate system ($K_m$, $5.2 \times 10^{-6}$ M; $V_{max}$, $8.9 \times 10^{-15}$ M · spore$^{-1}$ · h$^{-1}$). SG-1 spores did not oxidize Co(II) in the absence of oxygen, also indicating that oxidation was not due to abiological Co(II) oxidation on the surface of preformed Mn(III,IV) oxides. These results suggest that some microorganisms may directly oxidize Co(II) and such biological activities may exert some control on the behavior of Co in nature. SG-1 spores may also have useful applications in metal removal, recovery, and immobilization processes.**

Cobalt (Co) is a metal of biological, environmental, and economic importance. Co is an essential nutrient that is required by all organisms. It is the central metal cofactor in the corrine ring of vitamin $B_{12}$ (cobalamine) (23) and also plays an important role in the carboxypeptidase E reaction associated with the biosynthesis of many peptide neurotransmitters and hormones (17). At high concentrations Co can be toxic to living organisms, but in the environment cobalt concentrations rarely reach those toxic levels (>1 mg · kg of tissue$^{-1}$ or >1 mg · liter of water$^{-1}$) (29). However, the radionuclide $^{60}Co$ is an activation product in radioactive wastes (48, 65) and has been identified as a priority pollutant at various U.S. Department of Energy sites around the nation (59).

In the environment, Co has two naturally occurring oxidation states, Co(II) and Co(III) (11). Co(II) occurs either as a dissolved species in solution or in small amounts in different solid phases such as carbonates and sulfides (47). Soluble Co(II) is the form taken up by cells. Under conditions of high pH and $E_h$, Co(III) is the more thermodynamically stable form. Co(III) can exist in solution with strong organic complexing agents or can occur as solid hydroxide or oxyhydroxide phases which are not available to living organisms. Co(III) is enriched in ferromanganese nodules and crusts (24, 60). Most studies of Co have focused on the adsorption and oxidation of Co by manganese oxides or ferromanganese nodules (24, 37, 41–43, 50, 55, 61, 66). Co is greatly enriched in both freshwater and marine ferromanganese nodules. This enrichment is believed to be due to the reaction of cobalt with manganese and/or ferric oxides (4, 37, 50). Co(II) is first adsorbed onto the surface of Mn(IV) oxides and then oxidized to Co(III). In the open ocean, the concentration of Co is generally below 100 pM (5, 40).

The low concentrations of Co and limitations in current methodology have hindered our understanding of the geochemical cycle of cobalt, and thus, the biological contribution to cobalt cycling in natural environments is not well known. Although there are a few reports on the adsorption of Co by both live and dead phytoplankton (19, 34), algae (18, 39, 46), and bacteria (25), there are few reports about biological involvement in Co redox chemistry (56, 58).

Microorganisms that oxidize Mn(II) are implicated to be important in Mn(II) oxidation in nature. Mn(III,IV) oxides produced by such Mn(II)-oxidizing bacteria are strong oxidants that are capable of oxidizing a number of organic and inorganic chemicals. Since Mn(III,IV) oxides (or $MnO_x$, where $1 < x < 2$) can also oxidize Co(II), organisms that oxidize Mn(II) would be expected to oxidize Co(II), albeit indirectly, as shown in Fig. 1. In this indirect mechanism, the oxidation of Co(II) by the microbially produced $MnO_x$ could occur through a coupled redox reaction possibly via surface catalysis in which coordination of Co(II) on the surface of $MnO_x$ reduces the activation energy required for oxidation by $O_2$, perhaps in a manner similar to the oxidation of Mn(II) by the surface of $MnO_x$ (10, 13). Alternatively, because of the similarity of the

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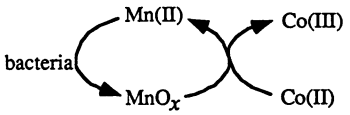


FIG. 1. Indirect mechanism of Co(II) oxidation.

redox potential of Co and Mn, it is possible that Mn(II)-oxidizing organisms could directly catalyze the oxidation of Co(II): $2Co^{2+} + 1/2O_2 + 3H_2O \rightarrow 2CoOOH + 4H^+$. In this direct mechanism, Co(II) is theoretically oxidized to Co(III) by direct catalysis involving some cellular component. The Co(III) then precipitates as a Co(III) oxyhydroxide. This might occur because of the similar redox properties of Co and Mn and because the reaction is thermodynamically favorable. The $E_0'$ for the Co(III)-Co(II) couple at 1 μM Co(II) is 0.59 V, while that for $MnO_2$-Mn(II) at 1 μM Mn(II) is 0.64 V (53). The standard free-energy change ($\Delta G^{\circ\prime}$) of this reaction at pH 7 is −14.4 kcal (ca. −60.2 kJ)/mol of Co(II).

The marine *Bacillus* sp. strain SG-1 is one of the better-characterized Mn(II)-oxidizing bacteria in culture (12, 26, 45, 49). In *Bacillus* sp. strain SG-1 it is the spores and not the vegetative cells that oxidize Mn(II) (49). Mn(II) oxidation is due to a component of the mature spore coats and apparently does not require metabolic energy (12, 56, 57). Because Mn(II) oxidation is a thermodynamically favorable process at the pH of seawater and spores rendered nongerminable still oxidize Mn(II) (49), SG-1 spores apparently act as a surface catalyst reducing the activation energy and, thus, accelerating the rate of the reaction. We report here the direct binding and oxidation of cobalt by the Mn(II)-oxidizing spores of the marine *Bacillus* sp. strain SG-1 in the absence of Mn(III,IV) oxides produced by the spores and propose that the metal precipitation properties of *Bacillus* sp. strain SG-1 may have useful biotechnological applications.

## MATERIALS AND METHODS

**Bacterial strains and media.** Three *Bacillus* strains were used: SG-1, a marine manganese-oxidizing bacterium originally isolated from a near-shore sediment (45); SG-1W, a spontaneous Mn(II) oxidation mutant of SG-1; and *Bacillus subtilis* W168. SG-1 and SG-1W were grown in K medium (2 g of peptone [Difco Laboratories, Detroit, Mich.], 0.5 g of yeast extract [Difco Laboratories], 0.2 g of $MnCl_2 \cdot 4H_2O$ [filter sterilized and added to cooled medium], and 20 ml of 1 M HEPES buffer [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.7 to 7.8; autoclaved separately and added to cooled medium] per liter of 75% aged natural seawater or per liter of artificial seawater [ASW; 0.3 M NaCl, 0.01 M KCl, 0.05 M $MgSO_4$, 0.01 M $CaCl_2$; pH 7.0] prepared with high-purity reagents). Cells were grown with constant aeration on a rotary shaker at 150 rpm and 23°C. *B. subtilis* W168 was grown in a medium containing the same nutrients, with distilled water. Usually more than 90% of the bacteria had endospores within 7 to 10 days of incubation.

**Isolation and purification of spores.** Completely sporulated cultures, as confirmed by phase-contrast microscopy, were harvested and purified to remove vegetative cell debris (22, 49). Spores were harvested and washed by centrifugation at 10,000 × *g* and 4°C for 10 min as follows. Cells were washed with Milli-Q water (Millipore Corp., Bedford, Mass.), suspended in 10 mM Tris buffer (pH 7.5), treated with 50 μg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml for 45 min to 1 h at 37°C to lyse the vegetative cells, washed once each with 1 M NaCl, 0.15 M NaCl, and 0.1% sodium dodecyl sulfate (SDS), and washed with Milli-Q water at least five times. With the exception of the final SDS and Milli-Q water washes, all the treatments were supplemented with 10 mM (final concentration) EDTA and 0.003% (wt/vol; final concentration) phenylmethylsulfonyl fluoride stock solution (6 mg · $ml^{-1}$ in ethanol stored at −20°C; pH 7.5) to inhibit possible protease activities. To remove any remaining manganese oxides, spores were treated with 0.1 mM ascorbate (pH 7.8; Sigma Chemical Co.) on a rotary shaker at 150 rpm for 15 min after SDS treatment. Ascorbate is a known reducing agent of Mn oxides (52). Purified spores were stored in Milli-Q water at 4°C and were usually used within 2 to 3 days. The number of spores in suspension was determined by direct counts with a Petroff-Hausser counting chamber (Arthur H. Thomas Co., Philadelphia, Pa.).

**Determination of Co(III) (oxy)hydroxide.** The colorimetric reagent leucoberbelin blue (LBB), which reacts with Mn(III, IV) oxides forming a blue color (35), was tested for its reactivity with Co(III) oxides. A 0.1% LBB solution was prepared in 1% acetic acid and used for spot tests. Cobalt(III) oxyhydroxide (CoOOH) was prepared by the methods of Glemser (21) and Hüttig and Kassler (31) with some modification. Two hundred milliliters of 0.1 N $CoSO_4$ was added dropwise to 210 ml of 0.2 N NaOH with stirring. After 1 day of stirring, the precipitate was washed with Milli-Q water five times. The precipitate was confirmed to be CoOOH by X-ray diffraction spectroscopy.

To compare the sensitivities of LBB with Mn oxides, we quantified the reaction of LBB with oxidized Co and Mn spectrophotometrically at 618 nm, basically as previously described (35). Standard curves for oxidized Mn and Co were made from aliquots of potassium permanganate and the synthetic CoOOH suspension (see above), respectively. The concentration of CoOOH in our suspension was determined from its dry weight. The Co(II) compounds $CoSO_4 \cdot 7H_2O$ and $Co(NO_3)_2 \cdot 6H_2O$ were also tested for reactivity with LBB.

Spot tests of Co(II) oxidation by the *Bacillus* spp. were performed by incubating $10^8$ to $10^9$ spores · $ml^{-1}$ in 1 μM or 10 mM Co(II) for 3 to 24 h in prefiltered HEPES-buffered ASW for SG-1 and SG-1W and in HEPES-buffered Milli-Q water for *B. subtilis* W168. One aliquot of SG-1 spores was washed with 0.1 mM ascorbate (pH 7.8) after incubation in 10 mM Co(II) to determine whether the oxides were ascorbate reducible. One milliliter of each incubated solution was dried on a glass slide for 1 h in a laminar flow hood. One drop of 0.1% LBB spot test reagent was added to each slide.

**Radioactive cobalt binding and oxidation experiments.** Cobalt(II) binding to spores was determined by measuring the partitioning of a radioactive tracer, $^{57}Co$(II) (New England Nuclear Co., Boston, Mass.), between the dissolved and particulate phases by filtration. Dissolved and reduced Co(II) and particulate and oxidized Co(III) are the major forms of Co existing in nature. Dissolved Co(II) passes freely through the filter, whereas oxidized Co(III) and Co bound to spores are retained. Since bound (adsorbed and/or attached) Co(II) and oxidized Co(III) are particulate forms which are trapped on the filters, controls without spores were included in experiments. We also attempted to distinguish the amount of the particulate $^{57}Co$ that was either adsorbed or oxidized by treating the $^{57}Co$ trapped on the filters with nonradioactive (cold) Co(II) and ascorbate, respectively, and then measuring the residual particulate $^{57}Co$ (i.e., $^{57}Co$ trapped by the filters). The nonradioactive Co(II) should exchange with the adsorbed fraction of $^{57}Co$ (releasing adsorbed $^{57}Co$ back into solution), while the ascorbate should bring about the reductive dissolu-

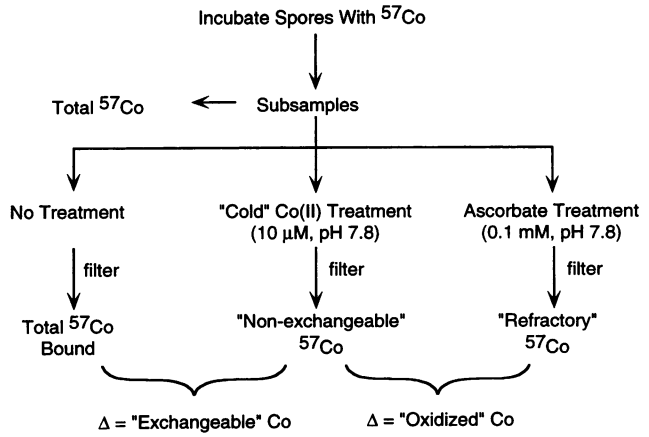


FIG. 2. Protocol used for the measurement of Co(II) oxidation using $^{57}Co$ as a radioactive tracer.

tion of Co(III) oxyhydroxides (releasing both oxidized $^{57}Co$ and $^{57}Co$ associated with oxide phases back into solution) (52, 53). Thus, by comparing these cold-Co treatments to the untreated control, a measure of the exchangeable fraction of $^{57}Co$ bound can be derived. Similarly, by comparing the ascorbate with the cold-Co treatment, the fraction of $^{57}Co$ that was oxidized can be estimated. The fraction of $^{57}Co$ remaining after ascorbate treatment, i.e., the refractory $^{57}Co$, is $^{57}Co$ that is probably tightly bound or taken up by the cells. The general protocol is shown in Fig. 2, and the experimental details are described below. It should be pointed out that a similar approach to this has been employed in studies of manganese oxidation (38, 54).

Spores were suspended in prefiltered (pore size, 0.22 μm) seawater (pH ≈7.8) supplemented with 0 to 100 μM $CoSO_4$ spiked with $^{57}CoCl_2$ (carrier free; specific activity, 8.844 Ci/g of Co). At various times, four to six replicate subsamples of 100 μl were obtained and filtered through 0.2-μm-pore-size Supor-200 membrane filters (Gelman Sciences Inc., Ann Arbor, Mich.) by using a 12-position 25-mm-filter-size Millipore manifold. The filters with trapped particulate material were rinsed twice with 5 ml of prefiltered seawater or ASW to minimize nonspecific $^{57}Co(II)$ adsorption to the filters and placed in gamma vials. In each vial, 4 ml of cold Co(II) solution (10 μM $CoSO_4$; pH 7.8) or 4 ml of ascorbate solution (0.1 mM; pH 7.8) was added (as part of the treatment to distinguish adsorbed Co and oxidized Co; see below), and the vials were counted for gamma radiation by using an LKB 1282 Compugamma LS gamma counter with a 3-in. (1 in. = 2.54 cm) NaI detector (Phamacia-Wallac Co., Turku, Finland). To determine the total counts per minute in each incubation, 100 μl of unfiltered subsample was placed in a vial with 4 ml of 0.1% (wt/vol) hydroxylamine hydrochloride ($NH_2OH$-HCl) solution, a strong reducing agent, in order to solubilize all the Co and to maintain a similar geometry for gamma counting, and counted for 1 to 5 min (error in counting, <0.1%). The percentage of the total radioactivity that was trapped by the filters was calculated from the means of the filter and total counts (unfiltered): 100 × (cpm of filters/total cpm).

The radioactive tracer, $^{57}Co(II)$, that is simply adsorbed onto the spore surface should exchange in the presence of excess nonradioactive Co(II) ions. Thus, to measure the exchangeable fraction of the total bound $^{57}Co$, an excess of cold Co(II) was added to displace the adsorbed $^{57}Co(II)$ until no further $^{57}Co$ is exchanged and equilibrium is reached. To accomplish this, the vials containing the filters with the 10 μM cold Co(II) solution (pH 7.8) and previously counted for determining total $^{57}Co$ binding (see above) were equilibrated for 12 to 18 h at 24°C. Separate time course experiments (data not shown) demonstrated that for the conditions of our experiments complete exchange had occurred by 12 h. After equilibration the cold-Co(II)-treated filters were removed and placed in a well of the filter manifold containing fresh filters. The cold-Co solution from the vial was then poured into the same well with the treated and new filters and filtered. The vials were washed twice with 4 ml of prefiltered seawater, and the seawater wash was also filtered through the same filters. The two filters were placed in the vials with 4 ml of $NH_2OH$-HCl (to solubilize the remaining particulate $^{57}Co$ and maintain counting geometry), and the gamma radiation was counted. The percentage of exchangeable cobalt could be determined from the difference between the means of total binding and the cold-cobalt treatment: percent total binding − 100 × (cpm of filters after cold-Co treatment/total cpm).

Treatment with ascorbate, a reducing agent, reduces oxidized Co(III) or Mn(III,IV) to soluble Co(II) or Mn(II) (52) and can, thus, be used to determine $^{57}Co$ associated with Co and Mn oxides (i.e., $^{57}Co$ that is either adsorbed to oxide phases or oxidized). The contents of the vials containing the filters with 0.1 mM ascorbate (pH 7.8; see above) were filtered and processed as described for the cold-Co treatments after 30 min (a time determined experimentally to be sufficient for complete reduction of the amount of Co(III) formed in our experiments). The percentage of cobalt oxidized was determined from the difference between the means of the cold-cobalt and ascorbate treatments: 100 × [(cpm of filters treated with cold Co − cpm of filters treated with ascorbate)/total cpm]. Co(II) oxidation rates were calculated from the initial slope of the time course (1 to 3 h) of the difference between the cold-Co(II) and ascorbate treatments.

**Effect of environmental factors.** The standard experimental conditions, unless specified otherwise, employed $10^8$ spores · $ml^{-1}$ in filtered (pore size, 0.2 μm; Nuclepore PC) seawater or ASW (pH ≈7.8) with 1 μM Co(II) (added as $CoSO_4 \cdot 7H_2O$), incubated at 24°C. Experiments were performed to characterize the optimal temperature or pH conditions for Co(II) oxidation, the effect of different Co(II) concentrations, and because the chemical mechanism for cobalt oxidation by Mn (III,IV) oxides does not require oxygen (9, 27), the effect of the presence and absence of oxygen. For the temperature optimum experiments, the incubation temperatures were varied between 4 and 70°C. The cobalt solutions were preincubated at each temperature for 30 min. The experiments were initiated with the addition of spores to temperature-adjusted media, and samples were removed periodically. The effect of pH on cobalt oxidation by SG-1 spores was examined in ASW in the pH range of 4 to 10. pH was adjusted by adding 1 N HCl and/or 0.1 N acetic acid or 1 N NaOH. The pH of each sample was checked at the end of each experiment to determine if it had changed. Co-oxidation was studied at various concentrations of Co(II), from 5 pM (addition of tracer only to ASW) to 1 mM Co(II) by adding known amounts of $CoSO_4 \cdot 7H_2O$. The kinetics of Co(II) oxidation was analyzed assuming Michaelis-Menten kinetics with Lineweaver-Burk (double reciprocal) and Eadie-Hofstee plots. For the oxygen–no-oxygen experiments, oxygen was removed from the medium by bubbling with $N_2$ gas (≈ 0.2 liter · $min^{-1}$) for 1 h before addition of spores, and $N_2$ gas was purged (≈ 0.1 liter · $min^{-1}$) through the medium during the entire incubation. Although oxygen concentration was not measured in these experiments, these

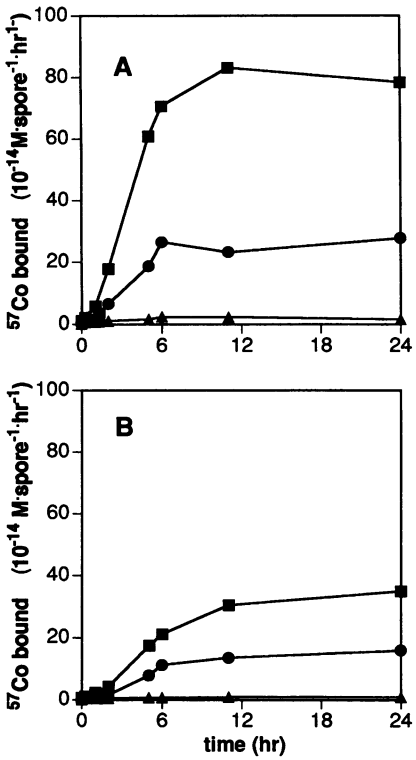


FIG. 3. $^{57}$Co binding by SG-1 in natural seawater before (■) and after treatments with nonradioactive cobalt (●) and ascorbate (▲). Spores ($10^7 \cdot ml^{-1}$) were incubated at 24°C in prefiltered aged seawater supplemented with 0.1 μM (A) or 1 μM (B) Co(II) spiked with $^{57}$Co.

TABLE 1. Reactivity of LBB with $KMnO_4$, CoOOH, $CoSO_4$, and $Co(NO_3)_2$

| Compound (μM) | $A_{618}$ |
|---|---|
| KMnO4[a] | |
| 0.1 | 0.036 |
| 0.5 | 0.055 |
| 1 | 0.124 |
| 2 | 0.227 |
| 5 | 0.739 |
| 10 | 1.578 |
| CoOOH[b] | |
| 0.3 | 0.033 |
| 0.5 | 0.045 |
| 2.7 | 0.09 |
| 5.4 | 0.209 |
| 27.2 | 0.43 |
| 54.4 | 1.14 |
| $CoSO_4 \cdot 7H_2O$[c] | |
| 1 | 0.035 |
| 10 | 0.039 |
| 100 | 0.035 |
| 1,000 | 0.036 |
| 10,000 | 0.037 |
| $Co(NO_3)_2 \cdot 6H_2O$[c] | |
| 1 | 0.036 |
| 10 | 0.033 |
| 100 | 0.037 |
| 1,000 | 0.035 |
| 10,000 | 0.035 |

[a] Mn(VII).
[b] Co(III).
[c] Co(II).

sparging procedures were designed to mimic the sparging procedures used for deoxygenating the reagents used for measuring the natural environmental oxygen concentrations across oxic-anoxic interfaces (3).

## RESULTS

**Cobalt binding versus Co oxidation.** The time courses of $^{57}$Co(II) removal from natural seawater by SG-1 with and without nonradioactive Co(II) and ascorbate treatments are shown in Fig. 3. The total binding of $^{57}$Co(II) was fairly linear over the first 6 h of incubation, after which the rates decreased. The removal of $^{57}$Co from solution could be due to Co(II) binding (or adsorption) to the spore surface, Co(II) oxidation, or a combination of both. To test for Co(II) oxidation, we found that the colorimetric reagent LBB, which reacts with Mn(III,IV) oxides forming a blue color (35), also reacts with synthetic Co(III) oxyhydroxide (CoOOH) (Table 1). The $A_{618}$ shows good correlations with the concentrations of CoOOH ($r^2$, 0.96) and $KMnO_4$ ($r^2$, 0.98). On a per-electron (equivalent) basis, the detection limits for oxidized forms of both Mn and Co were below 0.5 μM (microequivalents · $liter^{-1}$) and the reactivities (slopes of the lines of $A_{618}$ versus microequivalents · $liter^{-1}$) were about the same (0.03 and 0.02 absorbance units · μeq · $liter^{-1}$ for Mn and Co, respectively). LBB did not react with various Co(II) salts [$Co(NO_3)_2 \cdot 6H_2O$ or $CoSO_4 \cdot 7H_2O$]. A spot test of the purified and ascorbate-treated spores used in the experiments showed no reaction with LBB (data not shown), indicating that preformed Mn(III,IV) oxides were absent from the spore surface. After incubation of SG-1 spores in buffered ASW supplemented with 10 mM Co(II) for 1 day, the spot test had a positive blue color, indicating that Co(II) was indeed being oxidized (Fig. 4A). After treatment of these spores with 0.1 mM ascorbate, the LBB spot test was negative, indicating that ascorbate reduced all of the oxidized Co(III) from SG-1 spores (Fig. 4B). The oxidation of Co(II) by SG-1 in HEPES-buffered (pH 7.5) ASW containing 1 μM Co(II) was very rapid and could be easily detected within 3 h of incubation (Fig. 4C). A spontaneous Mn(II) oxidation mutant of SG-1, SG-1W, did not oxidize Co(II), nor did *B. subtilis* (Fig. 4D and E). A control without spores was also negative (Fig. 4F).

To measure the amount of Co(II) removed from solution that had been adsorbed versus oxidized, nonradioactive Co(II) and ascorbate treatments were carried out (Fig. 3). In these natural-seawater experiments, 30 to 50% of the total $^{57}$Co bound to SG-1 spores was exchangeable with non-radioactive Co(II) while virtually all of the $^{57}$Co(II) is solubilized by ascorbate. SG-1 had a much higher binding capacity for Co(II) than *B. subtilis* (Fig. 5). The Mn(II)-oxidizing mutant, SG-1W, also bound very little Co(II), apparently none of which was oxidized (Fig. 4 and 5). Binding of cobalt by SG-1W spores seemed to be simple adsorption because more than 90% of the adsorbed $^{57}$Co(II) was exchanged by cold-Co(II) ions (Fig. 5). In *B. subtilis* the portion of refractory Co remaining after ascorbate treatment was 78% of the total Co(II) binding.

**Effect of oxygen.** The binding and oxidation of Co(II) by SG-1 spores was significantly affected by oxygen (Fig. 6). The initial rate of Co binding by spores in the absence of oxygen was less than 5% of that under aerobic conditions (Fig. 6). When air was introduced to the anaerobic control, Co(II) binding proceeded at the same rate as the initial aerobic

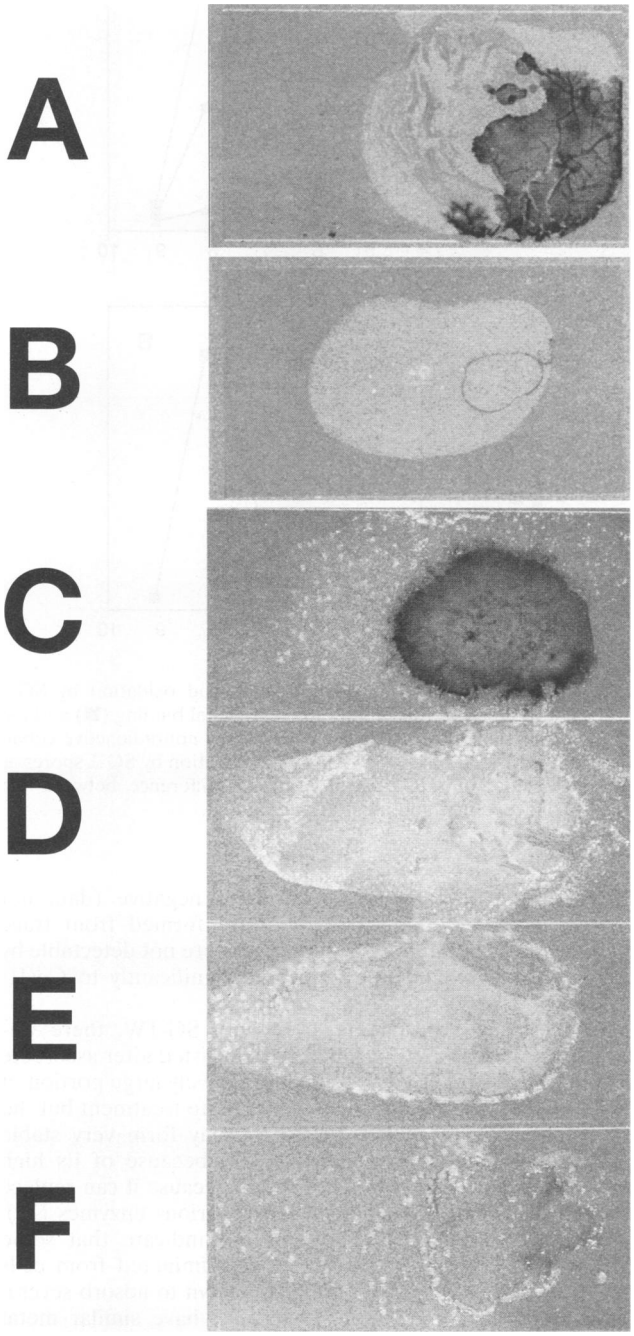


FIG. 4. Comparison of cobalt oxidation between SG-1, ascorbate-washed SG-1, SG-1W, *B. subtilis* W168, and a no-spore control using the LBB spot test. SG-1 and SG-1W were incubated in ASW; *B. subtilis* was incubated in distilled water. (A and B) Spores ($10^9 \cdot ml^{-1}$) were incubated in 10 mM Co(II) for 24 h. (C to F) Spores ($10^8 \cdot ml^{-1}$) were incubated in 1 μM Co(II) for 3 h. (A) SG-1 spores; (B) ascorbate-washed SG-1 spores; (C) SG-1 spores; (D) SG-1W spores; (E) *B. subtilis* W168; (F) no-spore control. Dark parts (originally blue) in panels A and C, positive results of the LBB spot test.

binding rate (data not shown). The difference between the cold-Co(II) and ascorbate treatments demonstrated that Co(II) was oxidized only when oxygen was present (Fig. 5). The rate of Co(II) oxidation in this experiment was $6.3 \times 10^{-14}$ M · spore$^{-1}$ · h$^{-1}$.

**Effect of pH.** Experiments conducted at various pH values in ASW demonstrate a clear optimum for both binding (Fig. 7A) and oxidation (Fig. 7B). The initial rate of cobalt oxidation by SG-1 spores showed a maximum rate around pH 8 (Fig. 7B). Binding and oxidation of Co(II) were insignificant below pH 6. The initial binding and oxidation rates at pH 9 were less than 3% of the maximal value. At higher pHs, the abiotic oxidation of Co(II) could be significant; however, results of control experiments without spores demonstrated that an insignificant amount of $^{57}$Co(II) was precipitated in 3 h (data not shown).

**Effect of temperature.** The temperature of incubation strongly affected cobalt oxidation by SG-1 (Fig. 8). The initial oxidation rates below 12°C were less than $1 \times 10^{-14}$ M · spore$^{-1}$ · h$^{-1}$. The maximal amount of cobalt oxidized by SG-1 spores occurred over a wide temperature range, 55 to 65°C. At 55°C, 0.8 μM Co(II) was oxidized by $10^8$ SG-1 spores in 1 h. The amount of Co(II) oxidized by spores at 25°C was only 25% of the amount oxidized at 55°C.

**Effect of Co(II) concentration.** Experiments were conducted to examine Co(II) binding and oxidation at various Co(II) concentrations (Fig. 9). The initial Co(II) oxidation rate reached saturation after about 10 μM (Fig. 9A). Cobalt oxidation could still be measured at the level of cobalt found in natural seawater (<250 pM of Co). Within 1 h, 95% of the natural level of Co (plus tracer) was oxidized by $10^8$ SG-1 spores · ml$^{-1}$ (data not shown).

## DISCUSSION

We have employed several approaches that demonstrate that the spores of the marine Mn(II)-oxidizing bacterium, *Bacillus* sp. strain SG-1, also oxidize Co(II). The positive reactions of LBB with both synthetic Co(III) oxides and SG-1 spores incubated in Co(II) solutions in the absence of added Mn(II) provide good evidence for Co(II) oxidation. The $^{57}$Co(II) radiotracer technique distinguishes the fraction of $^{57}$Co that is exchangeable from the fraction that is oxidized and is, thus, useful for understanding the environmental parameters that favor Co(II) oxidation by SG-1 and for studying the mechanism of Co(II) binding and oxidation.

The mechanism of Co(II) oxidation by SG-1 spores is not well understood, but it may be related to manganese oxidation. The fact that the Mn-oxidizing mutant, SG-1W, did not bind Co(II) well and did not oxidize Co(II) supports this inference. SG-1 could oxidize Co(II) to Co(III) via either a direct biochemical mechanism or an indirect mechanism involving Mn(III,IV) oxide as an intermediate. The results reported here strongly suggest that Co(II) oxidation occurs most likely via a direct oxidation pathway. Because the spores were treated with ascorbate to remove $MnO_x$ prior to the experiments and spot tests with LBB for confirmation of $MnO_x$ were also negative, it was unlikely that Co(II) oxidation could be catalyzed by preformed $MnO_x$. The fact that Co(II) was not oxidized in the absence of oxygen (Fig. 5) also supports this conclusion because Mn(IV) oxides and manganates are capable of oxidizing Co(II) directly (4, 9, 27). The clear pH and temperature optima (Fig. 6 and 7) and the fact that Co(II) oxidation obeys Michaelis-Menten kinetics (Fig. 8) strongly suggest that Co(II) oxidation is being catalyzed by a biological macromolecule, possibly a protein or polysaccharide, on the spore surface. In contrast, chemical oxidation of Co(II) to Co(III) on synthetic

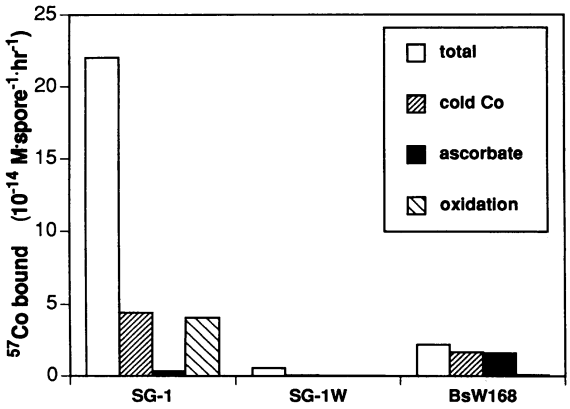


FIG. 5. Cobalt binding and oxidation by SG-1, SG-1W, and *B. subtilis* W168. Spores ($10^8 \cdot ml^{-1}$) were incubated in 1 μM Co(II) at 24°C for 1 h in prefiltered aged seawater for SG-1 and SG-1W and in 10 mM HEPES-buffered Milli-Q water (pH 7.5) for *B. subtilis* W168. The data represent total binding, the amount remaining after treatment with 10 μM nonradioactive cobalt or with 0.1 mM ascorbate and oxidized cobalt, calculated from the difference between the nonradioactive Co(II) treatment and ascorbate treatment.

birnessite occurs in the pH range of 4 to 7 (9, 14, 44). Auto-oxidation of Co(II) to Co(III) at higher pH was reported for several different conditions only after long incubation periods (28). The possibility that Co(II) oxidation could occur via a mechanism involving oxidation by $MnO_x$ if Mn(II) is present in the medium still exists. Although SG-1 oxidizes Co(II) even in pH-adjusted Milli-Q water and oxidation occurs in both artificial and natural seawater (Fig. 6 to 8), the complete elimination of Mn(II) from the experiments is very difficult, because Mn(II) is essential for sporulation in *Bacillus* spp. (6, 8, 15) and mature spores may contain more than trace amounts of Mn(II) as metal chelates. Thus, the spores themselves could be contaminating the medium with Mn(II). However, if this were the case, the Mn(II) concentration in the experiments would still be extremely low and the oxidation of Co(II) by $MnO_x$ would have to be extremely rapid to support the Co(II) oxidation rates we observed. However, the rate of chemical Co(II) oxidation by $MnO_x$ is slow relative to the rates we measure (28). Furthermore, incubations of SG-1 without

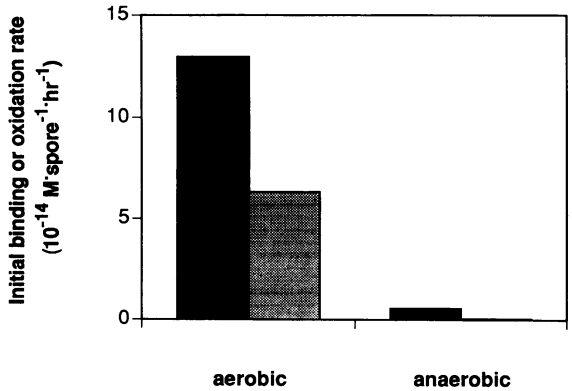


FIG. 6. Effect of oxygen on cobalt oxidation by SG-1. Initial rate of total binding (solid bars) or oxidation (shaded bar) in the presence and absence of oxygen in prefiltered aged seawater with 1 μM Co(II).

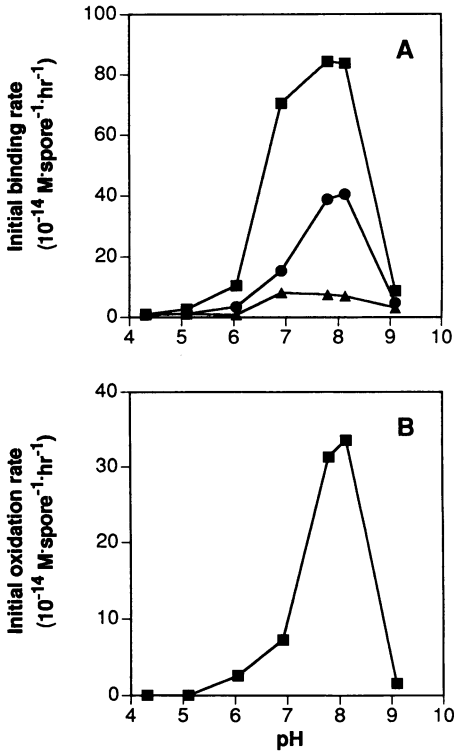


FIG. 7. Effect of pH on cobalt binding and oxidation by SG-1 spores in prefiltered artificial seawater. (A) Total binding (■) and the amount remaining after treatment with 10 μM nonradioactive cobalt (●) or 0.1 mM ascorbate (▲). (B) Cobalt oxidation by SG-1 spores as an initial oxidation rate derived from the difference between the cold-Co(II) treatment and ascorbate treatment.

Mn(II) or Co(II) additions were LBB negative (data not shown), indicating that, if $MnO_x$ were formed from trace amounts of Mn(II) in the medium, they were not detectable by LBB and likely would not contribute significantly to Co(II) oxidation.

During many experiments, even with SG-1W, there was some portion of refractory cobalt that persisted after ascorbate treatment. *B. subtilis*, which bound a relatively large portion of Co had ≈80% refractory Co after ascorbate treatment but did not oxidize it (Fig. 3 and 4). Co(II) may form very stable complexes with metalloproteins (7, 33) because of its high affinity for amino acid thiol groups and because it can replace other divalent cations as cofactors for various enzymes (33). Other studies with phytoplankton also indicate that some fraction of the total cobalt cannot be eliminated from cells (46). Cell walls of *B. subtilis* are also known to adsorb several heavy metals (16, 64). Spore coats may have similar metal binding properties that could explain the refractory Co.

Co(II) oxidation by SG-1 occurs under a variety of conditions representative of natural environments. The binding of Co(II) occurs over wide ranges of pH (pH 6 to 9) (Fig. 6A) and temperature (Fig. 7), suggesting that this mechanism of Co(II) oxidation could occur in natural seawater. The concentration of cobalt varies widely in nature. In marine environments, soluble Co(II) occurs between $10^{-12}$ and $10^{-10}$ M in the water column and 2 to 3 orders of magnitude higher in sediment pore waters (5, 32, 51). Abiotic oxidation of cobalt is favorable only under relatively high concentrations of Co(II), which are not often found in natural seawater. From thermodynamic calcu-

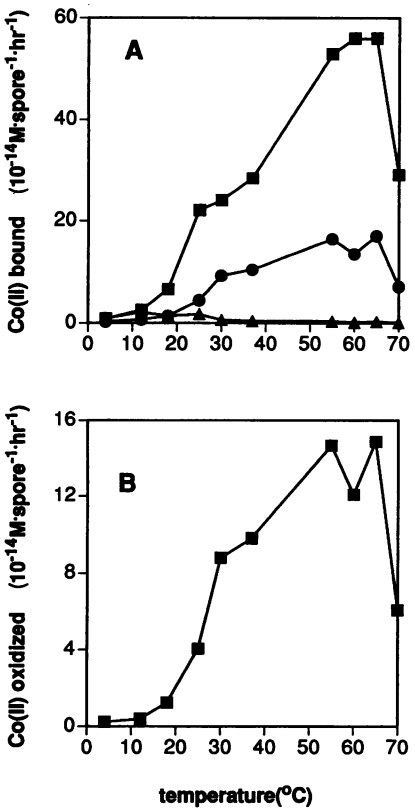


FIG. 8. Effect of temperature on cobalt binding and oxidation by SG-1 spores. (A) Total binding (■) and the amount remaining after treatment with 10 μM nonradioactive cobalt (●) or 0.1 mM ascorbate (▲). (B) Cobalt oxidation by SG-1 spores as an initial oxidation rate derived from the difference between the cold-Co(II) treatment and ascorbate treatment. Experiments conducted at 55, 60, 65, and 70°C used ASW; the remaining experiments used aged natural seawater.

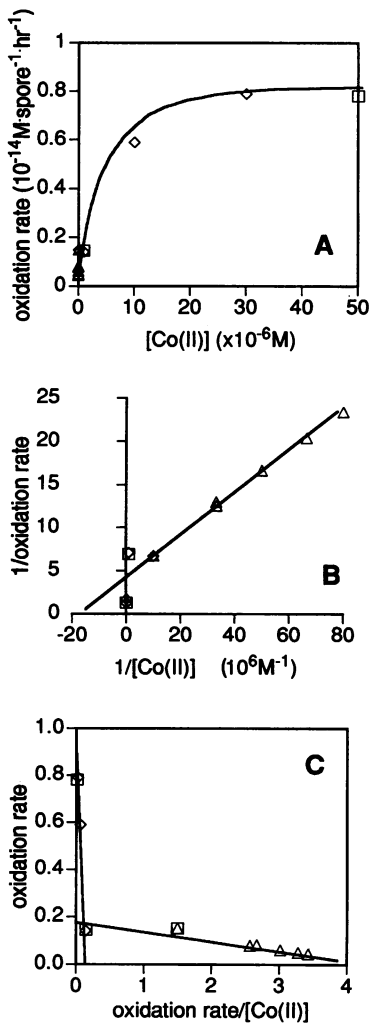


FIG. 9. Kinetics of cobalt oxidation by SG-1 spores. (A) Saturation of cobalt oxidation by high concentrations of Co(II). (B) Lineweaver-Burk plot of cobalt oxidation by SG-1 spores. (C) Eadie-Hofstee plot of the data. Experiments conducted with 5 pM (tracer only) and 10, 30, or 50 μM Co(II) used ASW; the remaining experiments used aged natural seawater. Different symbols represent separate experiments conducted on different days.

lations, Co(III) formation catalyzed by $FeOOH \cdot nH_2O$ requires cobalt concentrations in seawater of above $1.3 \times 10^{-8}$ M (4). SG-1 spores, on the other hand, were active over a broad range of cobalt concentrations from the trace levels present in our media to 100 mM. Measurements of cobalt oxidation by SG-1 spores at various Co(II) concentrations show that its kinetics obey Michaelis-Menten saturation kinetics (Fig. 9). From the Lineweaver-Burk plot ($r^2$, 0.94), the $K_m$ is estimated to be $6.6 \times 10^{-8}$ M and the $V_{max}$ is $2.6 \times 10^{-15}$ M · spore$^{-1}$ · h$^{-1}$. Because there was a noted discrepancy between the $V_{max}$ calculated from the Lineweaver-Burk plot and the apparent $V_{max}$ from the saturation curve (Fig. 9A), we also analyzed the data by using an Eadie-Hofstee plot (Fig. 9C). Such a plot places equal emphasis on all the data, whereas the Lineweaver-Burk plot overemphasizes data with low rates (30). The results of this analysis suggest that SG-1 possesses two different Co(II) oxidation systems. One system is characterized by having a high-affinity and low-oxidation rate ($K_m$, $3.3 \times 10^{-8}$ M; $V_{max}$, $1.7 \times 10^{-15}$M · spore$^{-1}$ · h$^{-1}$), and the second system has a low-affinity and high-oxidation rate ($K_m$, $5.2 \times 10^{-6}$ M; $V_{max}$, $8.9 \times 10^{-15}$ M · spore$^{-1}$ · h$^{-1}$). This suggests that microorganisms like SG-1 are able to oxidize Co(II) and decrease the turnover time of Co(II) even at the low concentrations of Co(II) found in natural environments.

In natural environments the concentration of Mn(II) is several orders of magnitude higher than that of Co(II). Thus, it is quite likely that, if an organism like SG-1 were active in the environment, Co(II) oxidation could occur via both a direct mechanism and an indirect mechanism involving oxidation by $MnO_x$. In aerobic environments, the relative importance of the direct mechanism versus the indirect mechanism for an organism like SG-1 will depend, in part, on the relative affinities of the cells for Co(II) and Mn(II). In suboxic environments the indirect mechanism of Co(II) oxidation by $MnO_x$ may be more important. Spore-forming bacteria like SG-1 may represent a significant portion of Mn-oxidizing bacteria in certain sedimentary environments (unpublished observations), and spore-forming *Bacillus* species can occur as 20 to 40% of the total colony-forming bacteria in certain aquatic environments and up to 80% in sediments (2). It is also highly likely that other microorganisms capable of oxidizing Co(II) may contribute to Co cycling in natural environments. These laboratory studies are consistent with our recent demonstration of microbially mediated Co(II) oxidation in oceanic surface waters (36) and

suggest that microorganisms may play a more significant role in Co cycling in nature than previously believed.

We have not yet identified the nature of the catalyst for Co(II) oxidation. In manganese oxidation, polysaccharide (20) and extracellular or surface proteins (1, 12) are usually implicated as the manganese-binding component. A preliminary study of SG-1 indicated that Mn(II) oxidation may be catalyzed by a spore coat protein (56). Both Mn(II) oxidation and Co(II) oxidation could be catalyzed by the same cellular component, as the spores of the Mn-oxidizing mutant, SG-1W, did not oxidize cobalt either. Recently, we have developed a genetic system in SG-1 and identified two regions of the chromosome involved in Mn(II) oxidation (63). Sequence analysis of these regions has identified eight open reading frames (genes), seven of which occur in one region in what appears to be an operon, with the eighth being in the second region (62). Two of the so-called *mnx* genes code for putative proteins that have regions of similarity to the family of multicopper oxidases and are, thus, good candidates for the Mn(II)-oxidizing [and perhaps Co(II)-oxidizing] protein(s). The observation based on kinetic analysis that we have two Co(II) oxidation systems in SG-1 is consistent with the notion that these two *mnx* genes are indeed coding for proteins involved in both Mn(II) oxidation and Co(II) oxidation. Further analysis of our transposon *mnx* mutants should help reveal the relationship between manganese oxidation and cobalt oxidation by SG-1 spores.

Our results demonstrate the potential for a direct contribution of biological activities to the geochemistry of Co. In addition, the unique and environmentally versatile metal binding and oxidizing properties of SG-1 spores suggest that they may have potential applications in a number of metal removal, recovery, and immobilization processes. For example, $^{60}Co$ has been identified as a major radionuclide pollutant at various Department of Energy sites in the United States. The Co binding and oxidation ability of the spores might be able to be incorporated into a remediation strategy for such sites. Alternatively, the spores might be used to recover Co from leachates of low-grade ore. The spores are dormant, and thus, growth does not have to be sustained. SG-1 is active over a wide range of environmental conditions (pH, temperature, metal concentrations, and salinity). Furthermore, spores rendered nonviable still oxidize Mn(II) (49), so one does not have to worry about unwanted growth of the organisms if nonviable spores are released into the environment. Because metal oxidation appears to be catalyzed by a protein, the tools of modern molecular biology can be used to enhance or alter the metal-precipitating characteristics of SG-1 spores. Thus, in addition to providing insights into the biogeochemical cycling of metals, SG-1 is a good model system for studying potential biotechnological applications of metal-precipitating microorganisms.

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