Indirect Oxidation of Co(II) in the Presence of the Marine Mn(II)-Oxidizing Bacterium *Bacillus* sp. Strain SG-1[∇]

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Cobalt(II) oxidation in aquatic environments has been shown to be linked to Mn(II) oxidation, a process primarily mediated by bacteria. This work examines the oxidation of Co(II) by the spore-forming marine Mn(II)-oxidizing bacterium *Bacillus* sp. strain SG-1, which enzymatically catalyzes the formation of reactive nanoparticulate Mn(IV) oxides. Preparations of these spores were incubated with radiotracers and various amounts of Co(II) and Mn(II), and the rates of Mn(II) and Co(II) oxidation were measured. Inhibition of Mn(II) oxidation by Co(II) and inhibition of Co(II) oxidation by Mn(II) were both found to be competitive. However, from both radiotracer experiments and X-ray spectroscopic measurements, no Co(II) oxidation occurred in the complete absence of Mn(II), suggesting that the Co(II) oxidation observed in these cultures is indirect and that a previous report of enzymatic Co(II) oxidation may have been due to very low levels of contaminating Mn. Our results indicate that the mechanism by which SG-1 oxidizes Co(II) is through the production of the reactive nanoparticulate Mn oxide.

Co is an essential nutrient for many organisms (it is the metal cofactor in cyanocobalamin, or vitamin B₁₂); however, at higher levels it can be toxic (3). In nature cobalt exists in two stable oxidation states: Co(II), which is soluble, and Co(III), which exists as relatively insoluble oxides or as organic complexes. Environmental cobalt cycling has been shown to be microbially mediated and related to the cycling of manganese (10, 12, 22, 26). Correlations between particulate Mn and Co have been found in various environmental systems. Cobalt was found to be associated with the Mn-rich particulate layer in the Black Sea, a permanently stratified anoxic basin (21), which was interpreted as the scavenging of Co onto the Mn oxides. Later, it was shown that adsorption of cobalt onto Mn oxides did occur and could account for the enrichment of Co found in the particulate layer of the Black Sea (13). Further examination of this particulate-bound cobalt by X-ray photoelectron spectroscopy (16) showed that it had been oxidized from Co(II) to Co(III) at the MnO₂ interface and that Mn(IV) was the oxidant (6).

The connection between cobalt precipitation and bacterial activity was made by examining bacterial activity from two anoxic fjords (26). Depth profiles of Mn and Co in the fjords correlated, suggesting similar cycling. Using radioisotopes as tracers of Mn(II) and Co(II), it was found that the precipitation of both metals was inhibited by bacterial poisons but not by specific inhibitors of photosynthesis (the oxic/anoxic interface was in the photic zone in one of the fjords). The inhibition rates of these processes by high levels of each metal were not

the same, suggesting that there might be different mechanisms involved for Mn and Co precipitation. Lee and Fisher (10) looked at the ability of consortia of coastal microbes grown on decomposing diatom cultures to increase the rates of oxidation of both Mn(II) and Co(II). They reported that the oxidation (as determined by particle association) increased over time independently of surface area, which led them to conclude that the oxidation of both Mn(II) and Co(II) by the microbes was direct.

SG-1 is a spore-forming Mn(II)-oxidizing bacterium isolated from sandy marine sediments in La Jolla, CA (20). The Mn(II)-oxidizing enzyme is located on the exosporium (the outer layer of the spores) (9) and forms Mn oxides while the cells are metabolically inactive. Based on genetic studies, this enzyme is thought to be a multicopper oxidase (MCO)-like protein, although it has never been purified (27). The function of Mn(II) oxidation is not known (24), but this ability is found in many different *Bacillus* species (7, 8) as well as numerous unrelated bacteria (23). Lee and Tebo (11) used cultures of the Mn(II)-oxidizing *Bacillus* sp. strain SG-1 to examine the oxidation of Co(II) under more-controlled conditions. Incubation of purified SG-1 spores with Co(II) resulted in a fraction of the radiotracer being taken up onto the spores. The immobilized Co was leukoerbelin blue positive (2, 11) and ascorbate reducible, indicating that it had been oxidized to Co(III) (11).

Like Co(II), Cr(III) in the environment is oxidized by manganese oxides, and the oxidation occurs faster in the presence of Mn(II)-oxidizing bacteria. However, it was recently shown that *Bacillus* sp. strain SG-1 and other Mn(II)-oxidizing bacteria require only very small amounts of manganese to indirectly oxidize Cr(III) to Cr(VI) (18), accelerating Cr(III) oxidation but not directly oxidizing Cr(III). Based on this finding, we chose to reexamine whether the apparent oxidation of Co(II) to Co(III) by SG-1 (11) was a result of the same indirect

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pathway. Previous studies of Co(II) oxidation by SG-1 suggested that the Co(II) oxidation was direct (11), but the methods used would not have detected the relatively low levels of Mn that are able to oxidize measurable amounts of Cr(III) and possibly Co(II). Here, we use a more-stringent cleaning protocol during spore preparation to determine whether Co(II) oxidation in this system is direct (enzymatic) or indirect [due to Mn(IV) oxides formed by the spores].

MATERIALS AND METHODS

Spore preparation. *Bacillus* sp. strain SG-1 was grown in a complex medium (0.5 g yeast extract, 2.0 g peptone, 750 ml filtered seawater, 250 ml water) with 1 mM Mn(II) (as MnCl₂) and buffered with 20 mM HEPES at about pH 7.8. After 95% of the cells had sporulated (as determined by visual counts with a Petroff-Hausser counting chamber under a light microscope), spores were harvested and purified according to the method of Rosson and Nealson (20), with several extra washing steps. To lyse any remaining vegetative cells, cultures were centrifuged, resuspended in 100 ml of 10 mM Tris-HCl, 10 mM EDTA, and 0.5 ml phenylmethylsulfonyl fluoride (PMSF) solution (6 mg/ml of ethanol) and 5 mg of lysozyme (50 µg/ml), and put on a rotary shaker for 1 to 2 h at room temperature. The spores were centrifuged and the pellet washed (five passes in a tissue homogenizer) with 100 ml of 1 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, and 0.5 ml PMSF. The spores were centrifuged again and the pellet washed with 100 ml of 0.15 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, 0.5 ml PMSF solution, and 0.035 g ascorbate. After washing with the ascorbate-containing buffer, the spores lost the dark brown color, indicating that the Mn oxides present had been reduced. The solution was resuspended in 0.1% sodium dodecyl sulfate overnight at room temperature. To remove any remaining salts, organics, or detergents, the following day the spores were centrifuged and washed with MilliQ water five times, washed another time with 0.035 g ascorbate in MilliQ water, and washed a final five times in MilliQ water. The spores were stored in MilliQ water at 4°C until they were used, except for the spores used for spectroscopy experiments, which were stored at -20°C.

Synthesis of abiotic Mn oxides. δ-MnO₂ was made according to the method of Murray (14). Briefly, 1 M MnCl₂ was heated to 95°C while stirring and made basic with 1 M NaOH. A solution of 0.127 M KMnO₄ was added dropwise while being stirred and heated. The resulting oxide particles were centrifuged, washed, and lyophilized. The surface area of the oxides was determined by standard five-point Brunauer-Emmett-Teller analysis using nitrogen as the adsorbate (Quantachrome NOVA 1000) to be approximately 110 m² per gram, and the average oxidation state was measured to be 3.75 by titration (15).

Colloidal Mn oxide was prepared according to the method of Perez-Benito et al. (19). Ten milliliters of 100 mM KMnO₄ was brought up to 50 ml with distilled water and titrated with 18.8 mM Na₂S₂O₃ while being stirred until the pink color of the permanganate was not visible in solution after filtration with a 0.02-µm syringe filter. The resulting colloid was brought to 1 liter with water and remained translucent and in suspension over several months of storage.

Co(II) and Mn(II) oxidation experiments. Incubations were conducted in tissue culture bottles with 15 ml of artificial seawater (ASW) (0.05 M MgSO₄ · 7H₂O, 0.01 M CaCl₂ · 2H₂O, 0.3 M NaCl, 0.01 M KCl) and 20 mM HEPES buffer (pH 7.5) and amended with various concentrations of Mn(II) (as MnCl₂ · 4H₂O) or Co(II) (as CoCl₂). *Bacillus* sp. strain SG-1 spores were added to a concentration of 2 × 10⁸ cells/ml. The bottles were shaken on a rotary shaker (150 rpm) at room temperature until the end of the experiment. Samples were incubated for 1 to 2 h. The time points were chosen to measure the initial oxidation rates; experience has shown that Mn(II) oxidation is linear for the first 12 to 24 h at these spore densities (data not shown).

Incubations were spiked with ⁵⁷Co(II) (carrier free; Amersham Biosciences) and/or ⁵⁴Mn(II) (carrier free; NEN Life Sciences). At the end of the incubation period, 1 ml of the solution was removed from the tissue culture bottle and counted to measure the total ⁵⁴Mn and ⁵⁷Co activities. The remaining 14 ml was filtered through Supor filters (0.2-µm pore size). A 2-ml sample of the filtrate was collected for counting. The filters and "total" samples were brought up to 2 ml in 0.1% NH₂OH to maintain counting geometry for all three sample types (filter, filtrate, and total). Samples were counted on a Wallac gamma counter. It was assumed that ⁵⁴Mn and ⁵⁷Co both acted as ideal tracers, so the fraction immobilized was representative of the total metal immobilized in the incubation. The percentage of Co(II) or Mn(II) oxidized was calculated as the counts per ml trapped on the filter divided by the counts per ml of the "total" with a correction for sorption to the filter (see below). To get an amount oxidized, the percentage

was multiplied by the dissolved Mn(II) or Co(II) added. The amount oxidized was divided by the time of the incubation to get a rate.

To account for removal by sorption on the spores and filters, control experiments were done to calculate sorption blanks. Tissue culture bottles were set up in the same way as the previous experiments with spores. The tracer was added and the bottles were shaken for 5 min. The bottles were sampled and filtered as above. While the filters were still in the manifold, 5 ml of 10 mM CuSO₄ was overlain on the filters and left to sit for five more minutes. The Cu solution was then filtered through the filters, and the filters and total samples were counted as above. The fraction of the isotope bound to the filter after Cu treatment was used as the control blank.

To measure both Mn(II) and Co(II) oxidation in a single sample, a double-labeling method was used. This eliminated the complications of using the formaldehyde colorimetric method for measuring dissolved Mn (5) with samples that contain Co(II), since formaldehyde was found to react with Co and interfered with the visible spectrum used to quantify the Mn (data not shown). On the gamma counter, the spectrum plots of each isotope were compared and the energies of each peak were shown not to overlap. This allowed us to set separate energy ranges that would capture each isotope and measure them independently in a single sample.

Sample preparation for XANES measurements. Stringently cleaned *Bacillus* sp. strain SG-1 spores (see "Spore preparation" above) were incubated with 10 µM CoCl₂ in 5 mM HEPES buffer (pH = 7.7 to 7.8), with no Mn present. The spores were concentrated by centrifugation, and the oxidation state of the Co was measured using X-ray absorption near-edge spectroscopy (XANES) as described below. This was compared to results for SG-1 spores incubated with both 1 µM Co and 10 µM Mn(II).

XANES measurements. All XANES measurements were performed on pelleted wet spores to ensure that the samples were in their native oxidation state. Samples were measured in a plastic (polychlorotrifluoroethylene) sample holder using 125-µm-thick polycarbonate sealed with Kapton tape as the window material. Standards used for Co oxidation state measurements were CoCl₂ (0.5 M) in solution for Co(II) and CoOOH (heterogenite) for Co(III). Co-K XANES spectra were collected in transmission at the Stanford Synchrotron Radiation Laboratory (SSRL) beamline 4-3 using a Si(220) monochromator with a harmonic rejection mirror installed with a cutoff energy of 9 keV. Energy was calibrated by defining the first derivative peak of a Co metal foil to be 7,709 eV. Spectra were collected over the range from 7,700 to 7,750 eV using a step size of 0.5 eV through the edge. All XANES data were normalized and analyzed using the software package SIXPACK (29).

RESULTS

Isotope tracer experiments were used to examine the effect of Mn(II) on Co(II) oxidation (Fig. 1A). Duplicate bottles of *Bacillus* sp. strain SG-1 in ASW medium were incubated with a range of Mn(II) (0 to 50 µM) at two levels of Co(II), 10 and 50 µM. Co(II) was not oxidized in the absence of Mn. Increasing the amount of Mn(II) present up to 25 µM increased rates of Co(II) oxidation asymptotically, approaching maximum values near 10 or 25 µM Mn(II) for 10 and 50 µM Co(II), respectively, after which little increase or inhibition was apparent. In the opposite experiments, Co(II) inhibited Mn(II) oxidation at all concentrations tested (5 to 100 µM) (Fig. 1B).

The types of inhibition of Co(II) and Mn(II) oxidation were examined using double-label experiments. Duplicate bottles of *Bacillus* sp. strain SG-1 in ASW medium were incubated with a range of Mn(II) (0 to 50 µM) at three separate levels of Co(II): 0, 10, and 50 µM. These experimental samples were double labeled with ⁵⁷Co and ⁵⁴Mn and allowed to incubate for approximately 3 h each, well within the linear range of Mn(II) oxidation (data not shown). The resulting Mn(II) and Co(II) oxidations were measured. Co(II) was shown to inhibit Mn(II) oxidation, and double-reciprocal (Lineweaver-Burk) plots (Fig. 2A) showed lines intersecting approximately on the y axis, suggesting competitive inhibition. Similar experiments were repeated with a range of Co(II) (0 to 100 µM) at two

FIG. 1. Effects of Mn(II) concentration on Co(II) oxidation (A) and Co(II) concentration on Mn(II) oxidation (B).

different Mn(II) levels. When plotted on a Lineweaver-Burk plot (Fig. 2B), these also intersected on the y axis, suggesting that the Mn(II) was a competitive inhibitor of Co(II) oxidation. Spores incubated with Co(II) in the absence of Mn were measured using XANES, and comparing the resulting spectrum with reference spectra showed that all the Co could be accounted for by Co(II), indicating that the spores were unable to oxidize Co(II) in the absence of Mn (Fig. 3). Abiotic experiments were conducted to examine whether the rates of Co(II) oxidation seen in the SG-1 incubations could be accounted for by the presence of Mn oxides (as opposed to enzymatic oxidation). A range of δ - MnO_2 was incubated with 50 μM Co(II) for one hour in the absence of spores and showed rates of oxidation much lower than those seen with the equivalent amount of Mn(II) and SG-1 spores present (Fig. 4). However, equivalent amounts of colloidal MnO_2 oxidized significant amounts of Co(II) much more quickly than the δ - MnO_2 , forming measurable Co(III) in less than a minute. After the initial few seconds, the colloidal MnO_2 aggregated, and the Co(III) production slowed (data not shown).

FIG. 2. (A) Double-reciprocal plot of Mn(II) oxidation rates as a function of Mn concentration at 0 μM Co(II) (filled triangles), 10 μM Co(II) (half-filled squares), and 50 μM Co(II) (open diamonds). (B) Double-reciprocal plot of Co(II) oxidation rates as a function of Co(II) concentration at 10 μM Mn(II) (open squares) and 50 μM Mn(II) (filled diamonds).

DISCUSSION

Bacillus sp. strain SG-1 causes the rapid biological oxidation of Mn(II) to Mn(IV) oxides (Fig. 5A) via the oxidation of Mn(II) to Mn(III) and then Mn(III) to Mn(IV) by a MCO-like enzyme (28, 31). The resulting Mn(IV) oxide can also catalyze the abiotic oxidation of Mn(II) to Mn(III, IV) oxides on its surface (4). The presence of SG-1 clearly increases the rate of oxidation of Co(II) compared to the rate with equivalent amounts of δ - MnO_2 , a synthetic Mn oxide that closely resembles the SG-1 biogenic Mn oxide (4) (Fig. 4). Previous research has shown that Co(II) oxidation did not occur in non-Mn(II)-oxidizing mutants of SG-1, demonstrating the involvement of the MCO (11). Our results (Fig. 1 and 3) indicate that Mn is required for the oxidation of Co(II) by *Bacillus* sp. strain SG-1, suggesting that the mechanism by which Co(II) is oxidized is indirect, most likely abiotic oxidation by oxidized Mn. At higher levels of Mn(II), the rates of Co(II) oxidation no longer

FIG. 3. Co K-edge XANES spectra of Co(II) added to purified SG-1 spores (solid line) compared to reference spectra of aqueous Co(II) [Co(II)_{aq}; dashes] and SG-1 incubated with both Mn and Co (dots).

increase and may even be inhibited. At the levels tested, Mn oxidation should not be inhibited by the Mn(II) present (18). Rates of Co(II) oxidation at 50 μ M Co(II) are higher than at 10 μ M Co(II) for a given Mn(II) concentration, suggesting competition between Mn(II) and Co(II) for oxidation by available oxidized Mn. The results from pure preparations of SG-1 spores are consistent with those seen in natural samples by Moffett and Ho (12), where Co(II) and Mn(II) seemed to act competitively. In that paper, the authors proposed that the mechanism of inhibition was competition for the same enzyme site in which either metal could be oxidized. Based on our results showing competitive inhibition (Fig. 2 and 3), Co(II) likely inhibits Mn(II) oxidation by binding to the enzyme and preventing the association of Mn(II) with the MCO (Fig. 5B). However, since Mn(II) is required for Co(II) oxidation, we propose that the Co(II) bound to the enzyme is not directly

FIG. 4. Comparison of cobalt(II) oxidized by δ -MnO₂ (1 h), spores with Mn(II) (1 h), and colloidal Mn oxide (less than one minute). Each treatment had the same amount of total Mn, although the oxidation states and forms differed.

FIG. 5. Schematics of potential pathways of inhibition of Co(II) and Mn(II) oxidation by SG-1. MnOx, Mn oxide.

oxidized or, if it is, it is not released from the enzyme as a Co(III) oxyhydroxide. We believe that Mn(II) inhibits Co(II) oxidation not by competing for an enzyme site but by competing for the highly reactive Mn oxides that are released by the MCO after oxidation. In this case, Co(II) could also compete with Mn(II) and inhibit Mn(II) oxidation at the second step (Fig. 5C).

While the traditional synthetic analog for biological Mn oxides (δ -MnO₂) seems to react too slowly to account for all the oxidation of Co(II) seen in the presence of SG-1 (Fig. 4), colloidal MnO₂ has been shown to be extremely reactive towards Co(II) as well as Cr(III) (18). Colloidal MnO₂ may be a better model for the initial reactive and nano-sized products of bacterial Mn(II) oxidation because of its fine particle size and dispersivity (18, 30). While the colloid reacts extremely fast but aggregates and becomes less reactive in ionic media under environmental conditions, the presence of SG-1 may provide a constant source of this highly reactive species that can encounter Co(II) and oxidize it to Co(III). This colloid species could be more reactive because of its relatively small size, ability to maintain a well-dispersed colloidal suspension, or higher mobility compared to larger particles. The presence of a smaller, more-reactive Mn species agrees with the proposed primary oxidation product in Mn(II) oxidation by SG-1, which is thought to be a nanoparticulate phyllosilicate with defects in the mineral lattice (4). As the product ages, the oxide becomes more crystalline and possibly less reactive. This may account for the changing Co(II) oxidation rates previously reported (9, 25): what was thought to be fast direct biological

Co(II) oxidation and slower abiotic Co(II) oxidation may have been faster oxidation due to the primary Mn oxide and slower oxidation by the more-crystalline aged product.

SG-1 is a convenient model organism for enzymatic Mn(II) oxidation due to the oxidation occurring while the cells are in a nonvegetative state excluding the complications from growth and metabolism. However, Mn(II) oxidation is widespread (23), and indirect Cr(III) oxidation has been shown in *Pseudomonas putida*, which oxidizes Mn(II) during late log phase (17, 32). It is possible that Co(II) could be indirectly oxidized by other Mn(II)-oxidizing bacteria. In fact, the competition and inhibition patterns seen in our cultures of SG-1 are consistent with results found by Tebo et al. (26), who examined Co(II) and Mn(II) removal in waters taken from a stratified fjord now known to have significant Mn(II)-oxidizing populations of bacteria. In their studies, high levels of Mn(II) inhibited Mn(II) oxidation and Co(II) oxidation, but Co(II) did not equally inhibit Mn(II) oxidation. Therefore, in a system where the Mn(II)-oxidizing enzyme(s) are unable to bind Co(II) [or have a much lower affinity for Co(II)] it can be expected that the Co(II) and Mn(II) would compete to be oxidized by Mn oxides, but the ability of the enzyme to oxidize Mn would be unaffected by the presence of additional Co(II).

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