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Cr(III) Is Indirectly Oxidized by the Mn(II)-Oxidizing Bacterium *Bacillus* sp. Strain SG-1

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Manganese oxides are the only known oxidants of Cr(III) in the environment, and predictions of the fate of Cr(III) have been based on Cr(III) oxidation rates with well-characterized Mn(III,IV) oxide minerals. Our research, however, indicates that the presence of Mn(II)-oxidizing bacteria may accelerate these rates through the production of very reactive Mn oxides or intermediates formed in the oxidation process. Experiments with the Mn(II)-oxidizing *Bacillus* sp. strain SG-1 show that this bacterium can accelerate Cr(III) oxidation compared to both abiotic and biologically produced Mn oxides. Initial rates of Cr(III) oxidation by biogenic oxides were approximately 7 times faster than Cr(III) oxidation rates by equivalent amounts of synthetic δ -MnO₂ and 25 times faster by SG-1 spores with Mn(II). Cr(III) oxidation by SG-1 is not direct; Mn is required, but only in small amounts, indicating that it is recycled. Cr(III) oxidation is inhibited above 5 μ M dissolved Mn(II), while Mn(II) oxidation is not, suggesting that the processes are controlled by different mechanisms. These results illustrate the need to consider bacterial activity and the concentration of Mn when predicting the potential for Cr(III) oxidation.

Introduction

Bioremediation and natural attenuation of environmental Cr(VI) pollution via Cr(VI) reduction depend on the less toxic Cr(III) being insoluble and relatively unreactive in soils and sediments. The only known environmental oxidants of Cr(III) are Mn oxides (1). The role of Mn and its contribution to chromium cycling is complicated by the role of bacteria in the cycle, as well as by the potential utility of Mn oxides in the remediation of other environmental pollutants (e.g., the *in situ* immobilization of Pb (2)). The addition or stimulation of Mn(II)-oxidizing bacteria has been proposed as a remediation strategy for some metals and organics (3). However, in the case of chromium, the hazard is increased in the presence of Mn oxides, because they are able to convert Cr(III) to Cr(VI), the more soluble, thermodynamically favored form.

Oxidation of Cr(III) by various manganese oxides has been studied in soils, in seawater, and with synthetic oxides (1, 4–6). Rates of Cr(III) oxidation with three different synthetic

Mn oxides were directly compared by Chung et al. (7). Hausmanite (Mn²⁺Mn³⁺₂O₄), the oxide with the highest Mn(III) content, was shown to oxidize Cr(III) much faster than either birnessite ((Na, Ca)Mn₂O₄·2.8H₂O) or pyrolusite (MnO₂). The rate was dependent on both pH and Cr(III) concentration, with inhibition occurring at high pH and high initial Cr(III). Nico and Zasoski (8) further investigated the hypothesis that the oxidation of Cr(III) by Mn oxides was due to the presence of reactive Mn(III). In these laboratory experiments, a synthetic birnessite was incubated with varying amounts of pyrophosphate to complex the available Mn(III) sites. Rates of oxidation of Cr(III) were slowed by pyrophosphate. These experiments were conducted for a range of pHs (from 3 to 6, with most experiments being run at pH 5), and under anaerobic conditions. The MnO₂ oxidized the Cr(III), but the lack of oxygen prevented the reoxidation of Mn(II) on the Mn oxide surface. Because of the complexity of the system, the mechanism of the reaction could not be determined.

Mn(II)-oxidizing bacteria are a diverse group and found in almost all environments, but a single ecological advantage of Mn(II) oxidation has not been found (9, 10). Hastings and Emerson (11) showed that bacteria oxidize Mn(II) up to 5 orders of magnitude faster than abiotic reactions. The biologically produced Mn oxides have an amorphous structure with a high surface area (9). The mechanism of Mn(II) oxidation by Mn(II)-oxidizing bacteria is not clear, although recent results with *Bacillus* sp. strain SG-1 have shown that a Mn(III) intermediate is enzymatically produced (12). The resulting Mn(III) is further oxidized by the enzyme to Mn(IV).

Using Mn K-edge X-ray absorption near-edge spectroscopy (XANES) and synchrotron-based X-ray diffraction (SR–XRD), the initial biooxide produced by SG-1 was determined to be a disordered hexagonal phyllosilicate with a small (less than 100 nm) particle size and many defects (13). As the initial biooxide aged, SR–XRD showed the formation of secondary products, including buserite and feitknechtite. The rate of formation and products formed were controlled by the thermodynamic properties of the system. Similarly, studies of *Pseudomonas putida*, a common freshwater and soil Mn(II)-oxidizing bacterium, using XRD, Mn K-edge XANES, and Mn K-edge X-ray absorption fine structure spectroscopy (EXAFS) showed that this bacterium also produced a poorly crystalline hexagonal layered oxide with significant vacancies (14). Comparisons to synthetic oxides showed that this oxide was more similar to vernadite and acid birnessite than to the more crystalline synthetic triclinic birnessite. The biological Mn oxides produced by *Pseudomonas putida* have been shown to oxidize Cr(III) to Cr(VI) (15, 16). Surprisingly, in growth medium with low levels of organics, the Cr(VI) produced was less toxic to the bacteria than the original Cr(III) (15).

Based upon the hypothesis that Mn(III) is the species that is primarily responsible for chromium oxidation and the observation that aged Mn oxides adsorb many other chemicals that potentially block the surface and makes them less reactive, we sought to discover whether bacterial activity may accelerate the transformation of Cr(III) to Cr(VI) in environmental systems. This could occur via the rapid production of a reactive Mn oxide, the production of a Mn(III) intermediate during bacterial oxidation, or the direct enzymatic oxidation of Cr(III).

Bacillus sp. strain SG-1 was chosen as a suitable organism for this study because in this organism it is the mature dormant spores that oxidize Mn(II). Growth and cell division

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are not variables during experiments, and potential toxicity of Cr is avoided. SG-1 was originally isolated from sandy nearshore marine sediments (La Jolla, CA), but spore-forming Mn(II)-oxidizing bacteria have been found in many environments including hydrothermal systems (17), suggesting that they could be relevant to the environmental oxidation of Cr(III). The Mn-oxidizing enzyme of SG-1 is a putative multicopper-oxidase-like protein (MCO) that has never been successfully purified, possibly because it exists as a complex (18). It is present in the exosporium which can be separated from the spore (18) and is active over a wide range of temperatures, Mn(II) concentrations, and ionic strengths. This bacterium has been well studied, and the genes thought to be responsible for Mn(II) oxidation (the *mnx* genes) have been identified, including one gene (*MnxG*) that encodes for an MCO-like protein. Non-Mn(II)-oxidizing *MnxG*-mutants have been generated (19), which allows for control experiments compared to the wild type. The enzyme thought to oxidize Mn(II) has been shown to be nonspecific, also oxidizing Co(II) (20); *MnxG* mutants lose this ability. In this paper, we demonstrate that the presence of the Mn(II)-oxidizing enzyme from *Bacillus* sp. strain SG-1 can increase rates of Cr(III) oxidation over the abiotic rates by synthetic and biologically formed Mn-oxides.

Materials and Methods

Spore Preparation. *Bacillus* sp. strain SG-1 was grown in a complex medium (0.5 g of yeast extract, 2.0 g of peptone, 750 mL of filtered seawater, 250 mL of water) with 1 mM Mn(II) (as MnCl_2) and buffered with 20 mM HEPES at about pH 7.8. After 95% of the cells had sporulated (as determined by visual counts under a light microscope), spores were harvested and purified as in Rosson and Nealson (21). 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 EtOH) and 5 mg of lysozyme (50 $\mu\text{g}/\text{mL}$) and put on a rotary shaker for 1–2 h at room temperature. The spores were centrifuged and the pellet was washed (5 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 was washed with 100 mL of 0.15 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, 0.5 mL of PMSF solution, and 0.035 g of ascorbate. After washing with the ascorbate-containing buffer, Mn oxides present were visibly reduced. The solution was resuspended in 0.1% SDS 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 of ascorbate in MilliQ water, and washed a final five times in MilliQ water. The spores were stored in MilliQ water at 4 degrees until they were used.

Synthesis of Abiotic Mn Oxides. δ - MnO_2 was made according to Murray et al. (22). Briefly, 1 M MnCl_2 was heated to 95 °C while stirring and made basic with 1 M NaOH. A solution of 0.127 M KMnO_4 was added dropwise while being stirred and heated. The resulting oxide particles were centrifuged, washed, and lyophilized. Surface area of the oxides was determined with N_2 -BET to be approximately 110 m^2 per gram, and the average oxidation state was measured to be 3.75 by titration (23).

Colloidal Mn oxide was prepared according to Perez-Benito et al. (24). Ten mL of 100 mM KMnO_4 was brought up to 50 mL with distilled water and titrated with 18.8 mM $\text{Na}_2\text{S}_2\text{O}_3$ 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 L with water and remained translucent and in suspension over several months of storage.

Cr(III) Oxidation by Mn Biooxides. Incubation experiments were conducted in an artificial seawater medium (ASW; 0.05 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 M NaCl, 0.01 M KCl) with 20 mM HEPES buffer (pH 7.5). Tissue culture bottles with 30 mL of medium were amended with additions of Mn-(II) (as a solution of MnCl_2) and/or Cr(III) (as CrCl_3 dissolved in unbuffered distilled water). Additions were made with concentrated solutions to keep total volumes approximately constant. At set time intervals, aliquots of the incubations were removed with a syringe and filtered through 0.2 μm Anotop filters, removing the cells and Mn oxides and quenching the Cr(III) oxidation reaction.

Cr(III) Oxidation by Mn(III) Complexes. Five complexes were made from Mn(III) acetate dissolved in an aqueous solution containing excess ligand and a pH 7.5 buffer to minimize disproportionation. They ranged from citrate and bis tris, which were expected to be weaker than the SG-1-Mn(III) complex to pyrophosphate and pyoverdine, which have been shown to be able to remove Mn(III) from SG-1, and desferrioxamine, which would also be expected to be stronger than the SG-1-Mn(III) complex. These were allowed to react with 50 μM Cr(III) for approximately 4 h before Cr-(VI) produced was measured.

Cr(III) Oxidation by Colloidal MnO_2 . A solution which contained a leucoberbelin blue (LBB)-reactive oxidized Mn colloid that was trapped on a 0.02 μm filter, but passed through a 0.2 μm filter, was synthesized. These characteristics were consistent with the characterization of the colloidal oxide by Perez-Benito et al. (24). We added this solution in a range of concentrations to a premixed aliquot of distilled water, HEPES buffer, and 100 μM Cr(III). The solution was inverted, and a sample was taken and filtered through a 0.02 μm filter to quench the reaction. The sampling procedure took less than 10 seconds.

Mn and Cr Measurements. Aliquots were taken from the tissue culture bottles with syringes and filtered through 0.2 μm Anotop syringe filters (except in the abiotic experiments with colloidal Mn oxide, as described above). Mn(II) oxidation was measured as the loss of dissolved Mn as determined using the colorimetric formaldoxime assay (25). Leucoberbelin blue (LBB) was used to test for oxidized Mn(III,IV) (26). Cr(III) oxidation was measured by the production of Cr(VI) as determined by the colorimetric diphenylcarbazide assay (27). All colorimetric measurements were made using a Perkin-Elmer Lambda Bio 20 spectrophotometer, and calibrated using growth medium to account for background interference.

Results and Discussion

Initial experiments sought to test whether the biogenic manganese oxide produced by *Bacillus* sp. strain SG-1 was able to oxidize Cr(III) to Cr(VI) under conditions simulating seawater and how this oxidation compared to δ - MnO_2 , a synthetic analog of the biogenic oxide. Results were compared to δ - MnO_2 and to spores with no Mn(II) or Cr(III) added (Figure 1). In this experiment, initial rates of Cr(III) oxidation were fastest in the absence of preformed biogenic oxides, with 2 days of oxide accumulation oxidizing Cr(III) slightly slower and the bottle with 7 days of preformed biogenic oxides slower still. However, all of these biological systems oxidized Cr(III) at least twice as fast as the δ - MnO_2 . Interestingly, the bottle with no added Mn(II) also oxidized Cr(III) faster than the 100 μM δ - MnO_2 , signifying that the bacteria were closely involved in Cr(III) oxidation. The no Cr(III) biological control served to ensure that nothing in the spore preparation reacted with the reagents used to measure Cr(VI). After 2 days, the rates of Cr(III) oxidation slowed to very similar rates in all bottles. This change in rate was consistent when examined with more closely spaced time points (data not shown). The results from this experiment ruled out our initial hypothesis,

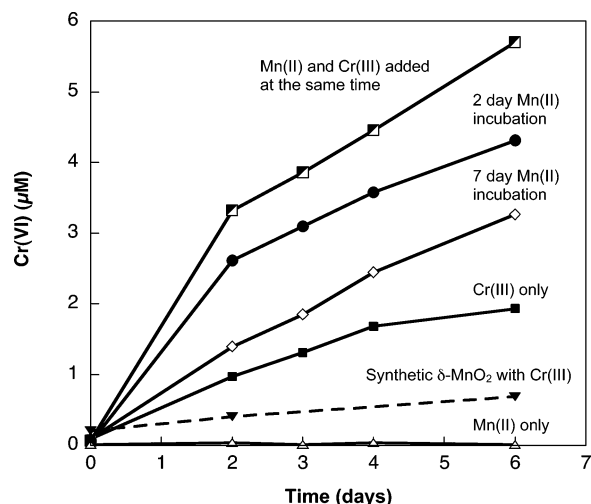


FIGURE 1. Effect of preformed Mn oxides on Cr(III) oxidation by SG-1. Half-filled squares represent 100 μM Cr(III) and 50 μM Mn(II) added at the same time; filled circles represent Mn(II) added 2 days before Cr(III); open diamonds represent Mn(II) added 7 days before Cr(III). Filled squares represent SG-1 with 100 μM Cr(III) and no added Mn(II), open triangles are 50 μM Mn(II) with no Cr(III). The dashed line is the reaction of 100 μM synthetic $\delta\text{-MnO}_2$ with Cr(III). All incubations were conducted in artificial seawater medium (ASW) buffered at pH 7.5 with 20 mM HEPES, at room temperature, and shaken on a rotary shaker at 150 rpm.

which was that the presence of preformed Mn oxides prior to the addition of Cr(III) would cause higher initial rates of oxidation of Cr(III) compared to conditions without, with the greater amount of preformed oxides (7 vs 2 d) oxidizing Cr(III) the fastest. This, along with the fact that all of the biological experiments were much faster than those with $\delta\text{-MnO}_2$, suggested that the increase in oxidation of Cr(III) in the presence of SG-1 can be attributed to more than just the reactivity of the biological oxides.

Several possible pathways could explain why Cr(III) oxidation is accelerated by SG-1. First, the Mn(II)-oxidizing enzyme of SG-1 may produce a very reactive form of oxidized Mn. This could be an enzyme-bound Mn(IV), a dissolved reactive Mn(IV), or a very small particle of MnO_2 . A second option is that a Mn(III) intermediate in $\text{Mn(II)} \rightarrow \text{Mn(IV)}$ oxidation may be reactive toward Cr(III), similar to the Mn(III) in Mn oxide minerals. Finally, the enzyme may be nonspecific, oxidizing Cr(III) directly. The decrease in rates of Cr(III) oxidation in the presence of higher amounts of Mn oxide could be due to a physical blocking of the surface of the spore where Cr(III) is oxidized, similar to what has been shown for Mn oxidation by the spores (11).

Spot checks of the spores in the experiment presented in Figure 1 showed that the spore preparations contained some residual Mn in solution—approximately 0.5 μM —prior to the start of the experiment. This was residual from the growth medium or was released from the spores during spore preparation, since *Bacillus sp.* strain SG-1 requires relatively high amounts of Mn(II) for sporulation and accumulates it in the spore cortex. For all subsequent experiments, a second and third ascorbate wash and 5 additional MilliQ water washes were added to our spore preparation protocol. The resulting spore preparations had Mn concentrations in 0.2 μM filtrates of approximately 10–25 nM, as measured by ICP-OES.

The stringently cleaned SG-1 spores were incubated in HEPES-buffered ASW with a range of Cr(III) concentrations and 10 μM Mn(II). The rates of Mn(II) oxidation and Cr(VI) formation were measured. Cr(III) concentrations greater than 5 μM inhibited both Mn(II) and Cr(III) oxidation (Figure 2A),

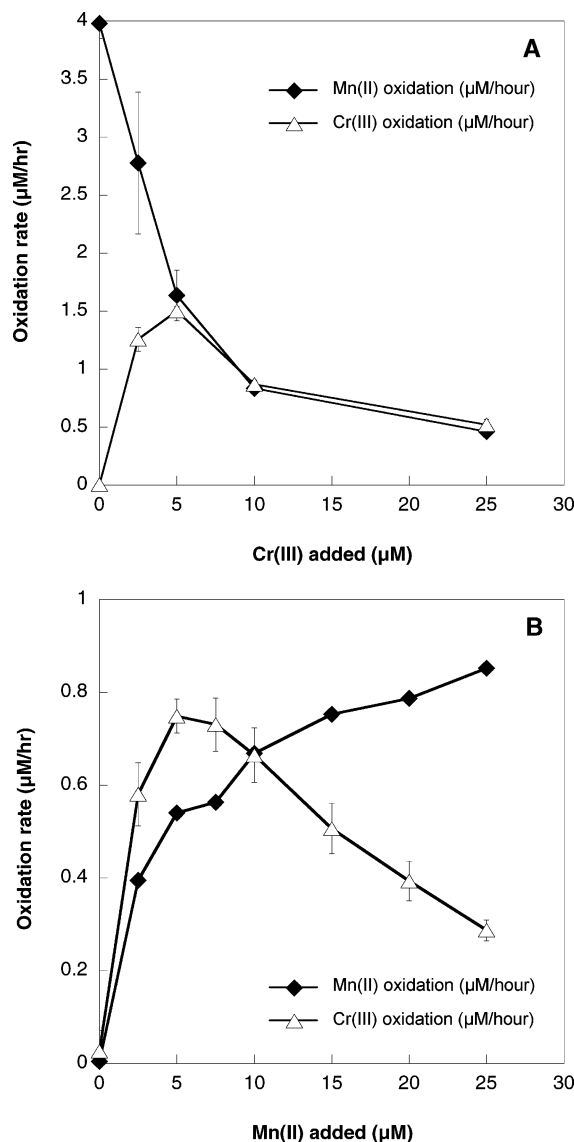


FIGURE 2. Changes in rates of Cr(III) and Mn(II) oxidation with Mn(II) and Cr(III) concentrations. (A) Cr(III) (open triangles) and Mn(II) oxidation (filled diamonds) are both inhibited with high amounts of Cr(III). (B) With increasing amounts of Mn(II), Cr(III) oxidation is inhibited but Mn(II) oxidation is not. Rates were determined from Mn(II) loss and Cr(VI) production over the first 2 h of incubation. All incubations were conducted in artificial seawater medium (ASW) buffered at pH 7.5 with 20 mM HEPES, at room temperature, and shaken on a rotary shaker at 150 rpm.

possibly by forming a precipitate or adsorbing to the surface and blocking the Mn(II)-oxidizing enzyme. Under all the conditions in this experiment, the solubility of Cr(III) was exceeded (28) and a visible precipitate formed upon addition to the experimental medium. There were no organic ligands in solution which would be expected to bind to and solubilize Cr(III). Over several hours, Cr(VI) continued to be produced indicating that more Cr(III) than should have been initially soluble was available to react, possibly through oxidative dissolution (29), or that the Cr(III) had not reached equilibrium during our experiments.

SG-1 spores were also incubated in buffered ASW with a range of Mn(II) concentrations and the initial (linear) rates of Cr(III) oxidation (Cr(III) added = 100 μM) measured (Figure 2b). The results showed an optimal Mn(II) concentration of approximately 5 μM , above and below which rates of Cr(III) oxidation were lower. The decrease in Cr(III) oxidation below

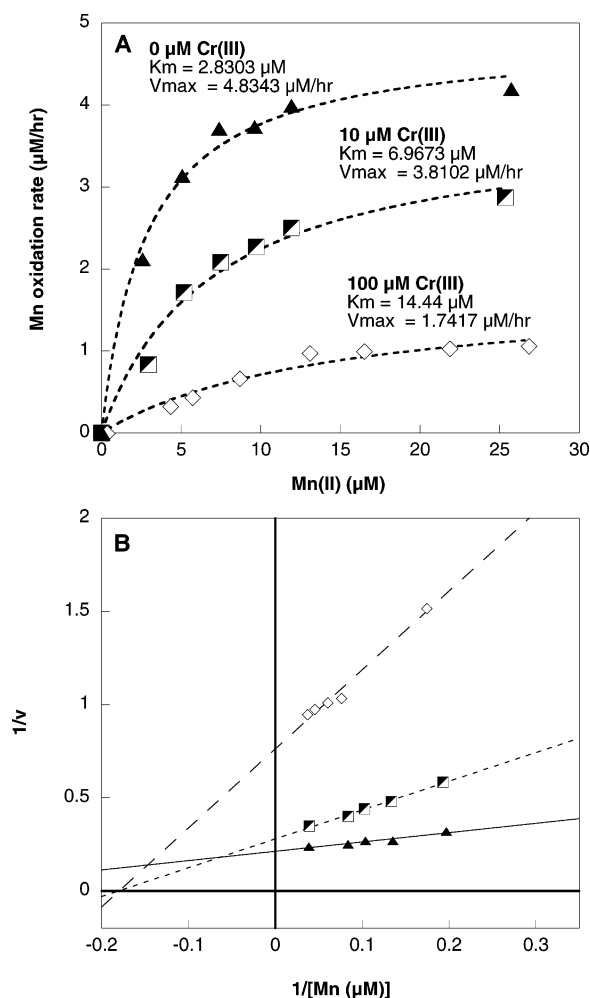


FIGURE 3. Inhibition of Mn(II) oxidation by three concentrations of Cr(III). (A) Curves of initial reaction velocities with Cr(III) = 0, 10, and 100 μM show Michaelis–Menten type kinetics and inhibition with increasing Cr(III). (B) Lineweaver–Burk linearizations of the data show changing intercept and slope, typical of noncompetitive inhibition. Rates were determined from Mn(II) loss or Cr(VI) production over the first 2 h of incubation. All incubations were conducted in artificial seawater medium (ASW) buffered at pH 7.5 with 20 mM HEPES, at room temperature, and shaken on a rotary shaker at 150 rpm.

5 μM Mn(II) shows that Mn(II) is required for Cr(III) oxidation, as opposed to Cr(III) being directly oxidized by the Mn(II)-oxidizing enzyme. Mutants in MnxG that do not oxidize Mn(II) do not oxidize Cr(III) either (data not shown), confirming the involvement of MnxG.

Because Cr(III) clearly had an effect on the rates of Mn(II) oxidation (Figure 2a) the kinetics of Mn(II) oxidation were measured at three different Cr(III) concentrations for a range of starting Mn(II) concentrations (Figure 3a). The resulting plots of initial Mn(II) oxidation velocities were fit to the Michaelis–Menten equation (dashed lines):

$$v = V_{\max}[S]/(K_m + [S]) \quad (1)$$

With increased Cr(III) concentration, the maximum velocity (V_{\max}) was decreased and the half saturation constant, K_m , was increased, indicating that Cr(III) was not simply competing with Mn(II) for the active site of the Mn(II)-oxidizing enzyme. Additionally, when Cr(III) oxidation rates were examined, high levels of Mn(II) inhibited this process (Figure 4). The same inhibition was not seen if all the Mn(II) was oxidized prior to the addition of Cr(III).

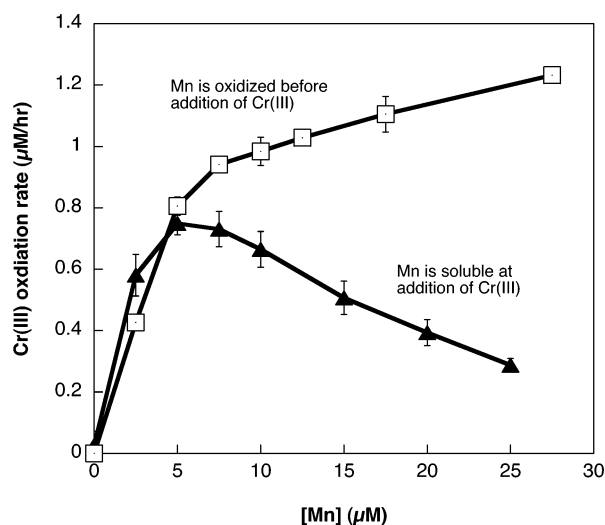


FIGURE 4. Inhibition of Cr(III) oxidation in the presence of SG-1 only occurs when the Mn is soluble (filled triangle). If all the Mn is oxidized prior to the start of the experiment (open squares), Cr(III) oxidation is not inhibited. Rates were determined from Mn(II) loss and Cr(VI) production over the first 2 h of incubation. All incubations were conducted in artificial seawater medium (ASW) buffered at pH 7.5 with 20 mM HEPES, at room temperature, and shaken on a rotary shaker at 150 rpm.

Based on experiments which recovered Mn(II)-oxidizing activity in a protein gel, the enzyme is thought to exist as a complex (18), leading to the possibility that Mn(II) could also act as a cofactor in addition to being a substrate. In this scenario, it is possible that both Cr(III) and Mn(II) bind to the enzyme at the same time. The Mn(II) may act to make the enzyme complex reactive toward Cr(III) or a second Mn(II). If this were the case, the Cr(III) should act competitively toward Mn(II), and high levels of Mn(II) would alleviate the inhibition of Mn(II) and Cr(III) oxidation caused by Cr(III). However, it can be seen that with 10 μM Cr(III), even much higher amounts of Mn(II) do not return the Mn(II) oxidation rates to the uninhibited state (Figure 3). Instead, Lineweaver–Burk plots showed changing slope and y-intercept (Figure 3b), trends typical of noncompetitive or mixed inhibition (30), which implies that the Cr(III) and Mn(II) are not simply competing for the same enzyme site.

A number of previous studies demonstrated a correlation between Mn(III) in a mineral and the ability of that mineral to oxidize Cr(III) (Table 1). Webb et al. showed that there is an Mn(III) intermediate during Mn(II) oxidation by SG-1, which can be trapped (12). SG-1 does not produce a solid-phase Mn(III) intermediate (31) and Mn(III) released into solution would not be stable with regard to disproportionation. However, Webb et al. (12) trapped the Mn(III) intermediate using pyrophosphate as a competing ligand, indicating that it remains bound to the enzyme before the second enzymatic oxidation step. To test whether an Mn(III) intermediate in Mn(II) oxidation by SG-1 could be responsible for Cr(III) oxidation, we prepared several different Mn(III) complexes with organic ligands. These Mn(III) complexes ranged from relatively weak (citrate) to very stable (pyoverdine) (Table 1). From the competition/trapping experiments of Webb et al. (12) we believe that these complexes should bracket the strength of the Mn(III)-enzyme complex in SG-1. From our results (Table 1), it does not appear that the Mn(III) from the enzymatic oxidation of Mn(II) by SG-1 is the likely cause of Cr(III) oxidation as none of the Mn(III) complexes oxidized Cr(III). This is consistent with Kim et al. (32) who did not find a correlation between Mn(III)

TABLE 1. Examination of Cr(III) Oxidation by Mn(III)

Mn(III) form	Cr(III) oxidation related to Mn(III)?	reference
synthetic δ -MnO ₂	yes—rates of Cr(III) oxidation slowed when pyrophosphate was used to chelate available Mn(III)	8 (Nico and Zasoski, 2000)
three synthetic minerals: birnessite, pyrolusite, hausmannite	yes—Cr(III) oxidation was fastest in the presence of minerals containing highest percentages of Mn(III)	7 (Chung et al., 1994)
seven Mn oxide minerals (six natural, one synthetic): hausmannite, manganite, romanechite, cryptomelane, lithiophorite, pyrolusite, synthetic birnessite	yes—Cr(III) oxidation rates correlate to high Mn(III) concentrations	35 (Weaver and Hochella, 2003)
Mn(III) (pH 3, aqueous)	no	33 (Weaver et al., 2002)
Mn(III)-PVD (pH 7.5, aqueous, excess ligand)	no	this study
Mn(III)-DFO (pH 7.5, aqueous, excess ligand)	no	this study
Mn(III)-pyrophosphate (pH 7.5, aqueous, excess ligand)	no	this study
Mn(III)-citrate (pH 7.5, aqueous, excess ligand)	no	this study
Mn(III)-Bis Tris (pH 7.5, aqueous, excess ligand)	no	this study

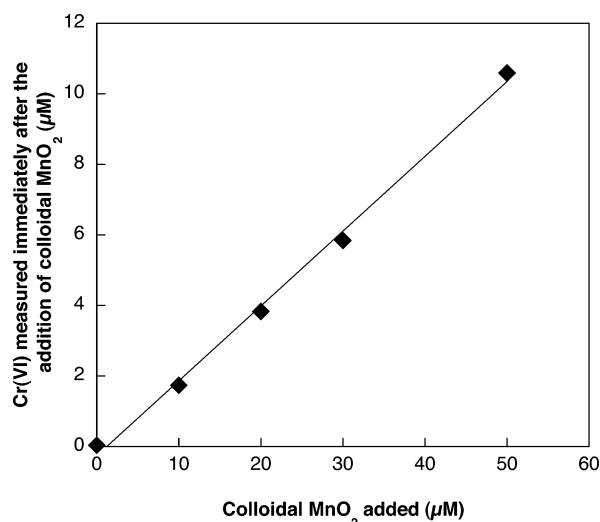


FIGURE 5. Cr(VI) produced by colloidal MnO₂ within the first 10 s of reaction with 100 μ M Cr(III) as a function of added MnO₂. Experiments were conducted in 20 mM HEPES buffer.

minerals and rates of Cr(III) oxidation and Weaver et al. (33) who showed that Mn(III) (uncomplexed) was not able to oxidize Cr(III) in aqueous solution until disproportionation began to occur. Although aqueous Mn(III) previously has been shown to oxidize Cr(III) (34), the acidic conditions used were not comparable to those found in our system (or most natural environments), and oxidation could have been due to Mn(IV) produced during disproportionation. The correlation between Mn(III) in minerals and Cr(III) oxidation could be due to structural deformities in the oxide surfaces leading to more reactive sites for Cr(III) oxidation (35).

The small size and relative lack of order in the biooxide could make the enzymatically formed Mn(IV) extremely reactive toward Cr(III). Small particles, such as the biooxide, or colloidal MnO₂ would have a relatively high surface area and be less limited by diffusion, leading to increased probability of contact between Cr(III) and the oxidized Mn and faster Cr(III) oxidation. In this case, the appropriate model compound for the reactivity of biological manganese oxides toward Cr(III) may not be the somewhat structured mineral δ -MnO₂, but a more reactive form. In experiments with a range of colloidal MnO₂ concentrations, approximately 20% of the Cr(III) was oxidized before the measurement could be made (Figure 5). This oxidized fraction was proportional to the MnO₂ concentration, suggesting that some fraction of the colloidal oxide was particularly reactive. After this initial timepoint, it was observed that the oxide was no longer

colloidal (brown Mn-oxide particles could be seen, and the LBB-reactive fraction was completely trapped on a 0.2 μ m filter) so no time course measurements could be made. The precipitation of the colloid was likely due to the higher ionic strength of the experimental solution and possibly due to the release of Mn(II) as the Mn oxide was reduced (36). The high reactivity of the colloid makes this a likely mechanism for the high rates of Cr(III) oxidation seen in the presence of SG-1 (and relative absence of oxidized Mn). It seems reasonable that the presence of an active Mn(III)-oxidizing enzyme functions to replenish the pool of small, reactive MnO₂ that is available to oxidize Cr(III) or Mn(II).

As the amount of Mn oxides increases, there could be several mechanisms that contribute to the slowed oxidation of Cr(III) seen at high levels of Mn(II) and longer time periods. First, as the biogenic Mn oxide begins coating the spores it could physically obstruct the enzyme, and the Cr(III) would be prevented from coming in contact with the reactive Mn(IV). Also, the formation of a mineral phase of Mn(IV) would lead to the abiotic oxidation of Mn(II) on the surface of the oxides. This could both slow the rate of enzymatic oxidation of Mn(II) (leading to less production of the reactive biooxide) and provide more surface area to which Cr(III) could adsorb, thereby decreasing the Cr(III) available for oxidation by the reactive fraction of the Mn oxides.

Initial experiments showed that the biological Mn(III,IV) oxide produced by *Bacillus* sp. strain SG-1 oxidized Cr(III) faster than the synthetic δ -MnO₂. The presence of Mn(II)-oxidizing bacteria further increased these rates through the production of very reactive Mn oxides or intermediates formed in the oxidation process. In this defined artificial seawater medium, SG-1 can recycle Mn quickly to oxidize large amounts of Cr(III) (e.g., Figure 1), which supports the idea that the presence of Mn(II)-oxidizing bacteria should be considered when determining the environmental fate of Cr—even when only trace amounts of Mn are present. In environmental systems which contain many more potential organic ligands for the Cr(III), Cr oxidation could be even faster, similar to what was seen with studies of *Pseudomonas* in two different freshwater media (15). Mn(II)-oxidizing bacteria are found in a variety of environments, and can oxidize Mn(II) even when levels of O₂ are extremely low (37), so any Cr(III) that is present has the potential to be rapidly, though indirectly, oxidized by these bacteria.

Acknowledgments

This research was supported by a Collaborative Research Activities in Environmental Molecular Sciences (CRAEMS) grant (CHE 0089208) from the National Science Foundation and the National Institute of Environmental Health Sciences

Superfund Basic Research Program (Grant ES10337 to the University of California San Diego). K.J.M. was supported in part by a STAR Graduate Fellowship from the U.S. Environmental Protection Agency and by the University of California Toxic Substances Research and Teaching Program. We thank Craig Anderson for comments on the final manuscript.

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Received for review June 26, 2006. Revised manuscript received October 16, 2006. Accepted October 30, 2006.

ES0615167