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Production of Manganese Oxide Nanoparticles by *Shewanella* Species

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ABSTRACT

Several species of the bacterial genus *Shewanella* are well-known dissimilatory reducers of manganese under anaerobic conditions. In fact, *Shewanella oneidensis* is one of the most well studied of all metal-reducing bacteria. In the current study, a number of *Shewanella* strains were tested for manganese-oxidizing capacity under aerobic conditions. All were able to oxidize Mn(II) and to produce solid dark brown manganese oxides. *Shewanella loihica* strain PV-4 was the strongest oxidizer, producing oxides at a rate of 20.3 mg/liter/day and oxidizing Mn(II) concentrations of up to 9 mM. In contrast, *S. oneidensis* MR-1 was the weakest oxidizer tested, producing oxides at 4.4 mg/liter/day and oxidizing up to 4 mM Mn(II). Analysis of products from the strongest oxidizers, i.e., *S. loihica* PV-4 and *Shewanella putrefaciens* CN-32, revealed finely grained, nanosize, poorly crystalline oxide particles with identical Mn oxidation states of 3.86. The biogenic manganese oxide products could be subsequently reduced within 2 days by all of the *Shewanella* strains when culture conditions were made anoxic and an appropriate nutrient (lactate) was added. While *Shewanella* species were detected previously as part of manganese-oxidizing consortia in natural environments, the current study has clearly shown manganese-reducing *Shewanella* species bacteria that are able to oxidize manganese in aerobic cultures.

IMPORTANCE

Members of the genus *Shewanella* are well known as dissimilatory manganese-reducing bacteria. This study shows that a number of species from *Shewanella* are also capable of manganese oxidation under aerobic conditions. Characterization of the products of the two most efficient oxidizers, *S. loihica* and *S. putrefaciens*, revealed finely grained, nanosize oxide particles. With a change in culture conditions, the manganese oxide products could be subsequently reduced by the same bacteria. The ability of *Shewanella* species both to oxidize and to reduce manganese indicates that the genus plays a significant role in the geochemical cycling of manganese. Due to the high affinity of manganese oxides for binding other metals, these bacteria may also contribute to the immobilization and release of other metals in the environment.

Members of the genus *Shewanella* are facultatively anaerobic, Gram-negative bacteria that are found in a wide range of environments but predominately in marine sediments and in association with fish (1, 2). The genus comprises mesophiles, psychrotrophs, and psychrophiles, some of which have been the subject of detailed studies due to their ability to use a wide range of electron acceptors in anaerobic respiration processes (1, 3). In particular, numerous *Shewanella* species are capable of reducing metals such as Mn(IV), Fe(III), V(V), Cr(VI), and U(VI), coupled to oxidation of organic and inorganic compounds (4–7).

Metal-reducing and metal-oxidizing bacteria are well recognized as playing important roles in the cycling of metals and organic matter in many environments (8–11). The majority of studies of metal reducers have centered on members of the *Shewanella* and *Geobacter* genera, even though metal reducers are generally a phylogenetically diverse group of bacteria. Fe(III) and Mn(IV) reducers like *Shewanella oneidensis* have been investigated extensively, due to their importance in carbon turnover in anoxic environments and their potential in biotechnology processes such as bioremediation (12–14). A wide range of Fe(III) and Mn(IV) forms, from soluble chelated types to poorly crystalline solid minerals, can be both reduced and produced by bacteria, depending on culture conditions. Electron transfer mechanisms in reductive processes have been investigated in detail, revealing a variety of mechanisms that can involve *c*-type cytochromes, extracellular electron shuttles, and direct interspecies electron transfer (15, 16).

Manganese exists as soluble or particulate Mn(II), Mn(III), or Mn(IV) compounds, with prevailing states being influenced by redox conditions and the presence of manganese-transforming microorganisms (17, 18). Understanding the role of manganese-transforming microorganisms in environmental manganese cycling is important because manganese is the second most abundant metal in the earth's crust, directly influences the cycling of other elements, and is an essential trace element for all living organisms. Similar to reductive processes, bacteria are known to be major contributors to manganese oxidation. The oxidation of Mn(II) to Mn(III/IV) is carried out by both phylogenetically diverse bacteria and fungi originating in a wide range of environments (18).

Numerous studies have indicated the requirement for multicopper oxidase (MCO)-like enzymes in the oxidation of Mn(II) (19, 20). Several model bacteria have been studied in detail, revealing the involvement of genes encoding MCOs, *mnxG* in spores of

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Bacillus strain SG-1 (21), *cumA* in *Pseudomonas putida* GB-1 (22), *mofA* in *Leptothrix discophora* SS-1 (23), and *moxA* in *Pedomicrobium* sp. ACM 3067 (24). In addition, laccases and peroxidases have been found to oxidize Mn(II) in fungi and several other bacteria (25–27).

In addition to their involvement in manganese cycling, Mn(II)-oxidizing bacteria are thought to have contributed in large part to the formation of natural manganese deposits around the world, including deep-sea manganese nodules (28, 29) and ore bodies (30, 31). Biogenic manganese oxides also have considerable potential biotechnological applications. The disordered oxide structures, with defects and cation vacancies, can act as depolarizers in electrochemical cells (32). The high affinity of biogenic manganese oxides for binding metals has led to the proposal of such oxides as a means of bioremediating metal contamination in waters and wastewaters (33–36). The current study extends the versatility of *Shewanella* species and describes manganese oxidation as a trait within the genus. Rates of oxidation, Mn(II) toxicity, and characteristics of the products were determined in the current study.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used for the current study were *Shewanella oneidensis* MR-1, *Shewanella putrefaciens* CN-32, *Shewanella putrefaciens* 200, *Shewanella loihica* PV-4, and *Shewanella denitrificans* OS217. Isolates were kindly provided by Kenneth Nealson at the University of Southern California. All strains are psychrotrophic facultative anaerobes and were maintained aerobically in PYE medium (37); PYE medium contained 1.0 g/liter peptone, 1.5 g/liter yeast extract, 7.5 g/liter NaCl, and 1.0 g/liter $(NH_4)_2SO_4$ in distilled H_2O, with 10 mM HEPES buffer (pH 7.5) added after autoclaving. All cultures were incubated on an orbital shaker (120 rpm) under aerobic conditions unless otherwise stated and were grown at 27°C.

Oxidation-reduction experiments. For manganese oxidation testing, the *Shewanella* strains were grown to mid-late log phase and 1 ml of each culture, diluted to an optical density at 600 nm (OD_{600}) of 0.1, was inoculated into a 100-ml volume of PYE medium amended with 3 mM $MnCl_2 \cdot 4H_2O$, in 250-ml Erlenmeyer flasks, and incubated at 27°C. Cultures were sampled after 0, 1, 2, 3, 4, 5, 7, 10, 15, and 20 days. Rates of oxide production were determined by measuring the amount of oxide produced at each time point. The extent of oxidation was the conversion of Mn(II) to Mn(IV) at each sampling time. Cell numbers were determined using the most probable number technique. Controls were set up as uninoculated medium and inoculated medium with 15 mM NaN_3 added. Controls were sampled for abiotic oxidation at the same time as inoculated cultures. All tests were done in triplicate. After 20 days of incubation, the manganese oxide products were recovered and analyzed. In separate cultures, all seven *Shewanella* strains were grown for 20 days in PYE medium amended with 3 mM Mn(II). After the manganese oxide was produced, cultures were tested to determine whether the bacteria were able to reduce the oxides. The cultures were transferred aseptically to sterile 125-ml serum bottles, 15 mM sodium lactate was added, and anoxic conditions were created by gassing the cultures and headspaces with sterile N_2. Manganese reduction was monitored, and the time taken for complete disappearance of the brown color was noted and confirmed using the leucoberbelin blue assay (38).

The effects of the initial Mn(II) concentration on oxidation by each of the *Shewanella* strains were tested in PYE medium amended with 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mM $MnCl_2 \cdot 4H_2O$. The bacteria were grown to mid-late log phase in PYE medium and 1 ml of each culture was inoculated into a 100-ml volume of PYE medium amended with the appropriate concentration of Mn(II), in 250-ml Erlenmeyer flasks. After 20 days of incubation, the amount of manganese oxide produced was measured.

Recovery of oxide products. The dark brown-black oxides produced were recovered by filtration. The solids were washed once in distilled water and then three times in dilute 0.03 M H_2SO_4, to remove inorganic residual matter as well as residual Mn(II) that might interfere with further analyses. The oxides were then washed with deionized water until filtrates were acid free.

Physical analyses. Fourier transform infrared (FTIR) spectroscopy (Spectrum Two FTIR spectrometer; PerkinElmer) was used to tentatively characterize biogenic oxides by comparison with standard analytical reagent-grade MnO_2 (Sigma-Aldrich). The FTIR spectroscopy technique for characterization of manganese oxides was well documented in previous studies (39–41).

A JSM-6510LV scanning electron microscope (JEOL USA) was used to visualize the biogenic oxides and analytical reagent-grade MnO_2, to compare the structures and to visualize detectable bacteria in the biological samples. Samples were processed with the assistance of Glenn Walker at the Australian National Fabrication Facility, Griffith University (Brisbane, Australia).

Chemical analyses. The presence of Mn(IV) was detected using the leucocrystal violet assay (42) and the leucoberbelin blue assay (38). Aliquots of 0.1 ml were taken, diluted as necessary, and analyzed using a Shimazdu UV-2550 UV spectrometer, at the appropriate wavelength for each assay. Absorbance values were plotted against a standard curve of known concentrations of potassium permanganate.

The O/Mn ratio of manganese oxide products was determined according to a modified iodometric method described by Murray et al. (43). Approximately 10 mg of each preprepared sample was weighed into a 50-ml beaker. A composite mixture of 10 ml demineralized water, 1 ml 20% (vol/vol) H_2SO_4, and 1 ml alkaline NaI solution (comprising 32 g NaOH and 60 g NaI in 100 ml demineralized water) was added to the sample and left for 18 h. Following this, the sample was filtered into a 100-ml beaker using a Millex GS 0.45-μm microfilter. A 1-ml aliquot of 1% (wt/vol) starch solution was added to the filtrate. To determine the total oxidizing equivalents, the I_2 liberated from each sample was titrated with 5 mM sodium thiosulfate, using a 50-ml burette. The titration was deemed complete when the blue color dissipated. The solution was brought up to 100 ml with demineralized water in a volumetric flask, and the manganese concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 8300 ICP-OES system; PerkinElmer). The average oxidation state was calculated and presented as MnO_x, where x is the aggregate average across all oxide states.

RESULTS

Manganese oxidation by *Shewanella* species. Oxidation of Mn(II) was investigated in five strains of *Shewanella* at 27°C (Table 1). All strains tested were able to oxidize Mn(II) to Mn(IV) under aerobic conditions, producing finely grained dark brown precipitates. The rates of oxidation varied considerably, with *S. loihica* being the most efficient and producing oxide at an average of 20.3 mg/liter/day. This was followed by the *S. putrefaciens* strains CN-32 and 200, which produced 13.9 and 15.9 mg/liter/day, respectively, and *S. denitrificans*, which produced 8.7 mg/liter/day. The least efficient oxidizer was the extensively studied metal reducer *S. oneidensis* MR-1, which produced 4.4 mg/liter/day. The maximum concentration of Mn(II) oxidized also varied between species. Generally, bacteria that oxidized higher maximum concentrations of Mn(II) were more rapid oxidizers of manganese; for example, *S. loihica* oxidized Mn(II) concentrations up to 9 mM but *S. oneidensis* only up to 4 mM. In addition, the efficiency of oxidation by *S. oneidensis* decreased substantially at concentrations above 1 mM. Figure 1 shows the optimal concentration of Mn(II) oxidized by the four *Shewanella* species. *S. loihica* and *S. putrefaciens* had optima around 3 to 5 mM, *S. deni-*

TABLE 1 Manganese-oxidizing properties of *Shewanella* strains[a]

Species and strain	Maximum Mn(II) concentration (mM)[b]	Rate of oxide production (mg/liter/day)[c]	Reduction of bioformed oxide[d]
S. denitrificans OS217	7	8.7 ± 2.7	+
S. loihica PV-4	9	20.3 ± 1.6	+
S. oneidensis MR-1	4	4.4 ± 1.2	+
S. putrefaciens 200	7	15.9 ± 3.9	+
S. putrefaciens CN-32	8	13.9 ± 3.1	+

[a] Cultures were incubated at 27°C.

[b] Mn(II) concentration at which complete inhibition of oxidation occurred.

[c] Oxidation at 3 mM Mn(II) (average of replicates ± standard deviation).

[d] Cultures were switched to anoxic conditions and replenished with 15 mM lactate.

trificans 2 to 4 mM, and *S. oneidensis* ≤1 mM. After oxidation occurred, the *Shewanella* species were tested, under anaerobic conditions, to determine whether they could reduce the preformed manganese oxides. In all cases, the oxides were reduced rapidly and completely within 2 days.

Figure 2 shows the time courses of oxidation and cell growth for two of the best oxidizers under the growth and incubation conditions used, namely, *S. putrefaciens* CN-32 and *S. loihica* PV-4. Log-phase growth for both bacteria commenced after 2 to 3 days; oxide formation began shortly thereafter and continued for several days, into the stationary phase of growth. Most oxide production by *S. putrefaciens* occurred after rapid growth began to slow, while oxide production was more closely aligned with growth for *S. loihica*. For both bacteria, oxide production slowed when around 70 to 90% of Mn(II) had been converted to an oxide. In a separate experiment, both *S. putrefaciens* and *S. loihica* were again shown to oxidize manganese (Fig. 3). Once oxidation had ceased after 10 days, cultures were transferred to anaerobic vessels, gassed with N_2, and sealed, and 15 mM lactate was provided. The bioformed manganese oxide products were reduced rapidly by the bacteria. After 1 to 2 days of incubation, there was no oxide evident in the cultures and there were concomitant increases in cell numbers.

Manganese oxide characterization. The manganese oxide products formed by *S. putrefaciens* CN-32 and *S. loihica* PV-4 were characterized. FTIR spectra revealed that both oxides had a major broad peak at around 530 wavenumbers (Fig. 4), which was typical of microbially formed disordered MnO_2 (32). Additional smaller peaks occurred at 1,600 and 1,000 wavenumbers, which were consistent with organic contamination (bacterial cells) and inorganic compounds such as medium precipitates. The control, i.e., standard analytical reagent-grade MnO_2, had a similarly broad peak at a slightly lower wavenumber, which was consistent with natural MnO_2 ores (44). Chemical analysis of oxide products revealed that, for both bacteria, the manganese had an oxidation state of 3.86 (Table 2), equating to a manganese oxide of $MnO_{1.93}$. The products were 92.0% and 91.6% manganese oxide for *S. putrefaciens* CN-32 and *S. loihica* PV-4, respectively. The non-manganese oxide component remaining was likely a combination of organic and inorganic impurities, as reflected in the FTIR spectra.

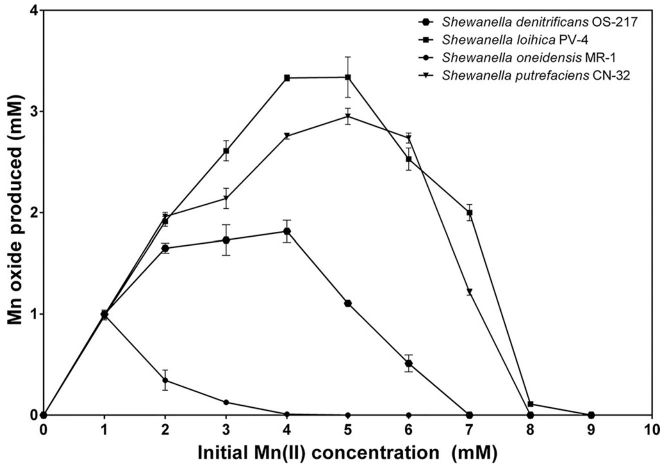

FIG 1 Optimal concentrations of Mn(II) oxidized by the *Shewanella* species *S. loihica*, *S. putrefaciens*, *S. oneidensis*, and *S. denitrificans*. No manganese oxides were formed in sterile controls or in poisoned controls with any of the *Shewanella* species.

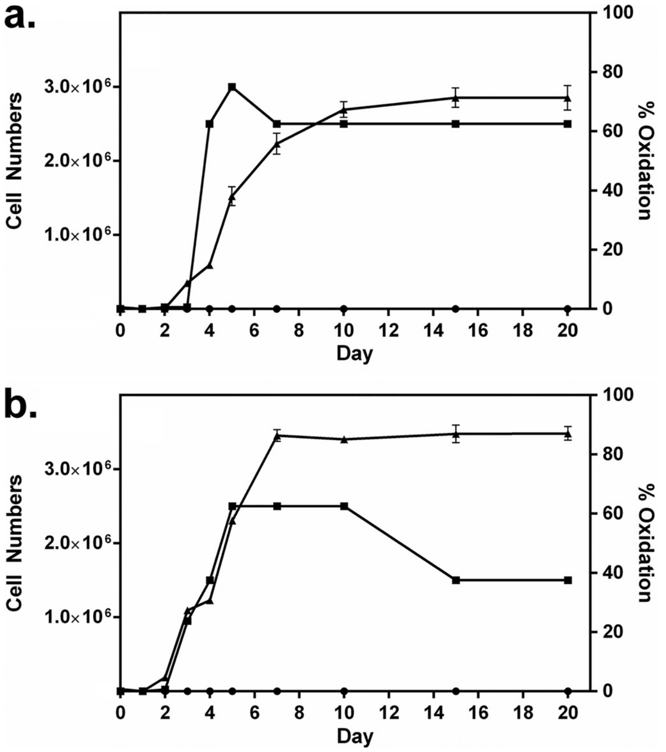

FIG 2 Extent of manganese oxidation (▲) and increases in cell numbers (cells per milliliter) (■) over time for *Shewanella putrefaciens* CN-32 (a) and *Shewanella loihica* PV-4 (b). Cultures were grown with 3 mM Mn(II). The extent of manganese oxidation in poisoned controls is also shown (●).

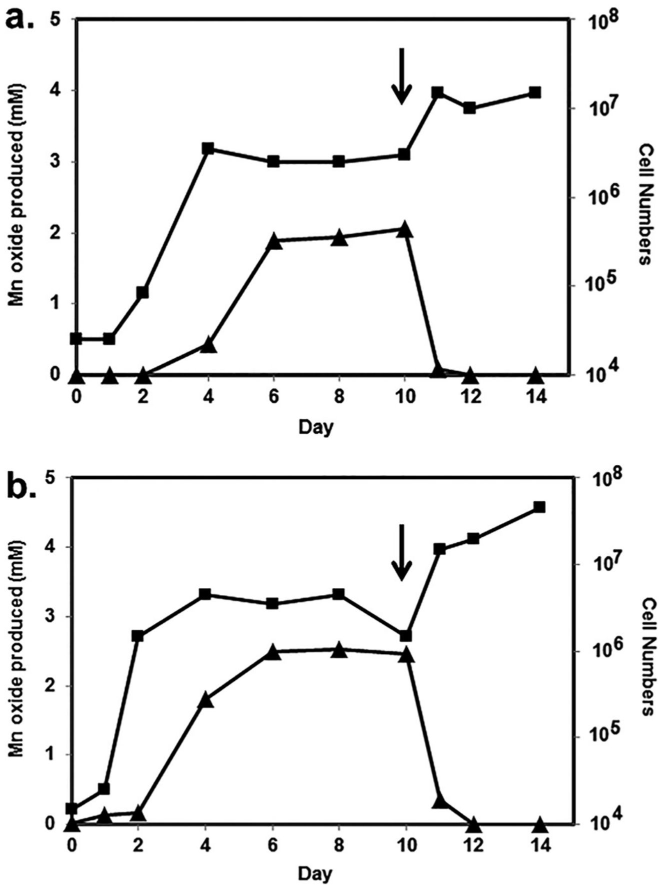

FIG 3 Manganese oxide formation (▲) and cell number (cells per milliliter) (■) variations over time for *Shewanella putrefaciens* CN-32 (a) and *Shewanella loihica* PV-4 (b), initially under aerobic conditions. After 10 days of incubation, conditions were changed to anaerobic (N_2 gas) with the addition of 15 mM lactate (arrows).

The oxidation state of the analytical reagent-grade oxide was very close to complete Mn(IV) at 3.98, equating to $MnO_{1.99}$.

Scanning electron microscopy revealed finely grained, nanosize, amorphous, round manganese oxide particles produced by both *Shewanella* strains (Fig. 5). Even after the precipitates were washed, bacteria were still seen clearly in the micrographs (Fig. 5, arrows). In contrast, the analytical reagent-grade MnO_2 particles were generally much larger crystalline particles, with no bacteria evident.

DISCUSSION

Bacteria play an important role in the cycling of manganese in a wide range of natural environments. The oxidation state of manganese in an environment is dependent on redox conditions, with oxidation being favored under oxic conditions and reduction being favored under anoxic conditions. Many bacteria are known either to oxidize or to reduce manganese, and there has been little investigation of bacteria capable of doing both. Members of the genus *Shewanella* have been known for some time to be involved in the anaerobic reduction of metals, including manganese, in a wide variety of environments (45). Unlike many dissimilatory metal-reducing bacteria, *Shewanella* species are able to use oxygen as a terminal electron acceptor in respiration. While manganese reduction in the *Shewanella* genus has been investigated extensively, the possibility of manganese oxidation has not been investigated in any detail. However, *Shewanella* species have been found as part of manganese-oxidizing communities and were recognized as being important in manganese cycling in the Columbia River in the United States (46, 47). Furthermore, Bräuer and colleagues (47) found that the manganese-oxidizing *Shewanella* isolates were most closely related to *S. denitrificans*, a moderate oxidizer in our studies. Similarly, manganese-oxidizing *Shewanella* isolates were found as part of the microbial community at Vailulu'u Seamount, Samoa (48). Blöthe and colleagues (49) found *Shewanella* isolates as dominant members of deep-sea manganese nodule communities and suggested that they had a key role in manganese cycling, including oxidation.

All *Shewanella* strains tested in the current study were able to oxidize manganese. The rates of oxidation by the *Shewanella* species compared favorably with those for species isolated previously from manganese and nonmanganese environments (40). In contrast to the current study, Chubar and colleagues (50) found that *S. putrefaciens* strain 200R precipitated Mn(II) as phosphates but not oxides. However, testing was performed in the absence of nutrients and cell growth. Studies in our laboratory have shown that the types and concentrations of nutrients directly influence the ability of the *Shewanella* strains to oxidize Mn(II) (A. C. Greene, unpublished data).

In the present study, *S. loihica* could oxidize Mn(II) concentrations up to 9 mM. Similarly, *Mesorhizobium australicum* strain T-G1 was found to oxidize Mn(II) concentrations up to 10 mM (51). In some of the earlier studies of manganese oxidation, Bromfield and David (52) found that an *Arthrobacter* soil isolate oxidized Mn(II) at concentrations up to 30 mM, with the maximum rate occurring between 0.5 and 6 mM. Xuezheng and coworkers (53) found several *Shewanella* isolates among 40 psychrotrophic and psychrophilic manganese bacteria isolated from the Arctic Ocean. The same authors found that the *Shewanella* species tolerated Mn(II) concentrations up to 10 mM with minimal inhibition.

Once the oxides had formed, changing conditions to anaerobic and providing an appropriate carbon source (15 mM lactate) resulted in all *Shewanella* species reducing these biogenic oxides. Indeed, the ability of *Shewanella* species not only to reduce and to oxidize manganese but also to readily utilize biogenic oxides as terminal electron acceptors indicates that the genus is likely to play a significant role in the geochemical cycling of the metal in environments that members inhabit. Several older studies reported bacteria that can both reduce and oxidize manganese. Bromfield and David (52) found that their *Arthrobacter* strain oxidized manganese under aerobic conditions and reduced it in deep static cultures, which presumably created anaerobic conditions. Indeed, the most studied manganese oxidizer, *Bacillus* strain SG-1, was reported to reduce Mn(IV) as well (54). The difference with respect to the *Shewanella* species tested in our study is that, for *Bacillus* strain SG-1, the spores oxidized manganese whereas the vegetative cells reduced it.

Typically, bioformed manganese oxides are poorly crystalline (18, 32, 34, 55). Electron micrographs confirmed the precipitation of finely grained particles similar to those formed by other bacteria (55, 56). Furthermore, the relatively low O/Mn ratio suggests that the bioformed oxides from *Shewanella* species are consistent with poorly crystalline, disordered manganese oxides (32). Reduction

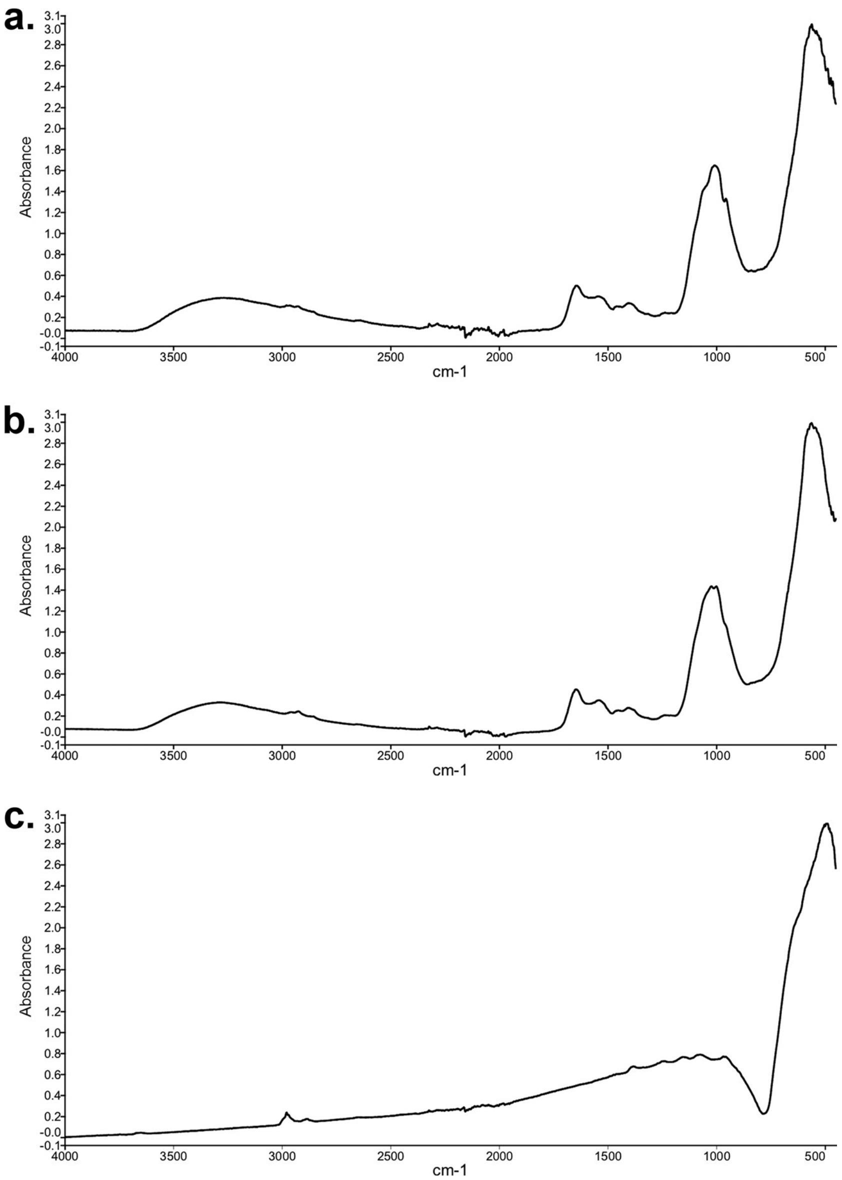

FIG 4 FTIR spectroscopy of manganese oxides formed by *Shewanella putrefaciens* CN-32 (a), manganese oxides formed by *Shewanella loihica* PV-4 (b), and analytical reagent-grade MnO_2 (c).

of the bioformed manganese oxides by *Shewanella* species was quick, usually completed within 1 to 2 days. The high rate of reduction was consistent with the much higher rate of reduction of poorly crystalline manganese oxides, compared with natural crystalline oxides (57). In fact, Burdige and colleagues (58) found that *S. oneidensis* MR-1 reduced highly crystalline pyrolusite substantially more slowly than amorphous, structurally disordered δ-MnO_2 (vernadite). It is likely that the characteristics of the poorly crystalline oxides, including greater surface area and structural defects, allowed more rapid transfer of electrons.

TABLE 2 Characteristics of manganese oxides produced by *Shewanella putrefaciens* CN-32 and *Shewanella loihica* PV-4, compared with analytical reagent-grade MnO_2

Manganese oxide origin	Oxidation state	Manganese oxide content (%)
S. putrefaciens CN-32	3.86	92.0
S. loihica PV-4	3.86	91.6
Analytical reagent-grade MnO_2	3.98	100

The mechanisms of oxidation have been investigated for numerous manganese-oxidizing bacteria. Evidence suggests that multicopper oxidase enzymes mediate manganese oxidation, and genomic investigations have indicated that the presence of multicopper oxidases is important for oxidation to occur (19, 20, 59). Laccases belong to the multicopper oxidase family and have been found to be present in *S. putrefaciens* (60) and *S. oneidensis* (61). In fact, laccases from fungi have been shown to oxidize Mn(II) enzymatically (25). The reason why bacteria oxidize Mn(II) is not

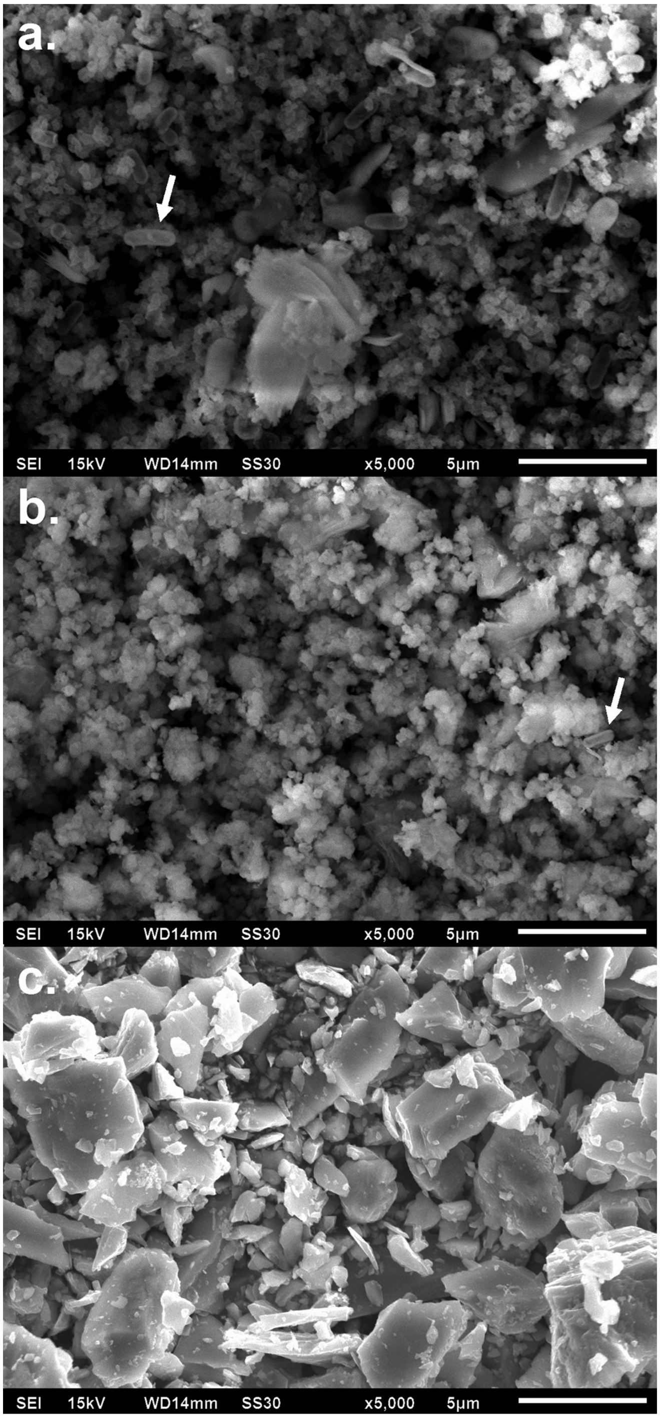

FIG 5 Scanning electron microscopy of manganese oxides formed by *Shewanella putrefaciens* CN-32 (a), manganese oxides formed by *Shewanella loihica* PV-4 (b), and analytical reagent-grade MnO_2 (c). Arrows indicate bacteria.

clear. Although it has long been suggested to be an energy-yielding process, no definitive evidence has ever been presented (19). It would make sense for *Shewanella* species to oxidize manganese as a strategy for storing electron acceptors that can be used when oxygen is depleted. Glasauer and colleagues (62) observed the synthesis of manganese nanogranules in the cytoplasm of *S. putrefaciens* during anaerobic growth.

The presence of Mn(II) and Mn(III/IV) in aquatic environments is largely influenced by interactions with the microbial flora and surrounding environmental conditions (57, 63). The ability of *Shewanella* species both to oxidize and to reduce manganese indicates that the genus plays a significant role in the geochemical cycling of the metal. Due to the facultative nature of the *Shewanella* species, it is likely that these bacteria contribute to the release of not only manganese but also other elements and metals in the environment, such as copper, cobalt, nickel, lead, iron, radium, uranium, and rare earth elements.

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