Production of manganese oxide nanoparticles by *Shewanella* species

Running title: Manganese oxidation 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 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 produce solid dark brown manganese oxides. *S. loihica* strain PV-4 was the strongest oxidizer, producing oxides at rates of 20.3 mg/L/day and oxidizing Mn(II) concentrations of up to 9 mM. In contrast, *S. oneidensis* MR-1 was the weakest oxidizer tested, producing 4.4 mg/L/day of oxide and oxidizing up to 4 mM Mn(II). Analysis of products from the strongest oxidizers, *S. loihica* PV-4 and *S. putrefaciens* CN-32, revealed finely grained nano-sized and poorly crystalline oxide particles with identical Mn oxidation states of 3.86. The biogenic manganese oxide products could subsequently be 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 have been detected as part of manganese oxidizing consortia in natural environments previously, the current study is the first clear instance of manganese reducing *Shewanella* species being able to oxidize manganese in aerobic cultures.

Importance

Members of the genus *Shewanella* are well known as dissimilatory manganese reducing bacteria. This study shows for the first time that a number of species from *Shewanella* are also capable of manganese oxidation under aerobic conditions. Characterisation of the products of the two most efficient oxidizers, *S. loihica* and *S.
*putrefaciens* revealed a finely grained nano-sized oxide. By changing culture conditions, the manganese oxide products could be subsequently reduced by the same bacteria. The ability of *Shewanella* species to both oxidize and reduce manganese indicates that the genus plays a significant role overall 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.
Introduction

Members of the genus *Shewanella* are facultatively anaerobic, Gram-negative bacteria 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 -oxidizing bacteria are well recognised 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 centred 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 potential in biotechnology processes such as bioremediation (12–14). A wide range of Fe(III) and Mn(IV) forms ranging from soluble, chelated types through to poorly crystalline solid minerals can be both reduced and produced by bacteria depending on culture conditions. In reductive processes, electron transfer mechanisms have been investigated in detail and revealed 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 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 as
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. Like 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 of 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 coding MCOs, mnxG in spores of
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).

Besides their involvement in manganese cycling, Mn(II) oxidizing bacteria are
believed to have contributed in a 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 their proposal 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. The rate of oxidation, Mn(II) toxicity and characteristics of 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 Professor Kenneth Nealson from the University of Southern California. All strains are facultative anaerobes, psychrotrophic and were maintained aerobically on PYE medium (37). PYE medium contained (per litre of distilled H2O): 1.0 g/L peptone, 1.5 g/L yeast extract, 7.5 g/L NaCl and 1.0 g/L (NH4)2SO4, and 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 grown at a temperature of 27°C.

Oxidation-reduction experiments. For manganese oxidation testing, the *Shewanella* strains were first grown to mid-late log phase and 1 mL of each culture, diluted to an OD_{600} of 0.1, was inoculated into a 100 mL volume of a PYE medium amended with 3 mM MnCl₂·4H₂O 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 interval. 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 media and inoculated media with 15 mM NaN₃ added. Controls were sampled for abiotic oxidation at the same time as inoculated media with 15 mM NaN₃ added. Controls were sampled for abiotic oxidation at the same time as inoculated...
cultures. All tests were done in triplicate. After 20 days incubation, the manganese oxide products were recovered and analysed. In separate cultures, all seven Shewanella strains were grown in PYE medium amended with 3 mM Mn(II) for 20 days. After the manganese oxide was produced, cultures were tested to determine if the bacteria were subsequently 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 culture and headspace with sterile N₂. Manganese reduction was monitored and the time taken for complete disappearance of the brown colour was noted and confirmed using the leucoberbelin blue assay (38).

The effects of initial Mn(II) concentration on oxidation by each of the Shewanella strains was tested in PYE medium amended with concentrations of either 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mM MnCl₂·4H₂O. The bacteria again were first grown to mid-late log phase in PYE medium and 1 mL of each culture was inoculated into a 100 mL volume of a PYE medium amended with the appropriate concentration of Mn(II) in 250 mL Erlenmeyer flasks. After 20 days 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 H₂SO₄ to remove inorganic residual matter as well as residual Mn(II) that may interfere with further analyses. The oxides were then washed with deionised water until filtrates were acid free.

**Physical analyses.** Fourier transform infrared (FTIR) spectroscopy (Spectrum 2 System FTIR spectrometer, Perkin Elmer) was used to tentatively characterize
biogenic oxides by comparison with standard analytic grade MnO2 (Sigma-Aldrich).

FTIR spectroscopy technique for characterization of manganese oxides is well
documented in previous studies (39–41).

A JSM-6510LV Scanning Electron Microscope (JEOL USA) was used to visualise
the biogenic oxides and analytical MnO2 to compare the structures as well as visualise
detectable bacteria within the biological samples. Samples were processed with the
assistance of Mr Glenn Walker of the Australian National Fabrication Facility,
Griffith University, Brisbane, Australia.

Chemical analyses. Presence of Mn(IV) was detected using the leucocystal violet
assay (42) and leucoberbelin blue assay (38). Aliquots of 0.1 mL were taken, diluted
as necessary and analysed using a Shimadzu UV-2550 UV spectrometer at the
appropriate wavelength for each assay. Absorbance was 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 of Murray et al. (43). Approximately 10 mg of each pre-prepared
sample were weighed out into a 50 mL beaker. A composite mixture of 10 mL
demineralised water, 1 mL 20% v/v H2SO4 and 1 mL alkaline NaI solution
(comprising 32 g NaOH and 60 g NaI in 100 mL demineralised water) was added to
the sample and left for 18 hours. Following this, the sample was filtered into a 100
mL beaker using a Millex GS 0.45 µm micro filter. A 1 mL aliquot of 1% (w/v) starch
solution was added to the filtrate. To determine the total oxidizing equivalents, the
liberated I2 from each sample was titrated with 5 mM sodium thiosulphate using a 50
mL burette. The titration was deemed complete when the blue colour dissipated. The
solution was brought up to 100 mL in a volumetric flask with demineralised water and
the manganese concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 8300 ICP-OES, Perkin Elmer). The average oxidation state was calculated and presented as MnOx where x was 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* the most efficient producing oxide at an average of 20.3 mg/L/day. This was followed by the two *S. putrefaciens* strains CN-32 and 200, which produced 13.9 and 15.9 mg/L/day, respectively, and *S. denitrificans* at 8.7 mg/L/day. The least efficient oxidizer was the extensively studied metal reducer, *S. oneidensis* MR-1 producing 4.4 mg/L/day. The maximum concentration of Mn(II) oxidized also varied between species. Generally bacteria oxidizing higher maximum concentrations of Mn(II), were the more rapid oxidizers of manganese, for example *S. loihica* oxidized Mn(II) concentrations up to 9 mM while *S. oneidensis* only up to 4 mM. In addition, the efficiency of oxidation by *S. oneidensis* dropped off 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 3-5 mM, *S. denitrificans* at 2-4 mM and *S. oneidensis* at or below 1 mM. After oxidation occurred, the *Shewanella* species were tested, under anaerobic conditions, to see if they could reduce the pre-formed
manganese oxides. In all cases, the oxides were reduced rapidly and completely within 2 days.

Figure 2 shows the time course of oxidation and cell growth for two of the best oxidizers under the growth and incubation conditions used, (a) *S. putrefaciens* CN-32 and (b) *S. loihica* PV-4. Log phase of growth for both bacteria commenced after 2-3 days and oxide formation occurred shortly after and continued for several days into the stationary phase of growth. With *S. putrefaciens*, most oxide production occurred after rapid growth began to slow down while oxide production was more closely aligned to the growth of *S. loihica*. With both bacteria, oxide production slowed when around 70-90% of Mn(II) had been converted to an oxide. In a separate experiment both *S. putrefaciens* and *S. loihica* again shown to oxidize manganese (Figure 3).

Once oxidation had ceased after 10 days, cultures were transferred to anaerobic vessels, gassed with N₂, sealed and 15 mM lactate was provided. The bioformed manganese oxide products were reduced rapidly by the bacteria. After 1-2 days incubation, there was no oxide evident in the cultures and there was a concomitant increase in cell numbers.

**Manganese oxide characterisation.** The manganese oxide products formed by *S. putrefaciens* CN-32 and *S. loihica* PV-4 were characterised. FTIR spectra (Figure 4) revealed that both oxides had a broad major peak at around 530 wavenumbers which was typical of microbially-formed disordered MnO₂ (32). Additional smaller peaks occurred at 1600 and 1000 wavenumbers which were consistent with organic contamination (bacterial cells) and inorganic compounds such as medium precipitates.

The control, standard analytical-grade MnO₂, had a similarly broad peak though at a slightly lower wavenumber which was consistent with natural MnO₂ ores (44). Chemical analysis of oxide products revealed that both bacteria the manganese had an
oxidation state of 3.86 (Table 2), equating to a manganese oxide as MnO$_{1.93}$. The product was 92.0% and 91.6% manganese oxide for *S. putrefaciens* CN-32 and *S. loihica* PV-4, respectively. The non-manganese oxide component remaining is likely a combination of organic and inorganic impurities as reflected in the FTIR spectra. The oxidation state of the analytical oxide was very close to complete Mn(IV) at 3.98 indicating MnO$_{1.99}$.

Scanning electron microscopy revealed finely-grained nano-sized amorphous round manganese oxide particles produced by of the both *Shewanella* strains (Figure 5). Even after washing the precipitates, bacteria are still seen clearly (arrows) in the micrographs. In contrast, the analytical grade MnO$_2$ particles are generally much larger crystalline particles and no bacteria are 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 of redox conditions, with oxidation favoured under oxic and reduction under anoxic conditions. Many bacteria are known to either oxidize or reduce manganese, and there is 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 has been investigated extensively in the *Shewanella* genus, 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 recognised as being important in manganese cycling in the Columbia River USA (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* as dominant members of deep-sea manganese nodule communities and suggested that they had a key role in manganese cycling, including oxidation.

All members of *Shewanella* tested in the current study were able to oxidize manganese. Rates of oxidation by the *Shewanella* species compared favourably with those isolated from manganese and non-manganese environments previously (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 done in the absence of nutrients and cell growth. Studies in our laboratory have shown that the type and concentration of nutrients directly influences the ability of the *Shewanella* strains to oxidize Mn(II) (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 out of 40 psychrotrophic and psychrophilic manganese bacteria isolated from 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 provision of an appropriate carbon source (15 mM lactate) resulted in all *Shewanella* species reducing these biogenic oxides. Indeed the ability of *Shewanella* species to not only reduce and oxidize manganese, but readily utilise 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 have reported bacteria that can both reduce and oxidize manganese. Bromfield and David (52) found that their *Arthrobacter* strain oxidized manganese under aerobic condition and reduced in deep static cultures presumably creating anaerobic conditions. Indeed, the most studied manganese oxidizer, *Bacillus* strain SG-1, was reported to reduce Mn(IV) as well (54). The difference with the *Shewanella* species tested in our study is that with *Bacillus* strain SG-1 the spores oxidized whereas the vegetative cells reduced manganese.

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 of the bioformed manganese oxides by *Shewanella* species was quick and usually completed within 1-2 days. The rapid rate of reduction was consistent with the much faster 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 the highly crystalline pyrolusite substantially slower than amorphous structurally-disordered $\delta$-MnO$_2$ (vernadite). It is likely that the characteristics of the poorly crystalline oxides,
including greater surface area and structural defects, allowed a more rapid transfer of electrons.

The mechanisms of oxidation have been investigated in numerous manganese oxidizing bacteria. Evidence suggests that multicopper oxidase enzymes mediate manganese oxidation and hence 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 enzymatically oxidize Mn(II) (25). The reason why bacteria oxidize Mn(II) is not clear. While it has long been suggested that it is an energy yielding process, no definitive evidence has ever been shown (19). It would make sense for *Shewanella* species to oxidize manganese as a strategy for storing electron acceptors that may be used when oxygen is depleted. Glasauer and colleagues (62) observed the synthesis of manganese nano-granules 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 to both oxidize and reduce manganese indicates that the genus plays a significant role overall 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 other elements and metals in the environment e.g copper, cobalt, nickel, lead, iron, radium, uranium and rare earth elements.
References


Figures

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

Figure 2. Extent of manganese oxidation (▲) and increases in cell numbers (cells/mL) over time (■) for (a) Shewanella putrefaciens CN-32, and (b) Shewanella loihica PV-4. Cultures grown with 3mM Mn(II). The extent of manganese oxidation in poisoned controls are shown as well (●).

Figure 3. Manganese oxide formation (▲) and cell number (cells/mL) variations over time (■) for (a) Shewanella putrefaciens CN-32, and (b) Shewanella loihica PV-4, initially under aerobic conditions. After 10 days incubation, conditions were changed to anaerobic (N₂ gas) with the addition of 15 mM lactate (indicated by arrow).

Figure 4. FTIR spectroscopy of (a) manganese oxides formed by Shewanella putrefaciens CN-32, (b) manganese oxides formed by Shewanella loihica PV-4, and (c) analytical grade MnO₂.

Figure 5. Scanning electron microscopy of (a) manganese oxides formed by Shewanella putrefaciens CN-32, (b) manganese oxides formed by Shewanella loihica PV-4, and (c) analytical grade MnO₂. Arrows show the presence of bacteria.
Table 1: Manganese oxidizing properties of *Shewanella* strains

<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Maximum Mn(II) concentration (mM)</th>
<th>Rate of oxide production (mg/L/day)</th>
<th>Reduction of bioformed oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. denitrificans</em> OS217</td>
<td>7</td>
<td>8.7 ± 2.7</td>
<td>+</td>
</tr>
<tr>
<td><em>S. loihica</em> PV-4</td>
<td>9</td>
<td>20.3 ± 1.6</td>
<td>+</td>
</tr>
<tr>
<td><em>S. oneidensis</em> MR-1</td>
<td>4</td>
<td>4.4 ± 1.2</td>
<td>+</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> 200</td>
<td>7</td>
<td>15.9 ± 3.9</td>
<td>+</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> CN-32</td>
<td>8</td>
<td>13.9 ± 3.1</td>
<td>+</td>
</tr>
</tbody>
</table>

1 Cultures incubated at a temperature of 27°C
2 Mn(II) concentration where complete inhibition of oxidation occurred
3 Oxidation at 3 mM Mn(II) with averages of replicates and standard deviation given
4 Cultures converted to anoxic conditions and replenished with 15 mM lactate
Table 2: Characteristics of manganese oxides produced by *Shewanella putrefaciens* CN-32 and *Shewanella loihica* PV-4, compared with analytical grade MnO₂

<table>
<thead>
<tr>
<th>Mn oxide origin</th>
<th>Oxidation state</th>
<th>Mn oxide content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. putrefaciens</em> CN-32</td>
<td>3.86</td>
<td>92.0</td>
</tr>
<tr>
<td><em>S. loihica</em> PV-4</td>
<td>3.86</td>
<td>91.6</td>
</tr>
<tr>
<td>Analytical grade MnO₂</td>
<td>3.98</td>
<td>100</td>
</tr>
</tbody>
</table>
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