Mn(II)-Oxidizing Activity of *Pseudomonas* sp. Strain MM1 is Involved in the Formation of Massive Mn Sediments around Sambe Hot Springs in Japan

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There has been an increasing interest in the application of Mn(II)-oxidizing microbes for Mn(II) containing waste water treatment, as well as biogenic Mn oxides for remediation of a number of toxic metals. Sambe hot spring in Shimane Prefecture, Japan is known for its Mn-rich spring water, and Mn oxide sediments are widely distributed over an area of 60 m × 80 m of this region. With an aim to find out Mn(II) oxidizing bacteria involved in this massive Mn(II) oxide deposit formation, culture enrichment was undertaken. *Pseudomonas* sp. strain MM1 was isolated as dominant Mn(II) oxidizing bacterium from the site. Biogenic Mn oxide formed by strain MM1 was shown to be poorly-crystalline birnessite, having the large specific surface area of 90 m²·g⁻¹. Mn(II) was readily oxidized by strain MM1 during the stationary phase and initial Mn(II) concentrations of up to 1.0 mmol·dm⁻³ (55 mg·dm⁻³) went well below the effluent standard of dissolved manganese (10 mg·dm⁻³; set by Japanese Ministry of Environment). Strain MM1 grew heterotrophically and Mn(II) did not serve as the sole electron donor. Nano-sized (30–50 nm) Mn oxide particles were produced by strain MM1, which later chained or aggregated to form larger Mn oxide minerals. Cells were observed eventually encrusted inside the Mn oxides. This study showed the important role of strain MM1 in the production of massive Mn oxide deposits at Sambe hot spring. The Mn(II) oxidizing ability of this strain is potentially applied for bioremediation of toxic metals.

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1. Introduction

Manganese (Mn) is an abundant transition element consisting of about 0.1% of the Earth’s crust. Mn concentration in soils is not uniform and can range from 0.002 to 10%, with its major accumulation form being oxides. In general, naturally found oxidation states of Mn are Mn(II), Mn(III) and Mn(IV), of which only Mn(II) exists as stable free cation in aqueous solution, whereas Mn(IV) is insoluble and precipitates as solid manganese oxide. Mn(III) is unstable and disproportionates into Mn(II) and Mn(IV), except in certain minerals or in soluble complexes.

Since Mn is an important trace element in biological systems, biogeochemical Mn cycle occurs as a result of diverse microbial metabolisms. Thermodynamically, chemical oxidation of Mn(II) to Mn(III)/Mn(IV) at circumneutral pH is limited and is mainly accelerated by Mn(II)-oxidizing microorganisms (such as bacteria and fungi), forming biogenic Mn oxide minerals in soils and sediments.

Mn(II)-oxidizing bacterial species so far identified are shown to be physiologically diverse, belonging to Proteobacteria, Actinobacteria and Firmicutes branches of bacterial domain, with the most well studied being *Pseudomonas putida* strains MnB1 and GI-B, *Leptothrix discophora* strain SS-1 and *Bacillus* sp. strain SG-1. Nevertheless, the physiological function of Mn(II) oxidation is yet to be clear.

Due to the characteristics of biogenic Mn oxides, such as large surface area and negative charges with structural defects, Mn oxides provide coordination sites for a variety of metal ions, playing key roles in their biogeochemical cycles. In fact, utility of biogenic Mn oxides has been proposed in a number of remediation applications for toxic metals (e.g., Pb(II), Zn(II), Co(II), Ni(II)), such as in water treatment, soil remediation and metal recovery.

Japan has a number of natural hot springs with some being rich in manganese: Microbial formation of Mn deposits has been described for hot springs in Hokkaido. One of the Mn-rich natural springs found in Honshu Island is located in the Sambe hot spring region of Shimane prefecture on the Sea of Japan, where precipitation of Mn oxides with a distinctive blackish color is distributed over an area of 60 m × 80 m. Two types of spring water streams are apparent in Sambe; (i) hot springs (HS) of 25–40°C and pH ~7, where precipitates of orange-colored Fe oxides and black-colored Mn oxides are concomitantly deposited, and (ii) cold springs (CS) of 10–25°C and pH ~6, where the deposit consists of black Mn oxide sediments of 20 cm thickness. Microbial community structure analysis indicated the presence of Mn(II)-oxidizing bacterium *Pseudomonas* sp. and Mn(II)-oxidizing fungi *Phoma* sp. and *Plectosphaerella* sp.

This study attempted to actually isolate the Mn(II)-oxidizing bacteria involved in formation of this massive Mn deposit at Sambe hot spring, and to characterize its Mn(II) oxidation efficiency as well as the resultant Mn-oxide product.

2. Experimental

2.1 Culture enrichment and single colony isolation

An aliquot of the black sediment collected at the HS and CS sites (Fig. 1) was inoculated in a 300 cm³ Erlenmeyer flask containing 100 cm³ of “Y” liquid medium (pH 6.6) composed of (g·dm⁻³); peptone 0.05, yeast extract 0.05, MgSO₄·7H₂O 0.6, CaCl₂ 0.07, PIPES 4.5, with 1 mmol·dm⁻³ MnSO₄. The flask was incubated at 25°C, shaken at 100 rpm. Liquid culture enrichment was repeated for a few times. For single colony isolation, “PYG” solid selective medium (pH 6.6) composed of (g·dm⁻³); peptone 0.25, yeast extract

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2.2 Determination of the 16S rRNA gene sequence and construction of phylogenetic tree

A single, black colony was carefully removed from the PYG solid selective medium and inoculated into a 100 cm³ Erlenmeyer flask containing 30 cm³ of Y liquid medium and incubated at 25°C; shaken at 100 rpm. After removing Mn oxide precipitates by gentle centrifugation, cells were harvested to extract genomic DNA using Ultra Clean Microbial DNA Isolation Kit (MO BIO). The 16S rRNA gene was amplified by PCR using universal primers (27f forward primer; 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492r reverse primer; 5'-TACGGYTACCTTGTTACGACTT-3') complementing positions 8 to 27 and 1510 to 1492 of Escherichia coli 16S rRNA gene, respectively. The PCR mixture (final volume 50 mm³) included 25 mm³ Premix Taq (TaKaRa BIO), 5 mm³ each of 27f and 1492r primers and 300 ng DNA template. The PCR product was determined by Research Support Center, Graduate School of Medical Sciences, Kyushu University. The 16S rRNA gene sequence was analyzed from the site by DGGE20) was not identical (94 %) to that of Pseudomonas sp. strain PCP. The 16S rRNA gene sequence of strain MM1 was analyzed using nitrogen gas adsorption (BET) method (BELSORP-mini II, BEL Japan).

3. Results and Discussion

In Y liquid medium inoculated with samples taken from HS or CS, black precipitates, typical of Mn oxides, were observed after 3 or 4 days incubation at 25°C. An aliquot of the liquid enrichment culture was then streaked onto Y solid selective media. After several times and the isolate was eventually named strain MM1. The 16S rRNA gene sequence of strain MM1 (1423 bp) was determined and shown to be 99% homologous to that of Pseudomonas sp. strain PCP. The 16S rRNA gene sequence (135 bp) of Pseudomonas sp. previously detected from the site by DGGE20) was not identical (94% homologous) to that of strain MM1, indicating that there is some phylogenetic diversity within the genus at the site. Consensus phylogenetic tree based on the 16S rRNA gene sequence shows the position of strain MM1 within the Pseudomonas branch and its relationship with other members of Mn(II) oxidizers (Fig. 2).

Previously, the DGGE analysis of the enrichment culture of a Sambe hot spring sample detected the presence of Pseudomonas sp. as the only Mn(II)-oxidizing bacterial species.20 In the present study, enrichment on selective solid media revealed only single type of black colored bacterial colonies, which turned out to be Pseudomonas sp. The results

Fig. 1 Map of the Sambe hot spring region, Shimane Prefecture, Japan (*HS and *CS indicate the two sampling points; partially re-drawn20).
indicate that this bacterial species plays an important role in the formation of massive Mn oxide deposits in Sambe hot spring. *Pseudomonas* sp. represents the most diverse, ecological significant group of bacteria on the planet and its distribution is universal.23) Therefore, Mn(II)-oxidizing activity by its members may significantly affect the biogeochemistry of Mn. In this study, no fungal colonies were formed on selective solid media. In contrast, in the previous attempt to isolate Mn(II)-oxidizing microbes from the same environmental sample, black fungal colonies grew readily on the same selective solid media whereas no single black bacterial colonies were visible (Sasaki, personal communication). A major drop in Mn(II) concentration at the initial Mn(II) concentrations of 0.1 and 1.0 mmol·dm$^{-3}$, compared to the previous sampling (HS; 0.9 mg·dm$^{-3}$ or Mn had inhibitory effect on cell viability. Formation of Mn oxides was accompanied by a decrease in culture pH (Fig. 3(b)), based on the stoichiometry of Mn(II) oxidation reaction, as typically written as follows:\(^\text{3)}\)

$$\text{Mn}^{2+} + 1/2\text{O}_2 + \text{H}_2\text{O} \rightarrow \text{MnO}_2 + 2\text{H}^+$$

At the initial Mn(II) concentrations of 0.1 and 1.0 mmol·dm$^{-3}$, Mn(II) was effectively oxidized by strain MM1 and the dissolved Mn(II) concentrations went below the detection limit by AAS (0.02 mg·dm$^{-3}$) in 2 days. Growth of strain MM1 was not inhibited at up to 8 mmol·dm$^{-3}$ (data not shown). Effect of glucose and yeast extract on Mn(II) oxidation by strain MM1 is shown in Fig. 4. No Mn(II) oxidation was observed in sterile control cultures (solid symbols) or activity of different Mn(II)-oxidizing microorganisms at the site: Fungal Mn(II) oxidation activity contributing more dominantly to the Mn deposit formation at higher Mn(II) concentrations, may have been lowered/lost due to the drop in Mn(II) concentration. Under such circumstance, bacterial Mn(II) oxidation activity may be seen as the dominant Mn deposit formation mechanism.

Mn(II) oxidation by strain MM1 is shown in Fig. 3. No Mn(II) oxidation was observed in sterile control cultures (data not shown). In all inoculated cultures, cell numbers increased quickly within 20 h up to $\sim 6.0 \times 10^7$ cells·cm$^{-3}$ (Fig. 3(a)). Mn(II) oxidation progressed during the stationary phase. Mn(II) oxidation was accompanied by a decrease in cell numbers, especially at higher initial Mn(II) concentrations (Fig. 3(a)), implying that cells were trapped inside the Mn oxides and/or Mn had inhibitory effect on cell viability. Formation of Mn oxides was accompanied by a

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Fig. 2 Phylogenetic tree showing the relationship of isolate MM1 to other known Mn(II)-oxidizing bacteria, based on 16S rRNA gene sequence homology (values in brackets are NCBI accession numbers). Asterisks (*) indicate non-Mn(II)-oxidizing bacterial isolates. Scale bar indicates the number of nucleotide substitutions per site. Numbers at nodes indicate bootstrap values for 1000 replicates of the original dataset. Bacterial phyla, Proteobacteria, Actinobacteria, Firmicutes, are indicated on the right-hand side of the phylogenetic tree. The tree was rooted with *Leptospirillum ferrooxidans* L15 as outgroup.

Fig. 3 Mn(II) oxidation by strain MM1. Changes in Mn(II) concentration (solid symbols)/cell density (open symbols) (a), and changes in pH during Mn(II) oxidation (b). Initial Mn(II) concentrations were 0 mmol·dm$^{-3}$ (□), 0.1 mmol·dm$^{-3}$ (○, △) and 1.0 mmol·dm$^{-3}$ (▲, △).
oxidation was observed in the absence of yeast extract (Fig. 4), due to lack of cell growth (data not shown). Addition of glucose resulted in increase in cell numbers (data not shown), consequently facilitating Mn(II) oxidation (Fig. 4). The results indicate that strain MM1 grows heterotrophically and is not capable of utilizing Mn(II) as the sole energy source. Although Mn(II) oxidation is thermodynamically favorable, there is no unequivocal evidence directly linking Mn(II) oxidation to microbial energy conservation. In most cases, growth in the presence of Mn(II) is reported to be either mixotrophic or heterotrophic. One of the advantages of Mn(II) oxidation may be accumulation of Mn oxides as the storage of an electron acceptor for later use in anaerobic respiration. Another possible role of Mn oxides is to degrade humic substances to microbially utilizable low molecular weight compounds. Strain MM1 may be another example of heterotrophic Mn(II) oxidizer which may take advantage of accumulation of Mn oxides in order to utilize refractory organic compounds.

Mn oxide precipitates formed by strain MM1 was collected and analyzed by XRD (Fig. 5). XRD peak set was assigned to poorly crystalline birnessite (Na₄Mn₁₄O₂₇·9H₂O/Na₀.₅₅-Mn₂O₄·1.₅H₂O), with the d(001) value of 0.73 nm (JCPDS 43-1456). These results were similar to those reported for well-characterized _Ps. putida_ Mnb1. Strain MM1 cells during Mn(II) oxidation were observed by TEM (Figs. 6(a)–6(e)). Cells produced nano-sized (30–50 nm) Mn oxides particles (Fig. 6(b)), which were then chained and aggregated around the cells (Fig. 6(c)). Cells were finally encrusted in Mn oxides (Figs. 6(d), 6(e)). SEM images show biogenic Mn oxide minerals having rough surface incorporating Mn oxide-encrusted cells (Fig. 6(f)). Some planktonic cells escaped from encrustation were observed attached onto the fractured mineral surface (Fig. 6(g)). Since it is unlikely that cells are able to escape from Mn oxides once they have been encrusted, it would be interesting to know the physiological mechanism of how they benefit from their own Mn(II) oxidation activity.
4. Conclusion

There has been an increasing interest in the application of Mn(II) oxidizing microbes for Mn(II) containing waste water treatment, as well as biogenic Mn oxides for remediation of a number of toxic metals. This study focused on the Sambe hot spring region in Shimane Prefecture, Japan where Mn oxide deposits are widely distributed over an area of 60 m × 80 m. With an aim to find out the Mn(II) oxidizing bacteria involved in formation of such massive Mn(II) oxide deposits, culture enrichment on Mn(II) was undertaken. As a result, we have successfully isolated Pseudomonas sp. strain MM1 as dominant Mn(II) oxidizing bacterium from the site. Biogenic Mn oxide formed by strain MM1 was shown to be poorly-crystalline birnessite with the $d(001)$ value of 0.73 nm and the large specific surface area of 90 m$^2$·g$^{-1}$. Strain MM1 was tolerant to up to 8 mmol·dm$^{-3}$. Mn(II) was readily oxidized by strain MM1 during the stationary phase and initial Mn(II) concentrations of up to 1.0 mmol·dm$^{-3}$ (55 mg·dm$^{-3}$) went below the AAS detection limit (0.02 mg·dm$^{-3}$) in 2 days, well below the effluent standard of dissolved manganese (10 mg·dm$^{-3}$; set by Japanese Ministry of the Environment). Growth of strain MM1 was shown to be heterotrophic and Mn(II) did not serve as the sole energy source. Strain MM1 also awaits further investigations to be used as useful material for bioremediation of toxic metals.

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