Characterization of Manganese Oxide-Biomineralization by the Psychrophilic Marine Bacterium, *Arthrobacter* sp. Strain NI-2 and Its Spontaneous Mutant Strain NI-2'[#]

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ABSTRACT

Metal pollution and metal shortage are a growing threat to the global environment and world high-tech industry. One of the promising strategies for removing and recycling metal-elements from the environments is applying Metal-Biotechnology based on metal-related biological activities, which include bioaccumulation, bioadsorption, and biomineralization. In this study, focusing on Manganese (Mn) pollution, we have isolated and analyzed the Mn(II)-oxidizing marine bacterium, *Arthrobacter* sp. NI-2 strain, from Imari Bay, Imari-shi, Saga, Japan. We have also isolated a spontaneous mutant, *Arthrobacter* sp. NI-2' strain with enhanced Mn(II)-oxidizing activity. Under the liquid culture condition at 30 °C, *Arthrobacter* sp. NI-2' strain could efficiently remove more than 96% of Mn(II) from the liquid culture media containing 0.4 mM Mn(II). Although Mn(II)-oxidizing activity of *Arthrobacter* sp. NI-2 strain is suppressed under the low temperature conditions, the increased Mn(II)-oxidizing activity of *Arthrobacter* sp. NI-2' strain was maintained when the growth temperature was shifted from 30 °C to 10 °C. Therefore, the *Arthrobacter* sp. NI-2' strain would be useful as a tool for Mn removal from low-temperature water, such as groundwater around the mining area.

1. INTRODUCTION

Metal pollution and metal shortage are a growing threat to the global environment and world high-tech industry. As summarized in Figure 1, using metal-related biological activities (i.e., biosensor, bioaccumulation, bioadsorption, biomineralization, and chemisorption), Metal-Biotechnology would be one of the promising strategies for monitoring, removing and recycling metal-elements in polluted environments (Ike et al., 2011).

As Manganese (Mn) is one of the important elements for both the ecosystem and the industry, environmental Mn pollution and industrial Mn-shortage are the matter of concern. In the environment, Mn(III/IV) oxides, found in various solid forms, play important roles in the global cycling of many major elements (C and S) and trace elements (Fe, Co, Pb, Cu, Cd, and Cr) as an oxidation catalyst and a metal scavenger (Namgung et al., 2018; Tebo et al., 2004). In water ecosystem, Mn(II) found as Mn^{2+} ions are further oxidized for the formation of Mn(III, IV) oxides mainly by the activity of microorganisms, because the rates of Mn(II)-oxidation catalyzed by microorganisms are much faster than that of the abiotic Mn(II) oxidation (Nealson et al., 1988; Tebo et al., 2004). The biogenic Mn oxides formation system, i.e., Mn oxide-biomineralization, could be a good tool for Mn removal from industrial wastewater (Barboza et al., 2016; Ike et al., 2011). Moreover, metal scavenger property of Mn oxides could be an additional tool for multiple heavy-metal removal for industrial wastewater (Figure 2, Chemisorption). Mn(III/IV) oxides are also recognized as powerful oxidants that are capable of oxidizing a wide range of compounds including organic contaminants (Remucal and Ginder-Vogel, 2014).

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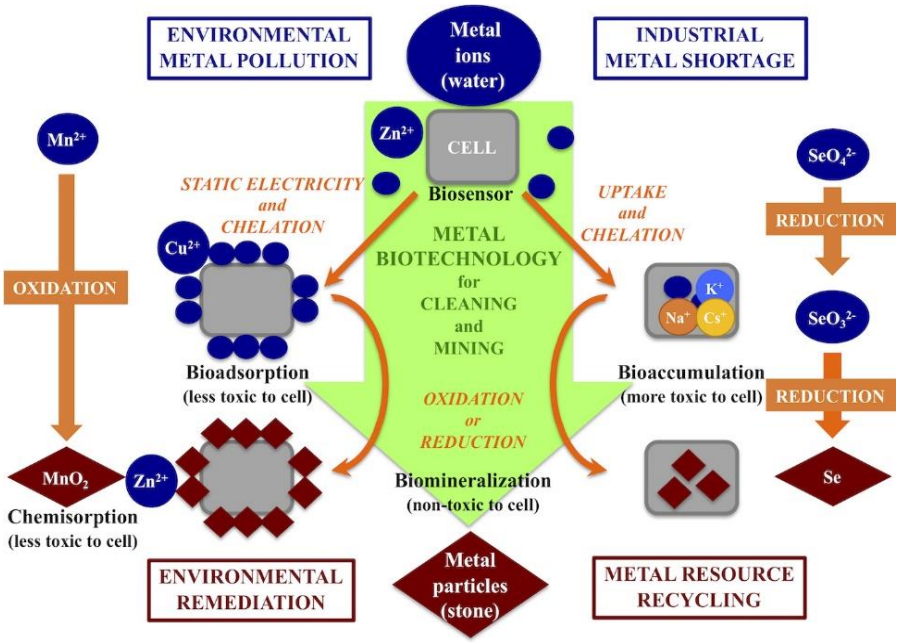


Figure 1. The overview of metal biotechnology including biosensor, bioaccumulation, bioadsorption, biomineralization, and chemisorption strategies.

As Manganese (Mn) is one of the important elements for both the ecosystem and the industry, environmental Mn pollution and industrial Mn-shortage are the matter of concern. In the environment, Mn(III/IV) oxides, found in various solid forms, play important roles in the global cycling of many major elements (C and S) and trace elements (Fe, Co, Pb, Cu, Cd, and Cr) as an oxidation catalyst and a metal scavenger (Namgung et al., 2018; Tebo et al., 2004). In water ecosystem, Mn(II) found as Mn^{2+} ions are further oxidized for the formation of Mn(III/IV) oxides mainly by the activity of micro-organisms, because the rates of Mn(II)-oxidation catalyzed by microorganisms are much faster than that of the abiotic Mn(II) oxidation (Nealson et al., 1988; Tebo et al., 2004). The biogenic Mn oxides formation system, i.e., Mn oxide-biomineralization, could be a good tool for Mn removal from industrial wastewater (Barboza et al., 2016; Ike et al., 2011). Moreover, metal scavenger property of Mn oxides could be an additional tool for multiple heavy-metal removal for industrial wastewater (Figure 2, Chemisorption). Mn(III/IV) oxides are also recognized as powerful oxidants that are capable of oxidizing a wide range of compounds including organic contaminants (Remucal and Ginder-Vogel, 2014).

As summarized in Figure 2, however, biogenic Mn(II)-oxidizing activities may be inhibited by environmental stresses such as osmotic stress caused by high salinity and thermal stress caused by dynamic changes of cold/hot weather. Therefore, in order to overcome the weakness of the biogenic Mn(II)-oxidation systems, we set out to isolate robust Mn(II)-oxidizing marine bacteria with ability to maintain the Mn(II)-oxidizing activities under high-salinity and/or low-temperature conditions. Previously, two Mn(II)-oxidizing marine bacteria strains NI-1 and NI-2 were isolated from Imari Bay, Imari-shi, Saga, Japan (Nakayama and Ikegami, 2009). As the NI-1 strain belonging to *Bacillus* sp., which exhibits Mn(II)-oxidizing activity even at 3% and 6% NaCl conditions, the *Bacillus* sp. NI-1 would be a good candidate for Mn-bioremediation of high-salinity wastewater such as contaminated seawater and concentrated seawater generated by desalination. Although the NI-2 strain does not possess Mn(II)-oxidizing activity under high-salinity conditions, we found that it thrives at low temperatures of 4 and 10 °C as a psychrophilic marine bacterium. As cold stress may inhibit Mn oxide-biomineralization system in cold wastewater such as drainage water of Mn mines in winter season or at cold climate regions, we further characterized NI-2 strain and its properties including Mn(II)-oxidizing activity under low-temperature conditions.

In this study, we have identified NI-2 strain as *Arthrobacter* sp. based on sequence analysis of the 16S rRNA gene in its genome. Moreover, we have

successfully isolated the spontaneous mutant *Arthrobacter* sp. NI-2' strain, which exhibits enhanced ability of Mn oxide-biomineralization at 10 °C condition. We conclude that the *Arthrobacter* sp. NI-2' strain would be useful for bioremediation of metal contaminated wastewater even at low temperature conditions.

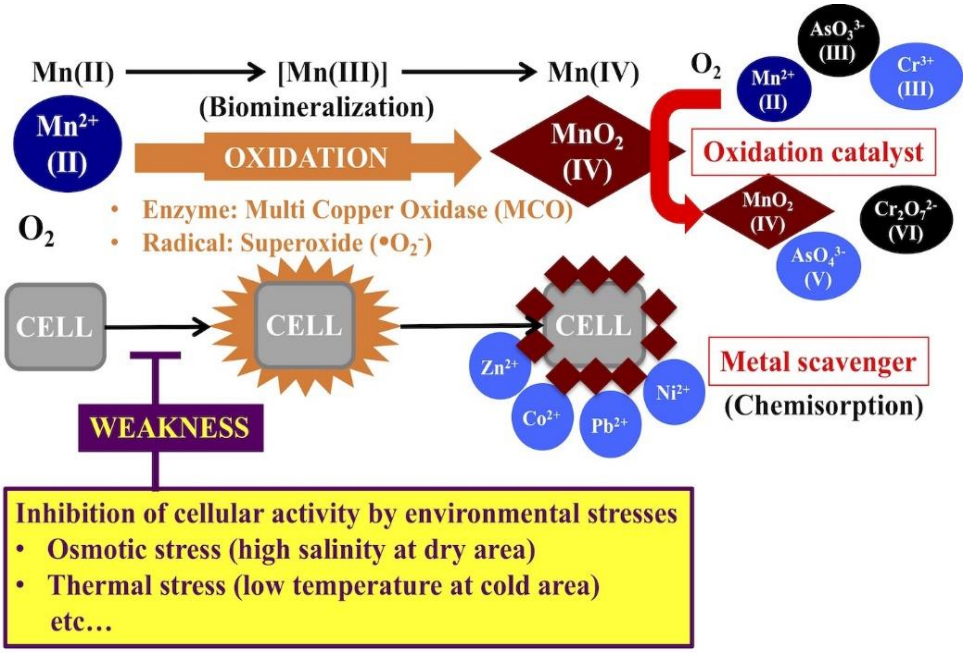


Figure 2. Mn oxide-biomineralization and chemisorption as a tool for heavy-metal removal from polluted water. Oxidation of Mn(II) to Mn(III/IV) is mediated by microbial cell activities, which include enzyme reaction by multi copper oxidase (MCO) and/or radical reaction by superoxide (O_2^-).

2. METHODOLOGY

2.1 Isolation of Mn(II)-oxidizing bacteria

In order to isolate Mn(II)-oxidizing bacteria (MOB), seawater and seafloor sediment samples collected from Imari bay, Imari-shi, Saga, Japan (Nakayama and Ikegami, 2009) were diluted in 3% NaCl, and 100 µL of each dilution was spread onto solid LEPT media (Boogerd and de Vrind, 1987) containing 0.2 mM $MnCl_2$ at $pH_{7.5}$ and incubated for 14 days at 30 °C in the dark.

MOB were identified by using leucoberbelin blue I (LBB), which reacts with Mn(III/IV) oxides and the LBB's color is changed to blue in colorimetric assays (Krumbein and Altmann, 1973). In brief, a drop or spray of the LBB reagent was applied directly to a brownish-black colony or to the whole surface of the growth medium, and the mixture was incubated for 5 min at room temperature in the dark prior to a visual inspection for color change. LBB-positive colonies were transferred and streaked for single colony isolation at least 3 times.

In this study, two isolates named NI-1 and NI-2 strains were used. The NI-1 strain, which was identified as *Bacillus* sp. (Nakayama and Ikegami, 2009) with Mn(II)-oxidizing activity is used as a positive control strain for Mn(II)-oxidizing activity. *Escherichia coli* DH5α, which possesses no Mn(II)-oxidizing activity is used as a negative control strain.

Spontaneous mutant of NI-2 strain with enhanced Mn(II)-oxidizing activity, named NI-2', was isolated by chance during routine subculture periods.

2.2 Identification of MOB isolates by 16S rRNA gene analysis

In order to identify MOB isolates, total genomic DNA was extracted and purified with ISOPLANT II kit (Nippon Gene Co., Ltd., Tokyo, Japan). The almost full length of 16S rRNA gene fragments (about 1.5 kbp) were PCR-amplified using the universal primer set, 27F (5'-AGAGTTTG-ATCCTGGCTCAG-3') and 1525R (5'-AAAGGAG-GTGA-TCCAGCC-3'), in a Takara Thermal Cycler (Takara, Shiga, Japan). The PCR products were subcloned with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA) following the manufacturer's

specifications. The sequence of the subcloned 16S rRNA gene fragment was determined using universal primer set, M13 Forward (-20) (5'-GTAAAACGA-CGGCCAG-3') and M13 Reverse (5'-CAGGAA-ACAGCTATGAC-3'), provided in the Kit. The sequences of the fragments were subjected to a homology search in the APORON database (Techno Suruga Laboratory, Shizuoka, Japan) and phylogenetic trees were constructed to ascertain the phylogenetic positions of the isolates.

2.3 Bioassay for Mn(II)-oxidizing activities under various conditions

In order to evaluate the effect of NaCl stress on the cell growth and biogenic Mn oxide formation, a series of solid LEPT media with 3% or 6% NaCl, and with or without 0.2 mM $MnCl_2$ were prepared. Then bacterial cells were streaked on the media and incubated for 14 days at 30 °C in the dark as a bioassay for Mn(II)-oxidizing activities under NaCl-stress conditions. In addition, to evaluate the effect of cold stress on the cell growth and biogenic Mn oxide formation, bacterial cells were streaked on the media and incubated for 14 days at 4 °C, 10 °C, or 30 °C in the dark as a bioassay for Mn(II)-oxidizing activities under cold-stress conditions.

2.4 Quantification of Mn in liquid culture media by ICP-OES analysis

In order to quantify Mn concentration in liquid culture media, bacterial cells were cultured in 6 mL of the half strength of liquid LEPT media (1/2 LEPT media) with or without 0.4 mM $MnCl_2$ for 14 days and 5 mL of liquid samples were collected from supernatant of the cultures after centrifugation at 3,000×g at room temperature for 10 min to pellet bacterial cells and Mn oxides. The liquid samples were transferred to 50 mL Polypropylene tubes with watch glasses as a lid (DigiTUBE, SCP Science, Quebec, Canada) and the tubes were settled in a digestion block (DigiPREP jr., SCP Science, Quebec, Canada). Digestion was done by adding 25 mL of Milli-Q water and 5 mL of 70% HNO_3 (Nacalai Tesque Inc., Kyoto, Japan) to each sample and heated the mixtures to 65 °C, maintained for 15 min, then heated up to 105 °C and maintained for 120 min before letting the mixture cool down to room temperature. After cooling down, 0.5 mL of 30% H_2O_2 (Fujifilm Wako Pure Chemical, Ltd., Osaka, Japan) were added to the mixtures and the digestion block was again heated to 105 °C and maintained at the temperature for 60 min. The digested solution samples were cooled down and filtered through 0.45 µm-pore-size Teflon® membrane filter (DigiFILTER, SCP Science, Quebec, Canada). After rinsing the filter with Milli-Q water, volume of each filtered samples was adjusted to 50 mL by adding Milli-Q water. Measurements were conducted on an ICP-OES (ICPS-7500, Shimadzu Corporation, Kyoto, Japan) following the instruction provided by the manufacturer.

3. RESULTS AND DISCUSSION

3.1 Identification of Mn(II)-oxidizing marine bacterium NI-2 strain

As shown in Figure 3, two Mn(II)-oxidizing marine bacteria strains NI-1 and NI-2 were isolated from seawater and seafloor sediment samples collected from Imari Bay, Imari-shi, Saga, Japan (Nakayama and Ikegami, 2009). We found that when Mn(II) is present in the medium, both NI-1 and NI-2 strains produce visible brown-colored particles (Figure 3(a) and 7). Production of biogenic Mn oxide was confirmed by blue color formation after LBB spray treatment (Figure 3(b)).

Based on 16S rRNA gene sequencing, the NI-1 strain was previously classified as *Bacillus* sp. (Nakayama and Ikegami, 2009), and the NI-2 strain is classified as *Arthrobacter* sp. in this study (Figure 4). The closest relative species of *Arthrobacter* sp. NI-2 strain is *Arthrobacter subterraneus* $CH7^T$, which was isolated as a new species from deep subsurface water of the South Coast of Korea (Chang et al., 2011). In the previous report, *A. subterraneus* $CH7^T$ was found to grow under low temperature conditions similar to deep sea environment (Chang et al., 2011). As shown in Figure 5, we found that *Arthrobacter* sp. NI-2 strain grow well under cold-stress condition (4 °C), while *Bacillus* sp. NI-1 strain cannot grow under this condition. The result indicates that *Arthrobacter* sp. NI-2 is a psychrophilic marine bacterium.

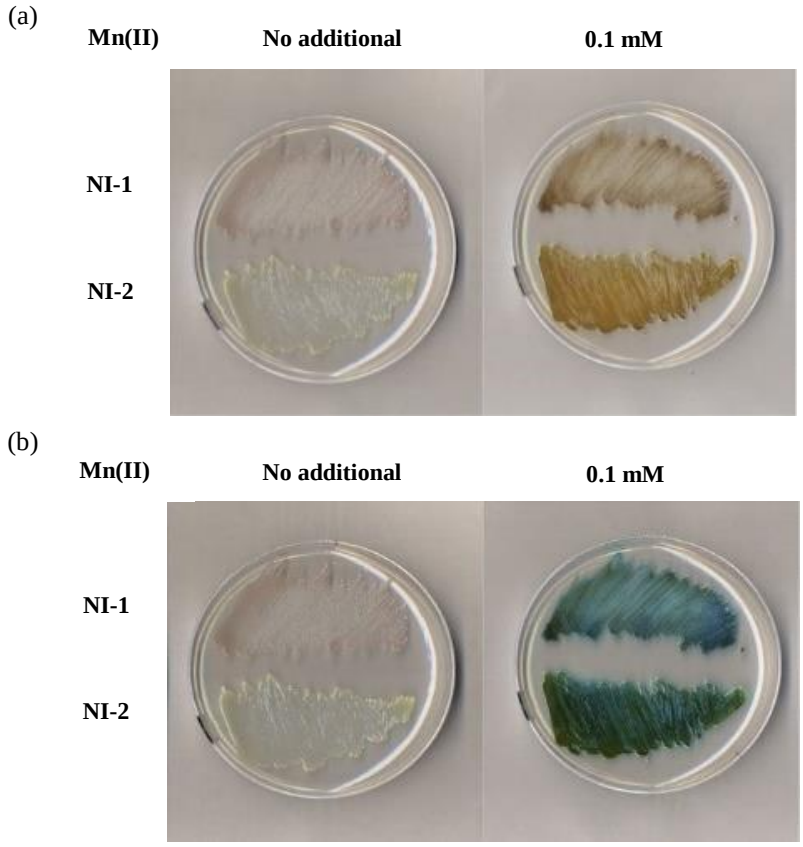


Figure 3. Mn(II)-oxidizing marine bacteria strains NI-1 and NI-2 isolated from Imari Bay, Imari-shi, Saga, Japan. (a), Both NI-1 and NI-2 stains were cultured for 14 days at 30 °C on LEPT media with or without $MnCl_2$ (pH 7.5). (b), Same samples as A, 5 min after LBB spray treatment.

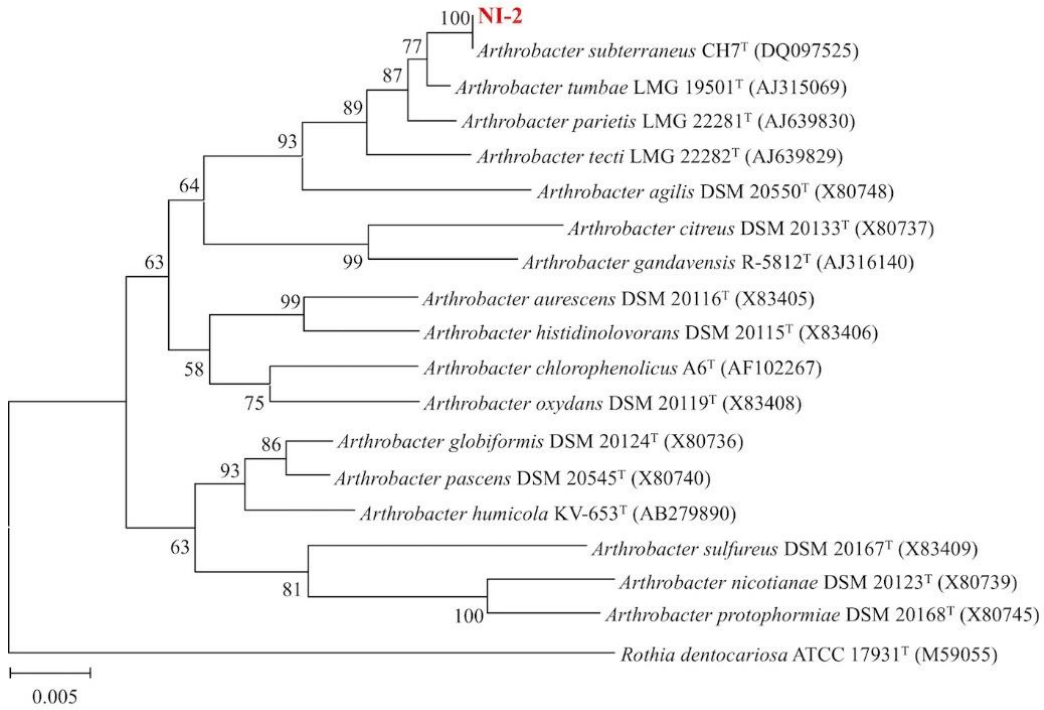


Figure 4. NI-2 strain is classified as *Arthrobacter* sp. in phylogenetic tree based on 16S rRNA gene sequencing by neighbor joining method.

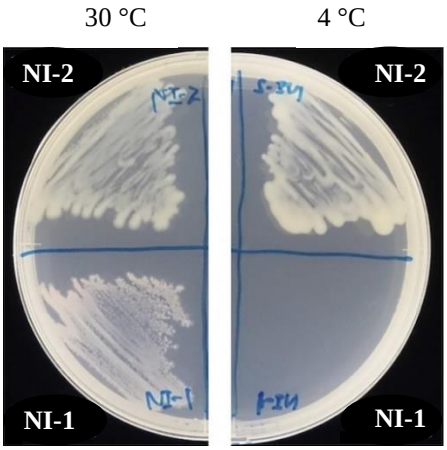


Figure 5. Growth of *Bacillus* sp. NI-1 and *Arthrobacter* sp. NI-2 under cold-stress condition.

3.2 Isolation of NI-2 derivative mutant with increased Mn(II)-oxidizing activity

Recently, *Arthrobacter* sp. strain QXT-31 was identified as a non-Mn(II)-oxidizing strain in monoculture condition, however, interaction (co-culture) with another non-Mn(II)-oxidizing *Sphingopyxis* sp. QXT-31 strain could induce cooperative Mn(II) oxidation by *Arthrobacter* sp. strain QXT-3 in an aquatic environment (Liang et al., 2016). Interestingly, during our screening and isolation process, we have also observed that *Arthrobacter* sp. NI-2 strain displayed improved Mn(II)-oxidizing activity when interacts with an unidentified non-Mn(II)-oxidizing bacteria (Figure 6). However, in contrast to *Arthrobacter* sp. QXT-31 strain, *Arthrobacter* sp. NI-2 strain showed Mn(II)-oxidizing activity even in monoculture at 30 °C (Figure 3 and Figure 7). This observation implicated that *Arthrobacter* sp. NI-2 strain is probably equipped with endogenous auto-activation system for Mn(II)-oxidizing activity.

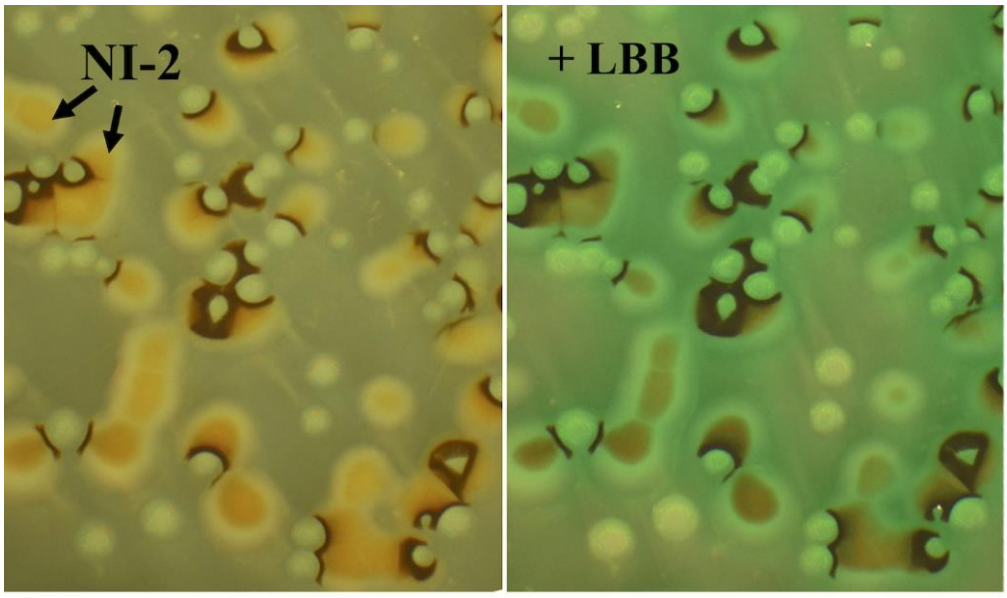


Figure 6. Enhanced Mn(II)-oxidizing activity by interaction with non-Mn(II)-oxidizing bacteria in *Arthrobacter* sp. NI-2 strain.

In this study, *Bacillus* sp. NI-1 and *E. coli* DH5α strains were used as a positive control strain and a negative control strain, respectively, for bioassay of Mn(II)-oxidizing activity. During the routine sub-culturing of NI-2 strain, we have identified a spontaneous mutant colony, which showed a faster and more intense accumulation of Mn oxide (brown-colored particles). As shown in Figure 7, this spontaneous mutant, which we named *Arthrobacter* sp. NI-2' possesses enhanced Mn(II)-oxidizing activity on the Mn(II)-containing LEPT medium at 30 °C.

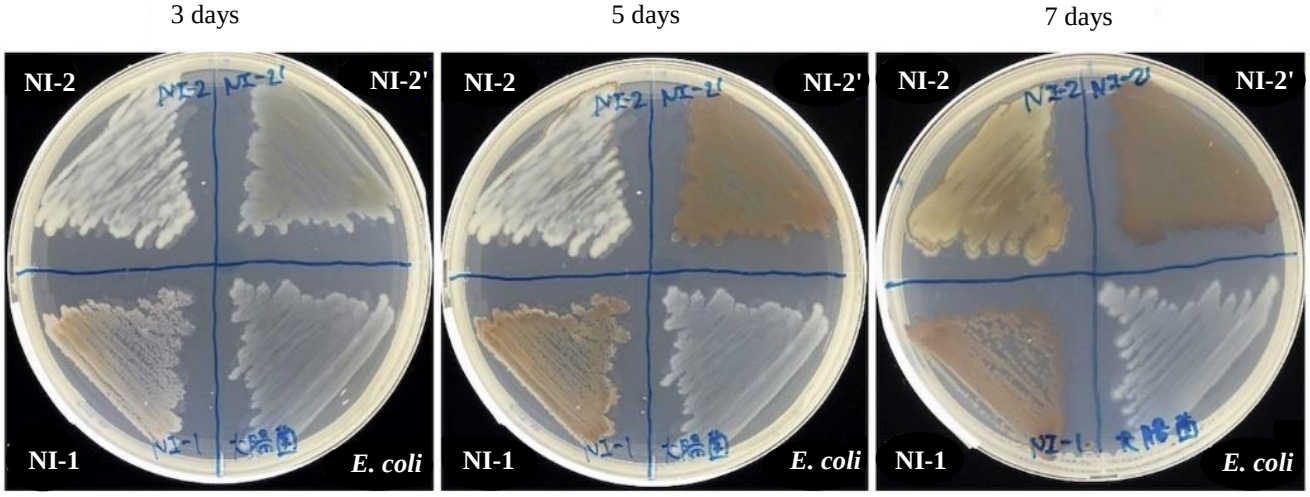


Figure 7. Mn(II)-oxidizing activity in *Arthrobacter* sp. NI-2 and its derivative mutant, *Arthrobacter* sp. NI-2'. *Bacillus* sp. NI-1 and *E. coli* DH5α were used as a positive and a negative control strains for Mn(II)-oxidizing activity, respectively.

3.3 Mn(II) removal by Mn oxide-biomineralization in liquid culture system

In order to evaluate ability of Mn(II) removal by Mn oxide biomineralization in liquid culture system, *Bacillus* sp. NI-1, *E. coli* DH5α, and *Arthrobacter* sp. NI-2' were subjected to bioassay of Mn oxide-biomineralization. Mn(II)-oxidizing activity seems to be mediated by multi copper oxidase (MCO) on the surface of spores of *Bacillus* sp. NI-1 and is found to be induced during spore formation triggered by starvation in other Mn(II)-oxidizing *Bacillus* sp. strains such as SG-1 strain (de Vrind et al., 1986; Francis and Tebo, 2002). Therefore, our bioassay was performed in half strength of liquid LEPT (1/2 LEPT) culture media, in which nutrients were reduced to accelerate the entering to the starvation phase. As shown in Figure 8a, both *Bacillus* sp. NI-1 and *Arthrobacter* sp. NI-2' were able to form brown-colored particles of Mn oxide efficiently in the culture medium containing 0.4 mM $MnCl_2$ (approx. 20 ppm Mn; doubled the concen-tration of Mn allowed in drainage water by Uniform Effluent Standard in Japan). Moreover, more than 96% of Mn(II) could be removed from liquid culture media through Mn oxide-biomineralization by *Arthrobacter* sp. NI-2' and *Bacillus* sp. NI-1 (shown in Figure 8(b)). While only 20% could be remove through biosorption or bioaccumulation by *E. coli* DH5α, which possessed no Mn(II)-oxidizing activity. The results suggest that both *Arthrobacter* sp. NI-2' and *Bacillus* sp. NI-1 strains would be useful for Mn-bioremediation of wastewater at 30 °C. As *Arthrobacter* sp. NI-2' was expected to grow under cold-stress conditions similar to the original *Arthrobacter* sp. NI-2 strain, we further investigated Mn(II)-oxidizing activity of each strain under cold-stress conditions.

3.4 Mn(II)-oxidizing activity under NaCl- or cold-stress conditions

Due to the fact that *Arthrobacter* sp. NI-2 and NI-2' strains can grow well under NaCl-stress condition similar to *Bacillus* sp. NI-1, we tested Mn(II) oxidizing activities of both *Arthrobacter* sp. NI-2 and NI-2' strains in medium containing 3% (sea water level) and 6% NaCl. Interestingly, Mn(II)-oxidizing activities of both strains were completely suppressed under these conditions (Figure 9). The results indicate that sea-water level of NaCl strongly affects Mn(II)-oxidizing activity of both *Arthrobacter* sp. NI-2 and NI-2' strains but not that of *Bacillus* sp. NI-1. Thus, *Bacillus* sp. NI-1 would be a useful strain for Mn removal in high-salinity wastewater including seawater and concentrated seawater as byproduct of desalination (Nakayama and Ikegami, 2009). Therefore, we decided to further investigate the ability of Mn oxide-biomineralization in *Arthrobacter* sp. NI-2' under low-temperature condition without NaCl stress.

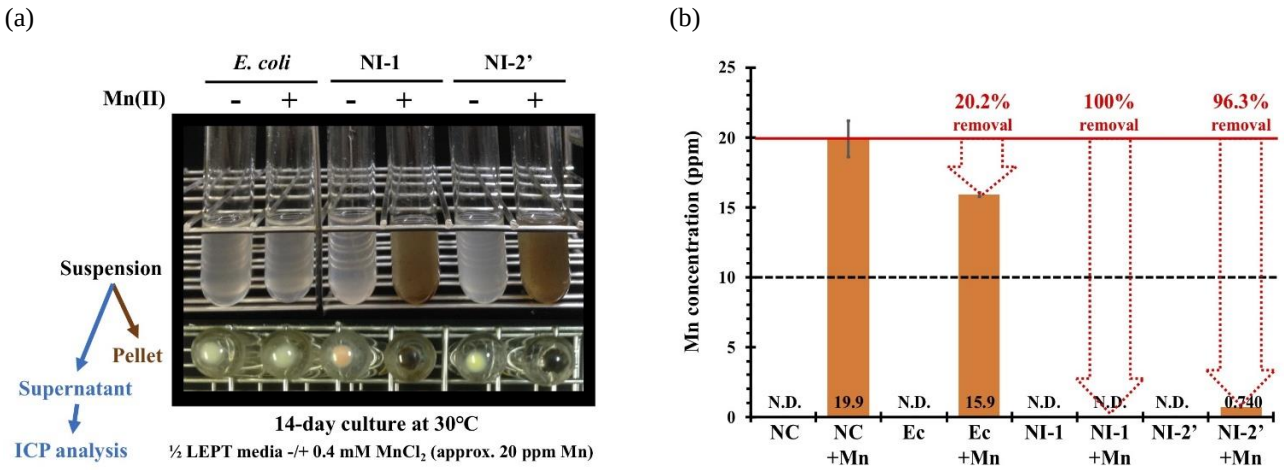
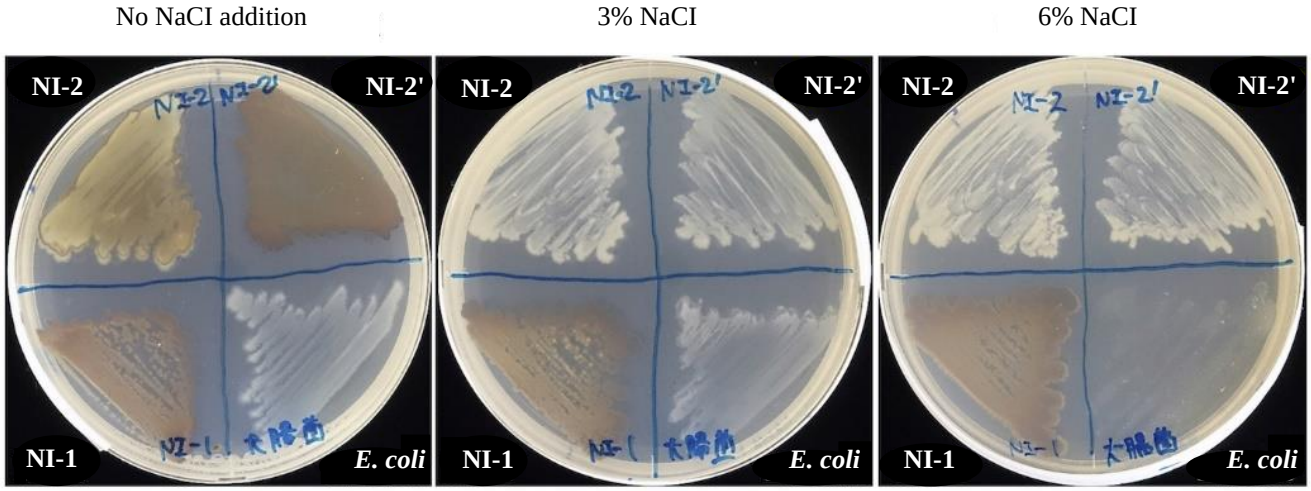


Figure 8. Bioassay of Mn oxide-biomineralization in liquid culture media using *E. coli* DH5α (Ec), *Bacillus* sp. NI-1 (NI-1), and *Arthrobacter* sp. NI-2' (NI-2'). (a), cell-suspension cultures are separated into pellets and supernatants by centrifugation and each supernatant was subjected to ICP analysis. (b), ICP-OES analysis of supernatant samples of suspension cell-cultures. N.D. indicates not detectable levels by ICP-OES because of low Mn concentration. NC indicates sample with no bacterial cells (no inoculation) as a control.



14-day cultural on LEPT media with 0.2 mM Mn(II) (pH 7.5, 30 °C)

Figure 9. Effect of NaCl stress on cell growth and Mn(II)-oxidizing activity of *Arthrobacter* sp. NI-2 and its derivative mutant, *Arthrobacter* sp. NI-2'. *Bacillus* sp. NI-1 and *E. coli* DH5α were used as a positive and a negative control strains of Mn(II)-oxidizing activity, respectively. The control plate (No NaCl addition) is exactly the same as the 14-days plate shown in Figure 7.

In order to evaluate Mn(II)-oxidizing activity of *Arthrobacter* sp. NI-2 and NI-2' under cold-stress conditions, these 2 strains together with *E. coli* DH5α and *Bacillus* sp. NI-1, were subjected to bioassay of Mn oxide-biomineralization on the Mn(II)-containing LEPT medium incubated at 30 °C, 10 °C, or 4 °C. As shown in Figure 10, the growth of *E. coli* DH5α and *Bacillus* sp. NI-1 strains were completely inhibited at 10 °C and 4 °C because of their sensitivity to cold stress, while both *Arthrobacter* sp. NI-2 and NI-2' strains thrived under these cold-stress conditions. Remarkably, *Arthrobacter* sp. NI-2' strain showed strong Mn(II)-oxidizing activity at 10 °C condition while *Arthrobacter* sp. NI-2 strain did not. These results suggest that Mn(II)-oxidizing activity may be connected to thermal-responsive signal transduction in *Arthrobacter* sp. NI-2 and NI-2'. Moreover, the *Arthrobacter* sp. NI-2' strain could be a superior strain for Mn-bioremediation of cold wastewater above 10 °C.

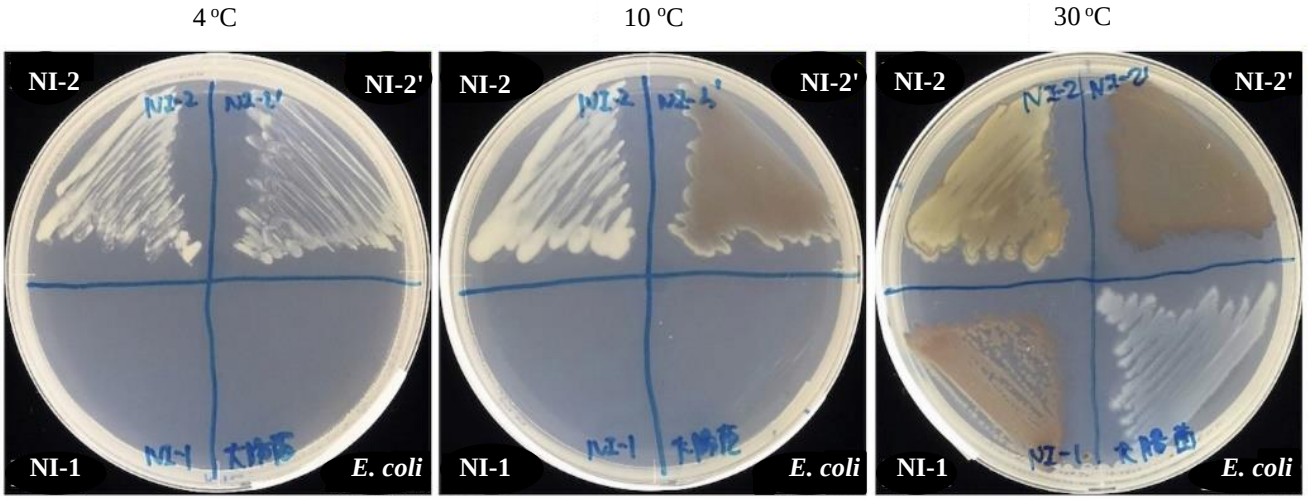


Figure 10. Effect of cold stress on cell growth and Mn(II)-oxidizing activity of *Arthrobacter* sp. NI-2 and its derivative mutant, *Arthrobacter* sp. NI-2'. *Bacillus* sp. NI-1 and *E. coli* DH5α were used as a positive and a negative control strains of Mn(II)-oxidizing activity, respectively. The control plate (30 °C) is exactly the same as the 14-days plate shown in Figure 7.

4. CONCLUSIONS

In the study, we identified and characterized Mn(II)-oxidizing marine bacteria, *Arthrobacter* sp. NI-2, and its derivative mutant *Arthrobacter* sp. NI-2' with an enhanced Mn(II)-oxidizing activity. In contrast to the previously identified *Bacillus* sp. NI-1, both NI-2 and NI-2' strains did not show Mn(II)-oxidizing activeity under NaCl-stress conditions. However, under cold-stress condition (10 °C), which inhibited the growth of *Bacillus* sp. NI-1 strain, the *Arthrobacter* sp. NI-2' strain can grow and actively oxidize Mn(II). Therefore, we conclude that, while *Bacillus* sp. NI-1 is a good strain for Mn-biomineralization under high salinity environments, the *Arthrobacter* sp. NI-2' strain can be used for Mn-bioremediation in low temperature environments, such as drainage water of Mn mines in winter or at cold climate regions.

To clarify molecular mechanisms underlying the activation of Mn(II)-oxidizing activity in *Arthrobacter* sp. NI-2' strain, we have currently established a transposon mutagenesis system for *Arthrobacter* sp. NI-2' strain with a modified method based on the methods previously described. The transposon-insertional mutants with suppressed Mn(II)-oxidizing activity obtained from the population will facilitate the identification of key genes in the mechanisms.

Chemisorption and oxidation catalyzing properties of Mn oxide (Figure 2) make Mn-biomineralization applicable for removal of multiple heavy metals and a wide range of compounds including organic contaminants from the environments. The finding of the key genes and factors in the mechanisms underlying the activation of Mn(II)-oxidizing activity will contribute to the development of efficient bioremediation technology for deconta-mination of wastewater even at low temperature conditions.

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