Isolation of a novel Co\(^{2+}\)-resistant bacterium and the application of its siderophore in Co\(^{2+}\) recovery from an aqueous solution

(Received September 21, 2018; Accepted December 25, 2018; J-STAGE Advance publication date: April 25, 2019)

Yukiko Shinozaki\(^{1,2}\)*, Hiroko Kitamoto\(^2\), Yuka Sameshima-Yamashita\(^{1,2}\), Aya Kinoshita\(^3\), Toshiaki Nakajima-Kambe\(^4\)

Research Fellow of the Japan Society for the Promotion of Science, 1-8 Chiyoda-ku, Tokyo 102-8472, Japan\(^1\); National Institute for Agro-Environmental Sciences (NIAES), 3-1-3 Kannondai, Tsukuba, Ibaraki 305-8604 Japan\(^2\); National Institute of Technology, Toyama college, 13 Hongo-machi, Toyama city, Toyama 939-8630 Japan\(^3\); Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba., Ibaraki 305-8572 Japan\(^4\);

*present address:

National Institute of Technology, Toyama college

Applied Chemistry and Chemical Engineering

13 Hongo-machi, Toyama city, Toyama 939-8630 Japan

Tel.: +81 76 493 5464

Fax: +81 76 492 3859

E-mail: shinozaki@nc-toyama.ac.jp

Keywords: siderophore, metallophore, cobalt, metal recovery.
Siderophores are considered to have a good potential as decontamination agents owing to their metal-chelating abilities. In order to confirm whether siderophores can be used in the recovery of metal ions, a siderophore (or metallophore) exhibiting Co\(^{2+}\)-chelating activity was screened to demonstrate its ability to recover Co\(^{2+}\) from an aqueous solution. A siderophore-producing bacterium, *Pandoraea* sp. HCo-4B, was identified from a screen of Co\(^{2+}\)-resistant bacteria grown in an aerobic enrichment culture with a Co\(^{2+}\)-supplemented medium. After incubation of the crude extracted siderophore in a Co\(^{2+}\)-containing solution, the Co\(^{2+}\)-siderophore complex was adsorbed on to a C\(_{18}\) column. The bound Co\(^{2+}\) was eluted from the column by the addition of 10 mM H\(_2\)SO\(_4\). The recovered amount of Co\(^{2+}\) was proportional to the amount of the added siderophore. We observed that the siderophore identified in this study binds to Co\(^{2+}\) at a 1 : 1 ratio.

**Introduction**

Siderophores are relatively low molecular weight chelating compounds, produced by bacteria and fungi under iron-deficient conditions (Neilands, 1995). The primary role of siderophores is to scavenge Fe\(^{3+}\), which is extremely insoluble under aerobic and neutral pH conditions, so as to make the mineral available to the microbial cell. The Fe\(^{3+}\)-siderophore complexes are incorporated into bacterial cells via specific membrane receptors (Schalk et al., 2011).

Although the stability constants of most Fe\(^{3+}\)-siderophore complexes are very high,
several siderophores also show strong affinities for other metals. They are also called metallophores (Johnstone and Nolan, 2015). It has been reported that siderophores complexed with metal ions other than iron have a lower uptake into cells than that of Fe$^{3+}$-siderophores by some specific membrane receptors (Schalk et al., 2011). These authors suggested that metallophores may contribute to heavy metal tolerance in bacteria, since toxic metals such as copper (Cu) and nickel (Ni) induce siderophore production in bacteria. They hypothesized that the metals present in the medium bind to siderophores, which then reduce the amount of metal ions diffusing into cells.

In other studies, siderophore-producing bacteria have proven to be effective in the phytoremediation of heavy metal-contaminated soils. Siderophores secreted by bacteria promoted metal uptake into plants (Rajkumar et al., 2010; Ma et al., 2011). Based on these findings on siderophore function, the usefulness of siderophore-producing bacteria in metal waste recovery, or remediation, has attracted recent attention (Johnston et al., 2013; Mosa et al., 2016).

We have been exploring siderophores (or metallophores) with the aim of developing a simple metal recovery method from wastewater environments and metal-contaminated soils. We report here the results of a screen, which identified a Co$^{2+}$-chelating metallophore, and the application of this metallophore in Co$^{2+}$ recovery from an aqueous solution.

**Material and Methods**

**Isolation of Co$^{2+}$-resistant bacteria**

As the sample source for screening, 10 soil samples were collected from the Ogasawara Islands
Co\textsuperscript{2+}-resistant bacteria were isolated using an aerobic enrichment culture technique according to the following protocol. Each soil sample (approximately 0.1 g) was separately added to 10 mL of nutrient broth (NB, Becton Dickinson Microbiology Systems, UK) containing 1 mM CoCl\textsubscript{2}·6H\textsubscript{2}O in a large test tube (ϕ 25 × 200 mm), and reciprocally shaken (70 times min\textsuperscript{-1}) at 30°C. When cell growth was observed, 10 µL of each culture was transferred into 10 mL of fresh medium. Following four rounds of enrichment, the culture was spread onto the same medium solidified with 1.5% (w/v) agar. After incubation at 28-30°C for 3 days, the grown colonies were isolated from the plate.

**Siderophore screening**

The siderophore-producing ability of the isolated Co\textsuperscript{2+}-resistant bacteria was evaluated by observation of a color change from blue to orange due to captured Fe\textsuperscript{3+}. The plate contained a modified minimal medium (4.0 g/L succinate, 1.0 g/L ammonium sulfate, 0.2 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.2 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, pH 7.0) with Chrome Azurol S (CAS) solution (Schwyn and Neiland, 1987) at a ratio of 9 : 1 and was solidified with 1.5% agar. The isolated bacteria were spread onto the CAS plate and incubated at 28-30°C for 3 days. When the siderophores secreted by bacteria remove Fe\textsuperscript{3+} from the Fe–CAS complex in the medium, the color of the medium around the colonies turns from blue to orange. Thus, the colonies that showed this apparent color change on the CAS plate were
selected for further experiments.

Identification of the selected bacterium by sequence of partial 16S rDNA

The isolated siderophore-producing bacterium, strain HCo-4B, was identified based on the homology of the partial 16S rDNA sequence, identified through a Basic Local Alignment Search Tool (BLAST) search against the DNA Data Bank of Japan (DDBJ) database. The 16S rDNA of HCo-4B strain was determined by a DNA analysis service (Tsuruga Bio, Toyobo Co. Ltd., Osaka, Japan), using the primer set 10F (5′-GTTTGATCCTGGCTCA-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′). The 16S rDNA sequences have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (accession number LC331267).

Siderophore production and preparation of crude siderophore extract

The isolated HCo-4B strain was grown for 3 days at 28-30°C in 100 mL of minimal medium in a 300 mL Erlenmeyer flask (6 sets), with shaking at 100 rpm. The cells were centrifuged (4,400 g for 15 min at room temperature) and the supernatant was filtered (0.45 µm PES filter unit, Nalgene). Siderophore in the supernatant was adsorbed on to a column (Sep-Pak®Plus C18, Waters, MA, USA), desalted by washing with 10 mL of purified water (Yamazen Pharmaceutical Co., Ltd., Osaka, Japan), and then the adsorbate was eluted with 3 mL of methanol. The eluate was vacuum-dried and resuspended in ultrapure water (Fujifilm Wako Pure Chemical Co., Osaka,
The siderophore activity of the culture supernatant and crude extract was evaluated by CAS assay (Schwyn and Neiland, 1987). The assay is based on the observation of color change and decrease in absorbance at 630 nm ($A_{630}$) that occurs as a result of Fe$^{3+}$ transfer from the Fe-CAS complex to the siderophore in a sample. One milliliter of CAS assay solution (15 µM Fe-CAS) and 1.0 mL of siderophore solution (diluted in 10 mM Tris-HCl buffer, pH 8.0) were mixed and left for more than 4 h at room temperature. A reference was prepared using the same buffer solution as used to dilute the sample. The equilibrium absorbance was measured at 630 nm. The activity was determined as the amount of Fe$^{3+}$ transferred from the Fe-CAS complex to the siderophore. The amount of Fe$^{3+}$ was calculated by using a calibration curve, with standard dilutions of 0, 5, 10, 15 µM Fe-CAS.

**Evaluation of the Co$^{2+}$-recovery ability of the crude siderophore extract by solid phase extraction**

An outline of the Co$^{2+}$-recovery method using a siderophore is shown in Fig. 1. The crude siderophore extracted from the culture supernatant of the selected HCo-4B strain was mixed with 10 mM Tris-HCl buffer (pH 8.0) containing CoCl$_2$·6H$_2$O (200 µM final concentration, total volume 10 mL). After incubation at room temperature for 30 min, the mixture was applied onto a column (Sep-Pak®Plus C$_{18}$, Waters) and the flow-through fraction was collected as Sample 1. Then, 5 mL of 10 mM H$_2$SO$_4$ was added to the column and the eluate was collected as Sample 2. The adsorbate
was eluted with 3 mL of methanol and collected as Sample 3. The Co\(^{2+}\) concentration in Samples 1 and 2 was determined using a colorimetric method with Nitroso-R salt (Zeiner et al., 2012). One milliliter of Sample 1 and 0.1 mL of reagent solution (0.25 g Nitroso-R salt (2-Hydroxy-1-nitroso-3,6-naphthalenedisulfonic acid disodium salt) in 100 mL ultrapure water) were mixed, and the absorbance was measured at 500 nm. Sample 2 (0.5 mL) was mixed with 0.5 mL of 200 mM Tris-HCl buffer, pH 8.0 and 0.1 mL of the reagent solution, and the absorbance was measured at 500 nm. In order to estimate the affinity of the siderophore for Co\(^{2+}\) and Fe\(^{3+}\), the Co\(^{2+}\) recovery rates with and without Fe\(^{3+}\) were compared (CoCl\(_2\), FeCl\(_3\), and the siderophore were each added at a final concentration of 200 µM). The Co\(^{2+}\) recovery rate was calculated from the amount of Co\(^{2+}\) recovered and the amount of Co\(^{2+}\) added. The siderophore activity in Sample 3 was evaluated by CAS assay as described above.

**LC-ESI-MS analysis**

To investigate the molecular weight of the siderophore in the crude extract, liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) was performed on an Alliance 2690 system (Waters) using an Inertsil ODS-3 column (ϕ 2.1 × 100 mm, 3 µm; GL Sciences, Japan). The mobile phase consisted of aqueous 50 mM acetic acid and methanol. A linear gradient of 5–30% methanol was used for 30 min at a flow rate of 0.2 mL/min. Mass spectrometry was performed using a Micromass ZQ system (Waters) equipped with an electrospray ion source.
Nitrogen was used as the sheath gas. The operating parameters were as follows: capillary voltage, 2.5 kV; cone voltage, 40 V; source temperature, 100°C; desolvation temperature, 350°C; cone gas flow, 50 L/h; and desolvation gas flow, 350 L/h.

**Results and Discussion**

**Screening of Co$^{2+}$-resistant and siderophore-producing bacteria**

Siderophore-producing bacteria exhibiting Co$^{2+}$-chelating activity are expected to grow in media containing high Co$^{2+}$ concentrations. However, some bacteria exhibit metal tolerance owing to the presence of an efflux pump (Nies, 2003), and not all metal-resistant bacteria produce siderophores. Hence, bacteria were screened for their ability to produce siderophores. As potential sources, we screened soil samples collected from the Ogasawara Islands, because we expected the samples to contain many endemic species. As a result, a Co$^{2+}$-resistant and siderophore-producing bacterium, strain HCo-4B, was isolated from Mukojima soil by an aerobic enrichment culture using a medium supplemented with Co$^{2+}$.

**Co$^{2+}$-recovery ability of the crude siderophore**

The siderophore activity (captured Fe$^{3+}$) of the culture supernatant of HCo-4B was 56.5 ± 10.6 nmol/mL. The siderophore was then partially purified and concentrated to 2.34 µmol/mL (total
volume of 8.5 mL) using a C\textsubscript{18} column.

The crude siderophore extract was added to Co\textsuperscript{2+} solution at various concentrations and the Co\textsuperscript{2+}-recovery ability was tested. As a result, the concentration of Co\textsuperscript{2+} in Sample 1 (flow-through fraction) decreased, while that of Sample 2 (eluted with H\textsubscript{2}SO\textsubscript{4}) increased, proportionally with the increase in the amount of siderophore added (Fig. 2). The recovered amount of Co\textsuperscript{2+} and the added siderophore activity were almost equal in the ratio (1 : 1). Similar to the findings of a previous report, where two siderophores with similar structures bound Fe\textsuperscript{3+} and Zn\textsuperscript{2+} at a ratio of 1 : 1 to the same location on the siderophore (Johnstone and Nolan, 2015), our data suggests that the siderophore identified in this study also binds Fe\textsuperscript{3+} and Co\textsuperscript{2+} in a ratio of 1 : 1. In general, siderophores have a high affinity for Fe\textsuperscript{3+} and a relatively low affinity for other metals (Schalk et al., 2011). In order to estimate the affinity of the siderophore for Co\textsuperscript{2+} and Fe\textsuperscript{3+}, the Co\textsuperscript{2+} recovery rate with and without Fe\textsuperscript{3+} was compared. The Co\textsuperscript{2+} recovery rate without Fe\textsuperscript{3+} was 97.7 \pm 0.5\%, and 94.2 \pm 1.0\% when Fe\textsuperscript{3+} was added. Since the decrease in the Co\textsuperscript{2+} recovery rate due to the addition of Fe\textsuperscript{3+} was small, the affinity of the siderophore for Co\textsuperscript{2+} is considered to be higher than that for Fe\textsuperscript{3+}. For further details, it would be necessary to perform a similar study using purified siderophore.

The measurement of siderophore activity of Sample 3 showed that 87.4 \pm 10.0 \% of the siderophore was recovered and found to be reusable (data not shown).

\textit{LC-ESI-MS analysis}
LC-ESI-MS analysis of the crude extracted siderophore revealed the presence of pseudomolecular ions at \( m/z \) 946 \([M - H]^-\) and \( m/z \) 948 \([M + H]^+\) (data not shown). The molecular mass was determined to be 947, which did not correspond to a molecular weight of any known siderophore (Lehner et al., 2013).

This study represents the first step towards the development of a model system for metal recovery using siderophores from an aqueous solution. We used a relatively low metal concentration for this evaluation. However, since the selected siderophore appeared to bind to \( \text{Co}^{2+} \) in a ratio of 1:1, it is expected that increasing the amount of siderophore would increase the amount of metal that can be concentrated. Since the identified siderophore did not lose its activity even when autoclaved, it appears to have a high thermal stability. In addition, the siderophore adsorbed on to a C\(_{18}\) column can be eluted with methanol and used repeatedly. Therefore, we believe that this siderophore can be used for practical applications in metal recovery. To the best of our knowledge, this is the first report of a simple method for \( \text{Co}^{2+} \) recovery using a siderophore. Future studies will focus on using wastewater from metal plating factories and investigating metal selectivity. The structure of the identified siderophore will be determined in the near future.

Acknowledgments

We thank Dr. Syuntaro Hiradate (NIAES, currently Professor at Kyushu University) for providing the soil samples from the Ogasawara Islands. We thank Dr. Tomoyuki Makino (NIAES, currently Professor at Tohoku University) and Dr. Masumi Ishizaka (NIAES) for the analysis of metal ions.
and LC-ESI-MS analysis, respectively. This work was supported by JSPS KAKENHI grant numbers 12J40219, 15K05590.

References


Figure legends

Fig. 1 Outline of the evaluation method for Co\(^{2+}\) recovery using the siderophore. See text for details.

Fig. 2 Co\(^{2+}\) recovery using a crude extract of the siderophore

Co\(^{2+}\) recovery using the siderophore produced by *Pandoraea* sp. HCo-4B was evaluated based on the method presented in Fig. 1. Values are expressed as mean (SD) at n = 3.
**Fig. 1**

Crude siderophore
200 μM CoCl$_2$·6H$_2$O
50 mM Tris-HCl (pH 8.0)

- Adsorbed on C$_{18}$ column
- Eluted with 10 mM H$_2$SO$_4$
- Eluted with 100% methanol
- Sample-1 (flow-through)
- Sample-2 (recovered Co$^{2+}$)
- Sample-3 (recovered siderophore)
Fig. 2

The graph shows the relationship between Co^{2+} concentration (nmol/mL) and siderophore activity (nmol/mL). There are two samples, Sample-1 and Sample-2, plotted on the graph. Sample-1 shows a decrease in Co^{2+} concentration with an increase in siderophore activity, while Sample-2 shows an increase in Co^{2+} concentration with a decrease in siderophore activity.