Appl Microbiol Biotechnol (2000) 54: 581–588

ORIGINAL PAPER

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Zinc biosorption by a zinc-resistant bacterium, *Brevibacterium* sp. strain HZM-1

Received: 1 February 2000 / Received revision: 31 March 2000 / Accepted: 1 May 2000

Abstract A zinc-resistant bacterium, *Brevibacterium* sp. strain HZM-1 which shows a high Zn^{2+}-adsorbing capacity, was isolated from the soil of an abandoned zinc mine. Kinetic analyses showed that Zn^{2+} binding to HZM-1 cells follows Langmuir isotherm kinetics with a maximum metal capacity of 0.64 mmol/g dry cells and an apparent metal dissociation constant of 0.34 mM. The observed metal-binding capacity was one of the highest values among those reported for known microbial Zn^{2+} biosorbents. The cells could also adsorb heavy metal ions such as Cu^{2+}. HZM-1 cells could remove relatively low levels of the Zn^{2+} ion (0.1 mM), even in the presence of large excess amounts (total concentration, 10 mM) of alkali and alkali earth metal ions. Bound Zn^{2+} ions could be efficiently desorbed by treating the cells with 10 mM HCl or 10 mM EDTA, and the Zn^{2+}-adsorbing capacity of the cells was fully restored by treatment of the desorbed cells with 0.1 M NaOH. Thus, HZM-1 cells can serve as an excellent biosorbent for removal of Zn^{2+} from natural environments. The cells could grow in the presence of significant concentrations of $ZnCl_2$ (at least up to 15 mM) and thus is potentially applicable to in situ bioremediation of Zn^{2+}-contaminated aqueous systems.

Introduction

Metallic zinc has been used for a variety of applications such as galvanization, the manufacture of brass and other alloys, and the fabrication of batteries (Barceloux 1999). The extensive use of zinc without its recovery has caused contamination of soil and freshwater habitats by this divalent cation (Jung and Thornton 1997; Talbot 1986; Vasconcelos and Tavares 1998). Also, due to the relatively low melting point of this metal, industrial plants that employ the roasting or heating of zinc compounds for metallic zinc production are potential sources of environmental zinc contamination (Barceloux 1999; Nies 1992). Although zinc is an essential nutrient as a trace element for animals, plants, and microorganisms, it is toxic to these organisms when present at millimolar concentrations (Barceloux 1999). For example, it is toxic for humans if incorporated at levels of 100–500 mg/day (Barceloux 1999; Fosmire 1990; Moore and Ramamoorthy 1984). A recent report shows that activated sludge samples from Sendai, Japan, contained 10^2–10^3 ppm of Zn^{2+} ion. Because this sludge is an important source of compost, lowering the levels of Zn^{2+} in the waste water is of environmental and agricultural importance.

The biosorption phenomenon is a metabolism-independent binding of heavy metals to biomass such as microbial and plant cells. The use of non-living microbial cells as biosorbents has been shown to be an effective means for removal or recovery of heavy metallic species from aqueous systems (Volesky and Holan 1995). This is exemplified by the removal of cadmium (Volesky et al. 1993), copper (de Rome and Gadd 1987), lead (Niu et al. 1993), and mercury ions (Tobin et al. 1984) from waste water and the recovery of gold (Kuyucak and Volesky 1989), silver (Tobin et al. 1984), and uranium ions (Bengtsson et al. 1995) from natural waters.

In this paper, we have isolated from the soil of an abandoned zinc mine at Hosokura, Miyagi, Japan, a zinc-resistant bacterium which can efficiently adsorb zinc. We analyzed the metal-binding properties of the bacterium and examined its usefulness as a biosorbent for zinc removal from aqueous systems. Because the cells of the isolate were viable at significant concentrations of

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zinc ion (>15 mM) and were found to be capable of adsorbing the metal ion under such conditions, the bacterium can potentially be used as an agent for zinc removal through an in situ bioremediation approach, in which zinc biosorption occurs during the bacterial growth at the contaminated site.

Materials and methods

Taxonomic studies on strain HZM-1

The methods used for preparation of chromosomal DNA and polymerase chain reaction (PCR) amplification of the 16S rRNA gene from chromosomal DNA and the primers used for PCR amplification have been described previously (Shida et al. 1996). Amplified 16S rRNA gene purified with a QIAquick spin PCR purification kit (Qiagen, Hilden, Germany) were used for the sequencing template. Sequencing was carried out as described by Sanger et al. (1977) using a Dye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer, Foster City, Calif.) and a model ABI 373A automatic DNA sequencer (Perkin Elmer). Sequences determined were compared with 16S rRNA gene sequences obtained from EMBL, GenBank, and DDBJ databases. Multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc} values; Kimura 1980), construction of a neighbor-joining phylogenetic tree (Saito and Nei 1987), and a bootstrap analysis for evaluation of the phylogenetic topology (Felsenstein 1985) were carried out with the CLUSTAL W version 1.5 program (Thompson et al. 1994).

Metal ion studies

$ZnCl_2$, $ZnSO_4$, $Zn(CH_3COO)_2$, NaCl, KCl, $MgCl_2$, $CaCl_2$, $CuSO_4 \cdot 5H_2O$, ethylenediaminetetraacetic acid (EDTA), and HNO_3 were of analytical grade and were obtained from Nacalai Tesque, Kyoto, Japan. The Milli-Q grade water was prepared with the Milli-Q Labo system (Millipore). Zn^{2+} content was determined by atomic absorption spectroscopy using a model AA-6700F apparatus (Shimadzu).

Preparation of the HZM-1 biosorbent

HZM-1 cells were grown in a medium (pH 6.8, 10 ml) containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, and 5 mM $ZnCl_2$ with shaking at 30 °C overnight. The culture was then inoculated into the same medium as above (1 l), except that $ZnCl_2$ was omitted and the cells were grown under the same conditions. Immediately after the optical density at 610 nm (OD_{610}) of the culture reached 1.0, the cells were harvested by centrifugation (5000 *g*). The collected cells were extensively washed with sterilized Milli-Q grade water, followed by lyophilization and were then used as a biosorbent.

Metal adsorption studies

The weighed quantities of lyophilized cells (typically 40 mg) were suspended in 10 ml of 1 mM HCl containing known concentrations (0–10 mM) of $ZnCl_2$. The pH was adjusted with 1 N HCl and 1 N NaOH as required. To avoid unknown effects of buffer components, buffering was not used. The mixtures were incubated at 30 °C for 12 h with gentle mixing, followed by centrifugation (5000 *g*) for 5 min. The collected cells were then lyophilized, treated with 70% HNO_3, and analyzed for Zn^{2+} by atomic absorption spectroscopy using a model AA-6700F apparatus (Shimadzu). The supernatants were also analyzed for equilibrium Zn^{2+} concentrations. Analyses of Cu^{2+} binding to HZM-1 cells were carried out using $CuSO_4 \cdot 5H_2O$ essentially in the same way as above.

Desorption and resorption studies

The cells (40 mg dry cells) adsorbing Zn^{2+} (0.56 mmol/g dry cells) were washed with an excess amount (20 ml) of one of the following solvents: H_2O (Milli-Q grade), 10 mM EDTA, 10 mM HCl at pH 2.0, 0.1% (w/v) Triton X-100, 50 mM sodium dodecyl sulfate, 0.1 mM cetyltrimethylammonium bromide, or 0.1 M Na_2CO_3. After collecting the cells by centrifugation, the Zn^{2+} content of the resultant cells was determined by atomic absorption spectrometry.

For resorption studies, the cells (approximately 40 mg dry cells) adsorbing Zn^{2+} (0.56 mmol/g dry cells) were washed with an excess amount (more than 20 ml) of 10 mM EDTA or 10 mM HCl, pH 2.0, followed by extensive washing with H_2O; and their Zn^{2+} adsorption capacities were analyzed as above. The desorbed cells thus obtained, either EDTA-treated or HCl-treated, were also washed with an excess amount (more than 20 ml) of 0.1 M NaOH followed by extensive washing with H_2O; and the Zn^{2+} adsorption capacities of the resultant cells were assayed as described above.

DNA sequence

The nucleotide sequence coding for 16S rRNA of *Brevibacterium* sp. strain HZM-1 has been submitted to the GenBank database under accession number AF229116.

Results

Isolation of bacterial Zn^{2+} biosorbent and its phylogenetic position

Microorganisms that could grow on a medium containing 5 mM $ZnCl_2$ were isolated and their Zn^{2+} adsorbing activities were analyzed. An isolate having the highest Zn^{2+} adsorbing capacity, strain HZM-1, was a Gram-positive rod; and the minimum Zn^{2+} concentration for inhibition (MIC) of its growth was 15 mM. For comparison, the MIC of the growth of a control bacterium, *Escherichia coli* strain JM109, was 2 mM under the same growth conditions.

To determine the phylogenetic relationship of the bacterium with known bacteria, the genomic DNA of the bacterium was extracted and the DNA coding for 16S rRNA was amplified by PCR. The nucleotide sequence of the amplified DNA was determined and compared with available 16S rRNA sequences to construct a phylogenetic tree (Fig. 1). The results clearly showed that the strain HZM-1 could be classified in the genus *Brevibacterium*.

Zn^{2+} biosorption by HZM-1 cells

It has been shown that the metal adsorption capacity of microbial biosorbents varies with their growth phase (Macaskie and Dean 1982; Volesky et al. 1993). Therefore, the relationship between the Zn^{2+} adsorption capacity and the growth phase of the bacterium was analyzed first. HZM-1 cells harvested at various phases during growth were incubated with 5 mM $ZnCl_2$ at pH 3.0 and 30 °C for 12 h at a cell concentration of 0.4% w/v; and the adsorbed Zn^{2+} on the cells from this

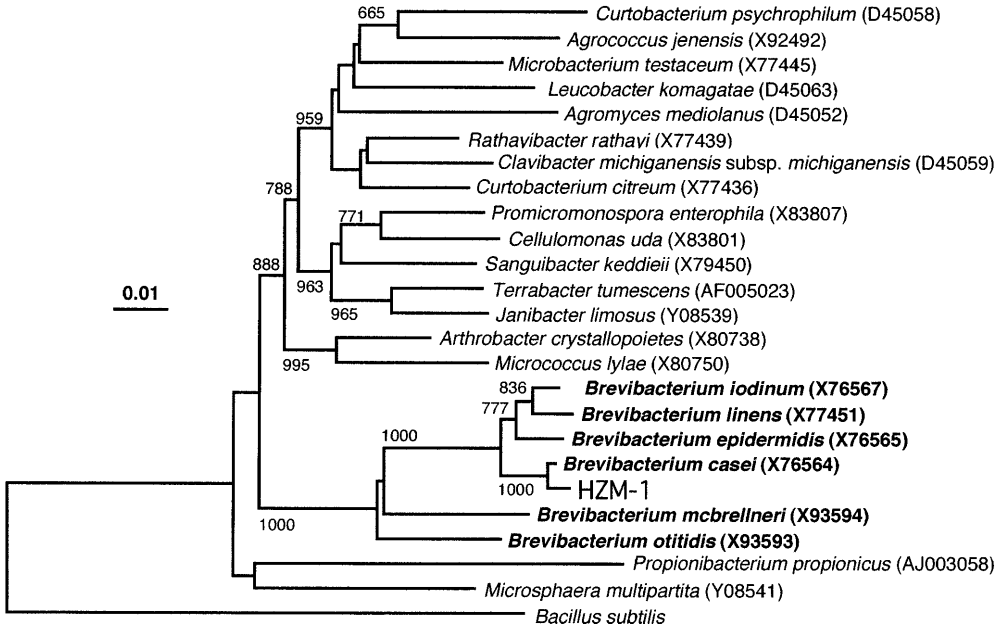


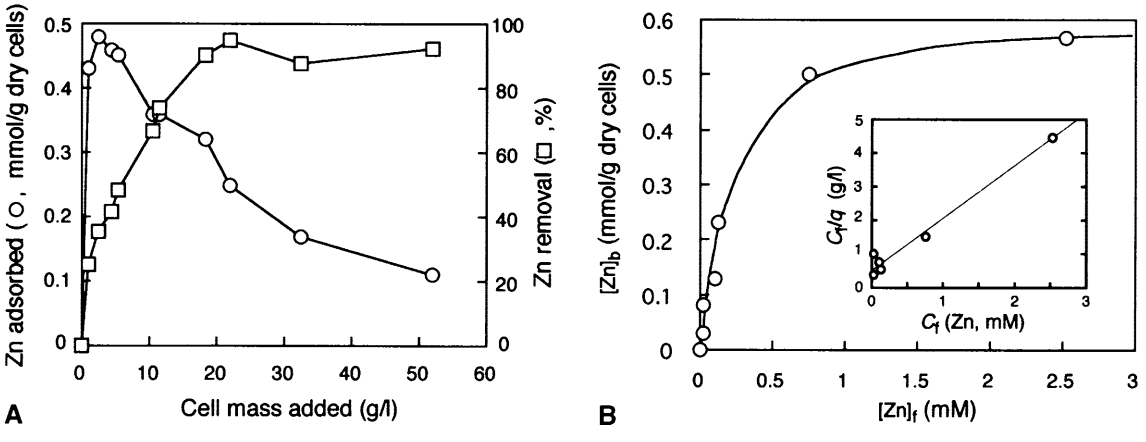
Fig. 1 Phylogenetic tree derived from 16S rRNA sequence data of strain HZM-1 and other related species. The GenBank accession number for each microorganism used in the analysis is shown *in parentheses* after the species name. Species belonging to the genus *Brevibacterium* are shown *in bold*. The *numbers* indicate bootstrap values greater than 600. *Bar* 0.01 nucleotide substitutions per site

incubation (see Materials and methods) was determined by atomic absorption spectroscopy. The results showed that the cells harvested during the logarithmic phase (OD_{610}, 1.0) showed the highest metal capacity (data not shown). Also, after lyophilization, HZM-1 cells showed 1.3-times higher Zn^{2+} adsorption capacity than the original wet form of the cells. Therefore, all biosorption studies described below were executed using the lyophilized form of exponentially growing cells.

Then the effects of cell concentration on both Zn^{2+} adsorbing capacity and extent of Zn^{2+} removal from the mixture (initial Zn^{2+} concentration of the mixture, 5 mM; Fig. 2A) during incubation were analyzed. The amount of Zn^{2+} adsorbed by the cells reached a maximum (0.49 mmol/g dry cells in this experiment) at cell concentrations of 0.3–0.5% (dry w/v) and then decreased with increasing cell concentrations, being simply due to dilution of the metal with the added cells. In contrast, the extent of Zn^{2+} removal from the mixture increased with increasing cell concentrations, and nearly all of the Zn^{2+} ions present in the mixture could be removed when the cell concentrations were higher than 2% (dry w/v), although the minimum cell concentration required for Zn^{2+} removal should depend on the initial Zn^{2+} concentration. All kinetic studies were done at a cell concentration of 0.4%, unless otherwise stated.

The time course of the Zn^{2+} adsorption by HZM-1 cells at 30 °C in 5 mM $ZnCl_2$ (pH 3.0) was also analyzed. HZM-1 cells rapidly adsorbed Zn^{2+} ions. The

Fig. 2 **A** Effect of cell concentration on Zn^{2+} adsorption to the cells (○) and the extent of Zn^{2+} removal from the aqueous phase (□). Varying amounts of cells were suspended in 5 mM $ZnCl_2$ in 1 mM HCl (pH 3.0, 10 ml) and were incubated at 30 °C for 12 h with gentle mixing. After incubation, Zn^{2+} contents in the cells and supernatant were analyzed as described. **B** Equilibrium-binding of Zn^{2+} to HZM-1 cells. The binding of Zn^{2+} to HZM-1 cells was measured after incubation at 30 °C with 1 mM HCl, pH 3.0, containing different concentrations of $ZnCl_2$ as described under Materials and methods. *Open circles* show the mean for three determinations and all errors are < 5%. *Inset* The equilibrium-binding data were plotted according to a linear format of the Langmuir isotherm equation: $[Zn]_f/[Zn]_b = [Zn]_f/Q_{max} + K_d/Q_{max}$, where $[Zn]_f$ is the free Zn^{2+} concentration, $[Zn]_b$ is Zn^{2+} adsorbed by the cells (mmol Zn^{2+}/g dry cells), Q_{max} is the maximum metal capacity of the cells (mmol/g dry cells), and K_d is the apparent metal dissociation constant



binding equilibrium was established within 15 min after the metal addition and was sustained over 10 h.

Kinetic characterization

The adsorption equilibria of Zn^{2+} on HZM-1 cells were analyzed (see Materials and methods). A constant amount of HZM-1 cells was repeatedly incubated with $ZnCl_2$ (0–5 mM) at pH 3.0 and 30 °C; and the amount of Zn^{2+} bound to the cells at equilibrium was determined as well as that in the supernatant. The binding data at equilibrium were seemingly fitted to the Langmuir isotherm equation (Fig. 2B):

$$[Zn]_b = Q_{max} \times [Zn]_f / (K_d + [Zn]_f) \quad (1)$$

where $[Zn]_b$ is the amount of metal adsorbed (mmol/g dry cells), Q_{max} is the maximum metal capacity of the cells (mmol/g dry cells), K_d is the apparent metal dissociation constant (mM), and $[Zn]_f$ is the equilibrium metal ion concentration (mM). A linear transformation of Eq. 1 (Fig. 2B, inset) allowed us to determine the Q_{max} and K_d values to be 0.64 mmol/g dry cells and 0.34 mM, respectively. When the experiment was executed with either $ZnSO_4$ or $Zn(CH_3COO)_2$ instead of $ZnCl_2$, the Zn^{2+}-binding kinetics were indistinguishable from those obtained with $ZnCl_2$, indicating that any effect of a counter anion on the Zn^{2+} biosorption should be negligible.

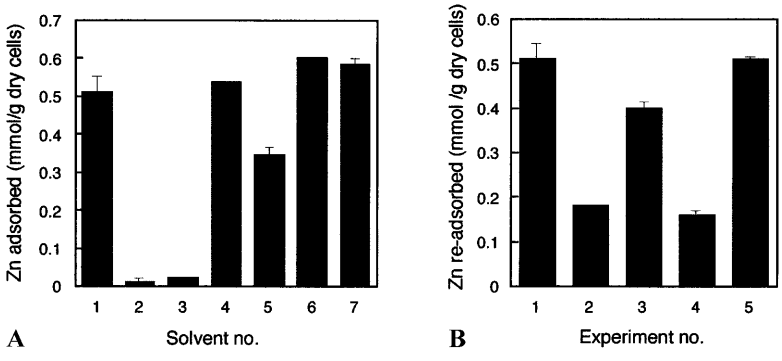
Effects of temperature and pH on zinc biosorption

Zn^{2+} biosorption by HZM-1 cells was virtually unaffected by temperature at the temperatures examined (5–70 °C); the maximum biosorption was obtained at 37 °C, but at 5 °C and 70 °C the cells still retained a capacity which was more than 80% of the maximum. However, the biosorption was found to be pH-dependent. HZM-1 cells showed a maximum Zn^{2+} adsorption at pH 3–7, but lost their Zn^{2+}-adsorbing activity at pH lower than 2. Consistently, the loaded metal could efficiently be desorbed by washing the cells with dilute acid (see below). In addition, the observed temperature- and pH-dependence profiles, along with results obtained from desorption/resorption studies (see below), provide a basis for the mechanistic consideration of Zn^{2+} biosorption by HZM-1 cells, which will be discussed in the Discussion.

Desorption/resorption studies

Many biosorbents can desorb their load of heavy metal ions by such procedures as acid treatment; and this feature is useful for recovering the bound metal ions (Inthorn 1996; Puranik and Paknikar 1997). In most cases however, biosorbents retain only insufficient biosorption capacities after the desorption procedure. These circumstances led us to analyze the Zn^{2+} desorption and resorption processes of HZM-1 cells. To find an efficient eluent for Zn^{2+} desorption, Zn^{2+}-bound HZM-1 cells were washed with a variety of aqueous solutions, which have thus far been used for desorption studies of other biosorbents (Puranik and Paknikar 1997), and the Zn^{2+} that remained bound to the cells was determined by atomic absorption spectrometry (Fig. 3A). Consistent with the results of the pH dependence studies (see above), 10 mM HCl was a very good eluent for Zn^{2+} desorption and allowed nearly

Fig. 3 **A** Desorption of Zn^{2+} from HZM-1 cells. The cells (40 mg dry cells) adsorbing Zn^{2+} (0.56 mmol/g dry cells) were washed with an excess amount (20 ml) of the solvents listed below. The Zn^{2+} bound to the resultant cells was determined by atomic absorption spectrometry. Solvents used were: *1* Milli-Q water, *2* 10 mM EDTA, *3* 10 mM HCl at pH 2.0, *4* 0.1% (w/v) Triton X-100, *5* 50 mM sodium dodecyl sulfate, *6* 0.1 mM cetyltrimethylammonium bromide, and *7* 0.1 M Na_2CO_3. *Error bars* represent mean ± standard error ($n = 3$). **B** Resorption of Zn^{2+} to HZM-1 cells. Experiments: *1* cells without prior desorption procedures were suspended in 10 mM $ZnCl_2$ in 1 mM HCl and incubated at 30 °C for 12 h with gentle mixing, and the amount of Zn^{2+} adsorbed onto the cells were determined, *2–5* Zn^{2+}-bound cells obtained in experiment 1 were then treated as described below (*experiments 2–5*). Finally, the cells were suspended in 10 mM $ZnCl_2$ in 1 mM HCl and incubated at 30 °C for 12 h with gentle mixing; and the amount of Zn^{2+} re-adsorbed onto the cells was determined. Treatments prior to the resorption process were as follows: *experiment 2* washing with 10 mM EDTA, *experiment 3* washing with 10 mM EDTA and subsequently with 0.1 M NaOH, *experiment 4* washing with 10 mM HCl, pH 2.0, *experiment 5* washing with 10 mM HCl and subsequently with 0.1 M NaOH. *Error bars* represent mean ± standard error ($n = 3$). For experimental details, see text



quantitative recovery of the loaded Zn^{2+}. Ten millimolar EDTA was also an efficient eluent, whereas nonionic and cationic detergents could not desorb the bound Zn^{2+}. After desorption with 10 mM HCl or 10 mM EDTA, the cells were found to have reduced Zn^{2+}-adsorbing capacities, which were approximately 30% of the original (Fig. 3B). However, treatment of the cells with 0.1 M NaOH after desorption could fully restore the Zn^{2+} biosorption capacity of the cells. It should be noted that more than a five-fold increase in Zn^{2+}-binding capacity upon NaOH treatment has been reported for fungal biosorbents (Fourest and Roux 1992). However, this was not the case for the HZM-1 biosorbent.

Binding of other heavy metal ions

It has been shown that several biosorbents adsorbing Zn^{2+} can also efficiently adsorb other heavy metal ions. To examine whether this is also the case for HZM-1 cells, the binding of Cu^{2+} ions was analyzed . The Cu^{2+} ion was selected as an example because its extensive use and increasing level in the environment are causes for concern (Chong and Volesky 1995). The results showed that HZM-1 cells could bind Cu^{2+} efficiently. Kinetic analyses showed that the binding of Cu^{2+} to the cells also followed the Langmuir isotherm kinetics (data not shown), and the Q_{max} and K_d values of Cu^{2+} binding to the cells (at pH 3.0, 30 °C) were 0.51 mmol/g dry cells and 0.45 mM, respectively.

Effect of alkali and alkali-earth metal ions

The alkali and alkali-earth metal ions such as Na^+, K^+, Mg^{2+}, and Ca^{2+} are ubiquitously present in the environmental waters. Thus, knowledge of the effect of these metal ions, termed here "coexisting cations", on Zn^{2+} biosorption is important for efficient application of the biosorbent. Adsorption of Zn^{2+} (initial concentration, 5 mM) by HZM-1 cells in the presence of one or all of the Na^+, K^+, Mg^{2+}, and Ca^{2+} ions (initial concentration, 0.1–10 mM) is presented in Fig. 4. The Na^+ and K^+ ions had virtually no effect on Zn^{2+} biosorption by HZM-1 cells. In contrast, the presence of Ca^{2+} ion caused a small but appreciable decrease in the Zn^{2+}-biosorption capacity. The capacity decreased with increasing concentrations of this divalent metal ion and was 47% of the control when the initial Ca^{2+} concentration was 10 mM. The presence of Mg^{2+} also caused a decrease in the Zn^{2+}-adsorbing capacity, but the effect was weaker than that of the Ca^{2+} ion. When equimolar amounts of the four coexisting cations (e.g., 5 mM for each metal) were simultaneously present in the mixture, the Zn^{2+}-adsorbing capacity of the cells was comparable to that obtained with the system containing 5 mM Ca^{2+} as a sole coexisting cation.

To examine whether or not HZM-1 cells could specifically remove relatively low levels (e.g., 10–100 ppm) of Zn^{2+} ions from aqueous mixtures even in the presence of a large excess of the coexisting cations, HZM-1 cells (2% w/v) were incubated with 0.1 mM Zn^{2+} ion containing an equimolar amount (2.5 mM for each) of one or a mixture of coexisting cations [Na^+, K^+, Mg^{2+}, and/or Ca^{2+} ions (as chloride)] in 1 mM HCl, pH 3.0, at 30 °C for 12 h; and the Zn^{2+} content of the cells and supernatants was determined. In the absence of coexisting cations, HZM-1 cells could remove 90% of the Zn^{2+} present in the mixture after incubation. However, in the presence of coexisting cations, the cells could still remove 60% of Zn^{2+} present in the mixture. Thus, the cells can effectively remove dilute Zn^{2+} ions from the mixture in the presence of a 10–100-times molar excess of coexisting cations.

Removal of Zn^{2+} during the growth of HZM-1 cells

One of the remarkable features of *Brevibacterium* sp. HZM-1 is that it can grow in the presence of significant concentrations (up to 15 mM, i.e., 981 ppm) of Zn^{2+}. If HZM-1 cells can adsorb the metal as they grow, the bacterium potentially serves as an agent for in situ bioremediation of Zn^{2+}-contaminated aqueous systems. To explore this possibility, HZM-1 cells were grown in a medium (pH 6.8, 100 ml) containing 5 mM $ZnCl_2$ with shaking at 30 °C; and both bacterial growth and the

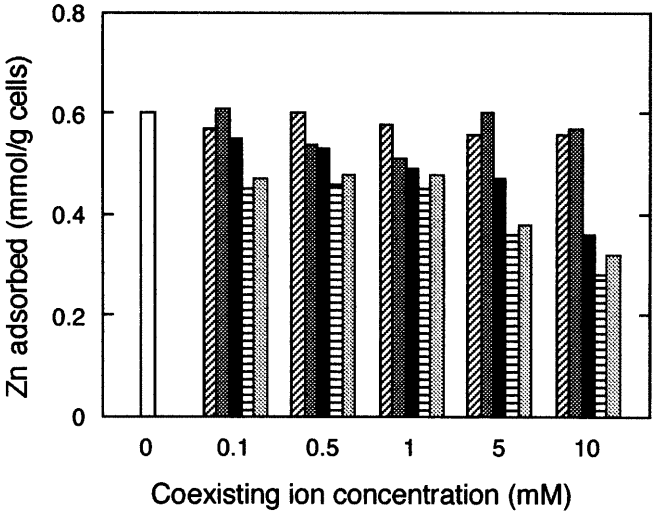


Fig. 4 Effect of coexisting alkali and alkali-earth metal ions, i.e., coexisting cations, on the Zn adsorption capacity of HZM-1 cells. The Zn^{2+} content of the cells was determined at 30 °C and pH 3.0; conditions were essentially identical to that described under Materials and methods, except that the incubation mixture (initial Zn^{2+} concentration, 5 mM) also contained one or all of the following coexisting cations (as chloride) at the concentration indicated: *diagonal hatching* Na, *stippling* K, *black* Mg, *horizontal hatching* Ca, *herring-bone hatching* all four coexisting cations simultaneously present, and *white* no added coexisting cation. Columns show the mean for three determinations and all errors are < 5%

Zn^{2+} content of the bacterial cells and culture supernatants were monitored during the cultivation (Fig. 5).

The results showed that HZM-1 cells could adsorb Zn^{2+} during their growth. Atomic absorption spectrometric analysis of the culture supernatant showed that 65% of the initial Zn^{2+} could be removed from the culture supernatant after 7 days of cultivation. The cells were harvested and subjected to desorption procedures (see above). Very interestingly, the cells retained more than 70% of the bound Zn^{2+} after extensive washing of cells with 10 mM HCl, pH 2.0, or 10 mM EDTA. This is in striking contrast with the observation that sorbent cells grown in the absence of Zn^{2+} lost almost all of the bound Zn^{2+} upon washing with these desorbing agents. These results suggest that desorbed Zn^{2+} (30% of the cell-associated Zn^{2+}) should arise from Zn^{2+} which had been adsorbed on the cell surface, whereas the residual Zn^{2+} remained incorporated within HZM-1 cells. It should also be mentioned that the estimated amount of Zn^{2+} adsorbed on the cell surface (after 7 days of cultivation) was 0.15 mmol/g dry cells, which was significantly lower than the maximum capacity (0.64 mmol/g dry cells) observed for lyophilized cells.

Discussion

Usefulness of HZM-1 cells as a microbial Zn^{2+} biosorbent

The Zn^{2+} ion occurs at relatively high concentrations in wastewaters (10–1000 ppm depending on the environments) and exerts its toxicity at much higher concentrations than do heavy metallic species such as cadmium, copper, lead, and mercury ions (Barceloux 1999). To find microorganisms which can efficiently remove such relatively high concentrations of Zn^{2+} from aqueous phase, Zn^{2+}-resistant bacteria were isolated and their Zn^{2+}-adsorbing capacities were analyzed. Among the isolates, strain HZM-1 (which was a Gram-positive bacterium isolated from the soil at an abandoned zinc mine) had a resistance to Zn^{2+} and was found to exhibit high Zn^{2+}-adsorbing capacity. Phylogenetic analyses showed that HZM-1 strain belongs to the genus *Brevibacterium*. To our knowledge, this is the first example of a *Brevibacterium* strain which can be used as a metal biosorbent.

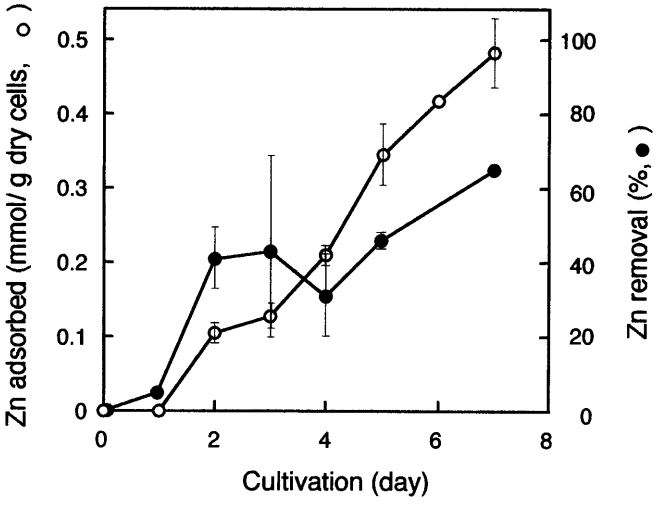


Fig. 5 Binding of Zn^{2+} to HZM-1 cells and its removal from medium during growth. HZM-1 cells were inoculated into a medium (pH 6.8, 100 ml) containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, and 5 mM $ZnCl_2$, cultivated with shaking at 30 °C; and the Zn^{2+} content of the bacterial cells and culture supernatants was monitored during the cultivation. ○ Zn^{2+} content of the cells, ● % removal of Zn^{2+} from the culture supernatant. *Error bars* represent mean ± standard error ($n = 3$)

The capacity and specificity of the metal-binding and desorption/resorption characteristics of HZM-1 cells showed that the cells should serve as a very good biosorbent for Zn^{2+}. The Q_{max} value for Zn^{2+} binding to HZM-1 cells was 0.64 mmol/g dry cells, corresponding to 42 mg Zn^{2+}/g dry cells, which is one of the highest values among those reported for Zn^{2+} adsorption by microbial cells. For comparison, the Q_{max} values (Zn^{2+} mmol/g dry cells) of metal binding reported thus far for microbial biosorbents are 0.56 (*Saccharomyces cerevisiae*; Volesky and May-Phillips 1995), 0.33 (*Streptoverticillium cinnamoneum*; Puranik and Paknikar 1997) 0.21 (*Rhizopus arrhizus*; Fourest and Roux 1992), and 0.08 (*Mucor miehei*; Fosmire 1990). Although a value of 0.97 mmol/g material (cell wall fraction) has also been reported for *Bacillus subtilis* (Doyle et al. 1980), this value is not based on the total cell weight and could not be directly compared with the Q_{max} value obtained in this study. HZM-1 cells could adsorb Zn^{2+} ions over a wide range of pH values (more than pH 3) and temperature (5–70 °C). In addition, the cells could specifically remove relatively low levels of Zn^{2+} ion (0.1 mM), even in the presence of a large excess (total concentration, 10 mM) of alkali and alkali-earth metals. Thus, HZM-1 cells can serve as an excellent biosorbent for the removal of Zn^{2+} from natural environments. Bound Zn^{2+} ions could be efficiently desorbed by treatment of the cells with 10 mM HCl or 10 mM EDTA; and the Zn^{2+}-adsorbing capacity of the cells was fully restored by treating the desorbed cells with 0.1 M NaOH. Thus, repeated use of the biosorbent will be possible; and HZM-1 cells may be used as an immobilized cell column for the continuous removal of Zn^{2+} from wastewater. It should be noted that HZM-1 cells could also adsorb Cu^{2+} with Q_{max} and K_d values comparable to those for Zn^{2+} ions, suggesting that HZM-1 cells may serve as a biosorbent for other heavy metal ions. However, this also implies that Zn^{2+} adsorption would be inhibited by Cu^{2+} ions when the concentrations of the coexisting Cu^{2+} ions are at sub-millimolar levels. Biosorption studies of a two-metal system containing Cu^{2+} and Zn^{2+} are currently underway.

One of the other remarkable features of strain HZM-1 is that it can grow in the presence of $ZnCl_2$ (at least up to 15 mM). This implies that it is potentially applicable

to in situ bioremediation of Zn^{2+}-contaminated aqueous systems.

Mechanistic consideration for Zn^{2+}-biosorption by HZM-1 cells

It has been shown that the mechanism of metal removal from aqueous systems by microorganisms can vary with the metal and microorganism involved, ranging from purely physicochemical interactions such as adsorption on cell walls, to metabolism-dependent mechanisms such as transport, internal compartmentation, and extracellular precipitation by excreted metabolites (Volesky and Holan 1995). In the case of HZM-1 cells, the mechanism for Zn^{2+} removal from aqueous systems could also vary with how the bacterium was used for this purpose.

When the lyophilized form of the bacterial cells was used for metal removal, the mechanism should be a metabolism-independent physicochemical adsorption process on the cell surface, judging from the following observations:

1. The cells retained a sufficient Zn^{2+}-binding capacity after treatment with 10 mM HCl and 0.1 M NaOH during desorption/resorption studies and also after the treatment at 70 °C for 12 h during temperature dependence studies; under such conditions, the bacterial cells were not viable (not shown). Thus, cell viability was not necessary for the Zn^{2+}-adsorption process. In addition, the bound Zn^{2+} could be quantitatively recovered by washing the cells with dilute acid and chelating agents, strongly suggesting that the Zn^{2+}-binding matrices exist on the surface of the bacterial cells.
2. Zn^{2+} biosorption by HZM-1 cells was virtually unaffected by temperature. It has been shown that the rate of metabolism- or energy-dependent metal adsorption (or incorporation) processes by microbial cells are generally dependent on temperature. In contrast, many energy-independent biosorption processes have been shown to be unaffected by temperature.

HZM-1 cells significantly lost their biosorption capacity at pH less than 2; and such a pH-dependence of Zn^{2+} binding can be at least in part explained in terms of the ion exchange principle: the degree of ionization of the anionic ligands in the Zn^{2+}-binding matrices of the cells decreased with decreasing pH and the ligand lost the ability to bind Zn^{2+}. Specificity studies showed that Zn^{2+} (0.1 mM) can bind to the cells in the presence of significant concentrations of Na^+, K^+, Mg^{2+}, or Ca^{2+} ions (10 mM), so that specific sites for Zn^{2+} must also be considered on the bacterial cell surfaces.

The hard/soft acid/base interaction between Zn^{2+} and the metal ligand(s) may also account for these observations. Efficient restoration of Zn^{2+}-binding capacity with 0.1 M NaOH can be consistently explained in terms of ion exchange of the ligands at the specific sites, which could be regenerated from their electrically neutral forms to anionic forms by alkaline treatment. Thus, carboxyl, hydroxyl, thiol, sulfate, and phosphate groups would be possible candidates for the Zn^{2+} ligands during biosorption. The fact that HZM-1 cells retain significant metal-binding activity at 70 °C, where most bacterial proteins of this mesophile would be denatured, indicates that the matrices are heat stable. Therefore, participation of metal-chelating proteins during biosorption would be less likely. Also, efficient desorption with EDTA strongly suggests that the adsorbed zinc existed as a divalent cation, rather than as a deposit of a metal crystalloid, as exemplified by metal deposition in the cell walls of *Bacillus subtilis* (Beveridge and Murray 1980). It should be noted that the structure of the zinc complexes also depends on pH; the dominant species are $[Zn(OH_2)_6]^{2+}$ at acidic pH (pH $<$ 7), $Zn(OH)_2$ (precipitate) at pH 7–9, and $[Zn(OH)_4]^{2-}$ at pH $>$ 9. However, because the $[Zn(OH_2)_6]^{2+}$ form predominantly exists at the pH range examined in this study (pH 2–7), the effect of other Zn^{2+} complex species on biosorption is negligible.

Removal of Zn^{2+} during bacterial growth

Importantly, strain HZM-1 can also remove Zn^{2+} from aqueous systems during its growth and, in this case, it was strongly suggested that the majority of the cell-associated Zn^{2+} should be present within the bacterial cells. This observation could not be simply explained in terms of the adsorption process, as discussed above. Rather, this probably arose from a metabolism-dependent incorporation of the metal during bacterial growth. Such a metabolism-dependent process, "bioaccumulation", should be related to the observed resistance of strain HZM-1 to Zn^{2+}. In addition, the growing cells seemingly exhibited a decreased biosorption on the cell surface compared with lyophilized cells. This may be, in part, because metabolic activities of the growing cells inhibited the metabolism-independent adsorption of zinc onto cell surfaces. More detailed studies of the suggested bioaccumulation process of the cells are currently underway to address this issue.

In the use of the strain HZM-1 for in situ bioremediation, metal capacity of the cells may vary with bacterial growth conditions and it could be possible that cells grown under conditions with some nutritional limitation would show lower metal capacities. Therefore, some nutrients should be supplemented at the remediation site in order to promote the growth of HZM-1 cells. Also, a fraction of Zn^{2+} present in aqueous environments should be bound to organic and inorganic matter; and this may be less efficiently removed by a biosorption process than free Zn^{2+}, which is a major metal species in the Milli-Q-based system. Under these circumstances, the suggested "bioaccumulation process" may play an important role in removing metal from the environment.

Acknowledgements We thank Dr. N. Sakai and Ms. M. Narumi, Research Institute for Environmental Science, Tohoku Turbo

Industry Co., Sendai, Japan, for their valuable suggestions on the determination of zinc by atomic absorption spectrophotometry. This work was supported by a grant from the CREST Noike project entitled "Energy recovery and recycling of resources from municipal wastes by advanced technologies for environmental protection".

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