The Journal of Microbiology, February 2008, p. 23-28 Vol. 46, No. 1
DOI 10.1007/s12275-007-0131-6
Copyright ⓒ 2008, The Microbiological Society of Korea

Removal of Heavy Metals by an Enriched Consortium

Eun Young Lee[2], Joung Soo Lim[2], Kyung Hwan Oh[1], Jae Yeon Lee[1], Seog Ku Kim[3], Yoo Kyung Lee[4], and Keun Kim[1]*

[1]Department of Bioscience and Biotechnology, [2]Department of Environmental Engineering, University of Suwon, Hwaseong 445-743, Republic of Korea
[3]Korea Institute of Construction Technology 2311, Goyang 411-712, Republic of Korea
[4]Polar BioCenter, Korea Polar Research Institute (KORDI), Incheon 406-840, Republic of Korea

(Received August 3, 2007 / Accepted December 27, 2007)

An enriched consortium obtained from lake-sediment was developed for the removal of heavy metals such as Cu, Pb, Cr, Ni, and Zn from heavy metal-contaminated water. The removal efficiency of heavy metals in a shaking condition was generally higher than that in the static state. After the fifteenth enrichment with assorted heavy metals, the removal efficiencies in the shaking and static condition at an average concentration of 100 mg/L of each heavy metal were approximately 99~100% and 95~100%, respectively, depending on the type of heavy metal. An aerobically grown, pure culture isolated from an enriched culture was analyzed by 16S rRNA sequencing and identified as *Ralstonia* sp. HM-1. This strain was found to remove various heavy metals with an efficiency of approximately 97~100% at an average concentration of 200 mg/L of each heavy metal.

***Keywords*: removal of heavy metals, enriched consortium, *Ralstonia* sp.**

Environmental pollution due to an increase in industrial activity is one of the most significant problems over the last century and is the corollary of economic growth. Toxic heavy metal production from the manufacture of plastic, electroplating, pigments, fertilizers, mining, and metallurgical processes in waste streams and the associated accumulation in living tissue throughout the food chain can pose serious health hazards (Iqbal and Edyvean, 2004; Akar and Tunali, 2005). Heavy metals in aquatic environments can be divided into two classes: 1) the bioavailable form (soluble, nonadsorbed, and mobile) and 2) the nonbioavailable form (precipitated, complexed, sorbed and nonmobile) (Maier *et al.*, 2000). The environmental hazards posed by heavy metals are directly linked to their mobility and thus their concentrations in solution. The processes traditionally used for removing heavy metals from industrial effluents have included chemical precipitation and filtration, chemical oxidation or reduction, electrochemical treatment, reverse osmosis, ion exchange, adsorption, and evaporation. Of these, the precipitation of metals using lime [$Ca(OH_2)$] was the most frequently used method (Park, 1987). While this method was economical and effective, metal solubility is affected by changes in pH, thus causing metals to be redissolved when the pH of the medium departed from the optimal range. In addition, these conventional processes were sometimes restricted due to technical or economical constraints (Volesky, 2001). Recent studies have focused on the biological treatments capable of removing heavy metals from contaminated water (Bang *et al.*, 2001). The surfaces of bacteria are analogous to mineral surfaces in that they possess surface functional groups that can adsorb to cationic species such as metals (Warren and Haack, 2001; Goyal *et al.*, 2003). Microbially facilitated removal of metals from contaminated water is based on the ability of microorganisms to complex and precipitate metals, resulting in both detoxification and removal of metals from the water column. This method seems to be a suitable alternative to the existing methods (Volesky and Holan, 1995) since microbial cell walls possess several carboxyl, hydroxyl, sulfhydryl and amino functional groups, which have the ability to bind metal ions (Veligio and Beolchini, 1997). Furthermore, the method utilizes different forms of microorganisms such as live, immobilized, and pretreated forms. The potential adsorptive capacity and affinity of bacteria to various types of metal depends on the type and density of the functional groups associated with the cell. A recent growing interest exists for the use of bacterial cells as biosorbents of metals in solution since their effectiveness as solid phase sorbents has increasingly become evident (Volesky, 1990; Volesky and Holan, 1995; Schiewer and Volesky, 2000).

In this study, an enriched consortium from lake sediment was shown to remove heavy metals such as copper, lead, chromium, nickel, and zinc. In particular, one type of bacterium, identified as *Ralstonia* sp. HM-1 by 16S rRNA gene sequencing, demonstrated the ability to remove heavy metals efficiently.

* To whom correspondence should be addressed.
(Tel) 82-31-220-2344; (Fax) 82-31-220-2344
(E-mail) kkim@suwon.ac.kr

Materials and Methods

Enrichment cultures used to remove heavy metals and isolation of a culture

The enriched cultures used for the removal of heavy metals originated from a biosorption medium (Ismail *et al.*, 2005) consisting of a 20 g/L glucose in a basal salt (BS-1) medium. In turn, BS-1 was composed of 3.0 g/L of $CaCO_3$, 2.0 g/L of $(NH_4)_2HPO_4$, 1.0 g/L of K_2HPO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of KCl, and 0.01 g/L of $FeSO_4 \cdot 7H_2O$. Prior to mixing, the glucose solution and BS-1 were autoclaved separately at 121°C for 15 min. The pH of the growth medium was adjusted to 5.5 with 1 M HCl. The development of the enriched consortium for heavy metal removal was prepared by collecting a 10 g sediment sample from below the Kwangdong bridge of Kyungan lake and placing it into a 500 ml serum bottle containing 90 ml of sterilized water and subsequently incubated the solution at 30°C for the 10 days. Next, a 10 ml aliquot of culture broth (without sediment) was transferred into each of the two bottles containing 90 ml of the BS-1 medium supplemented with 20 g/L of glucose and various metals. The control experiments were performed under the same conditions without inoculating the sediment or culture media. The final concentrations of heavy metals of the control were equivalent to the initial concentrations. One thousand mg/L of each stock aqueous metal solution made up of either $CuSO_4 5H_2O$, $PbSO_4$, $CrK(SO_4)_2 \cdot 12H_2O$, $ZnSO_4 \cdot 7H_2O$, or $NiSO_4 \cdot 6H_2O$ were diluted by BS-1 medium to proper concentration. One bottle was incubated at 30°C on a rotary shaker at 180 rpm, while the other was incubated at 30°C without shaking (static condition). The heavy metal concentrations in the enrichment culture were increased from 10 to 100 mg/L. All experiments were performed in duplicate.

After 15 successive culture transfers in the two experimental bottles, the cultures were plated onto biosorption agar medium containing the metals. Next, the cultures were incubated at 30°C for 10 days with one dominant colony appearing on a biosorption agar plate, which was subsequently isolated. Repeated transfers of the cells to fresh biosorption agar plates containing the metals resulted in a pure culture.

DNA extraction and PCR amplification

Total genomic DNA was extracted from a single colony grown on a biosorption agar plate using an AccuPrep genomic DNA Extraction kit (Bioneer Co. Ltd., Korea). Next, a nearly full-length 16S rRNA gene sequence was amplified by PCR using primers 27F; 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R; 5'-TAC GGY TAC CTT GTT ACG ACT T-3'. The PCR mixture consisted of 5 μl of 10× PCR buffer (final concentrations: 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3), 0.2 mM of each dNTP, 4 μl of each primer, 5 μl of template DNA, and 2.5 units of *Taq* polymerase (TaKaRa Ex Taq®, TaKaRa Bio Inc., Japan), in a final volume of 50 μl. The PCR reaction was performed in a thermal cycler (GeneAmp® PCR system 2700, Applied Biosystems Inc., USA) at 94°C for 5 min, followed by 35 denaturation cycles at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis and purified with an AccuPrep PCR purification kit (Bioneer Co. Ltd., Korea).

Sequence analysis

The full-length sequence of amplified 16S rRNA genes was deposited in the GenBank database of the National Center for Biotechnology Information (accession number DQ99783). The sequence was submitted to the Advanced BLAST search program to identify the sequences of any closely related organisms. The related sequences were preliminarily aligned with CLUSTAL W (Thompson *et al*., 1994), and manually adjusted with the aid of a 16S rRNA secondary structure information using PHYDIT (Chun, 1995). The phylogenetic analyses were performed using PHYLIP (Felsenstein, 1993), and the phylogenetic trees were determined by using the neighbor-joining method with Kimura's two-parameter model (Saitou and Nei, 1987). The resultant tree topology was evaluated using a bootstrap analyses of the neighbor-joining method based on 1,000 resamplings.

Analysis of heavy metal concentration

To measure the concentration of heavy metals in a solution, 10 ml of sample was centrifuged (10,000 rpm) for 15 min, followed by filtering of the supernatant. To determine the initial concentration of heavy metals in the sediment, a finely ground dewatered sediment (0.1 g) or culture sample (10 ml) was suspended in a Teflon vessel containing a mixed acid solution containing 2 ml of nitric acid and 7 ml of hydrogen peroxide. The solution was then adjusted to a final volume of 100 ml with distilled water. The vessel was heated to 200°C at 200 psi in a microwave (Microwave Accelerated Reaction System, XP-1500 Plus, USA) to ensure a complete digestion of the sediment. The concentrations of heavy metals were measured by ICP-MS (inductively coupled plasma mass spectrometer, HP 4500, USA).

Assay of heavy metal removal by an isolate

The heavy metal removal by an isolated culture was assayed by growing cells in 500 ml serum bottles containing 100 ml of biosorption medium supplemented with final concentration of 200 mg/L of each heavy metal (Cr, Ni, Cu, Pb, and Zn). Following the incubation of the culture in a rotary shaker at 30°C for 10 days, the cells were harvested by centrifugation at 10,000 rpm for 15 min. The resultant supernatant was then analyzed for heavy metal concentrations by ICP-MS.

Transmission electron microscopy

A LIBRA 120 (Carl Zeiss, Germany) analytic Transmission Electron Microscopy (TEM) was used for TEM analysis. Specimen preparation for the transmission electron microscopy consisted of primary fixation with 2% (v/v) paraformaldehyde and 2% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2 at 4°C for 2~4 h. After repeatedly washing with 0.05 M sodium cacodylate buffer on 3 occasions at 4°C, a 2% osmium tetraoxide solution in 0.05 M sodium cacodylate buffer was added for post-fixation at 4°C for 2 h. After briefly washing the mixture twice with distilled water at room temperature, En bloc staining was conducted at 4°C overnight. Next, the specimen was dehydrated with

ethyl alcohol, infiltrated with propylene oxide, polymerized at 70°C for 24 h, and sectioned with ultramicrotome (MT-X, RMC, USA).

Results and Discussion

Enrichment cultures for removing heavy metals

The percent removal of heavy metals by an enriched microbial consortium in a culture media containing different concentrations of heavy metals was investigated for samples on a shaker and in the static condition (no shaking) (Fig. 1 and 2). The changes in pH of the culture medium were minor (from 5.5 to 5.1), and could be ruled out. The average initial concentration of each heavy metal in a culture medium containing various heavy metals was set up at 10 mg/L, and subsequently increased to 50 mg/L and 100 mg/L. The concentrations of Cu for the 5th, 10th, and 15th culture of the medium was decreased by an enriched microbial consortium from the initial medium concentrations of 9.56, 63.1, and 104.3 mg/L, to final concentrations of 0.29, 0.3, and 4.9 mg/L, respectively in the static condition (Fig. 1). The metal concentrations of Cr, Ni, Cu, Pb, and Zn for the 15th enrichment cultures in the static condition were also decreased by the enriched microbial consortium, from 199.5, 95.5, 104.3, 50.0, and 50.0 mg/L to 10.2, 4.2, 4.9, 0.4, and 0.1 mg/L, respectively (Fig. 1). The removal efficiencies of heavy metals (Cr, Ni, Cu, Pb, and Zn) of the 5th culture via an microbial consortium in the static condition were determined to be 93.2%, 92.0%, 97.0%, 88.1%, and 98.4%, respectively. The removal efficiencies increased to near 99.0% and to approximately 100.0% at the 10th enrichment, but decreased slightly as the initial concentration increased to 100 mg/L.

The concentrations of Cu on the shaker for the 5th, 10th, and 15th cultured media were also lowered by the enriched microbial consortium to 0.2, 0.2, and 0.2 mg/L, respectively (Fig. 2). Compared with the results of the static conditions,

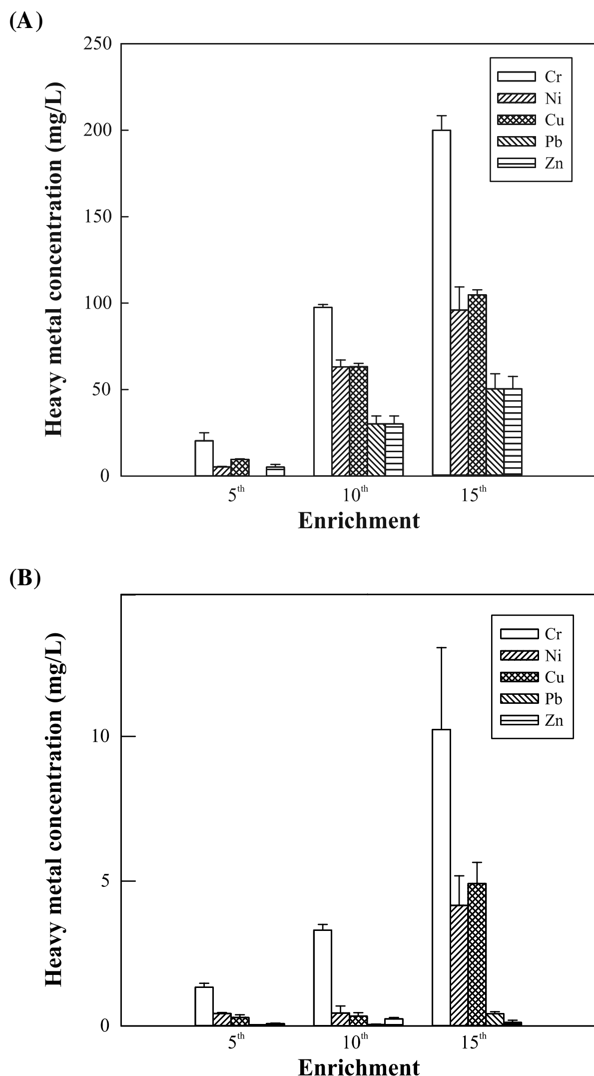

Fig. 1. Heavy metal concentrations in the medium without or with the inoculated culture, under the static condition. (A) Without inoculated culture, (B) With inoculated culture.

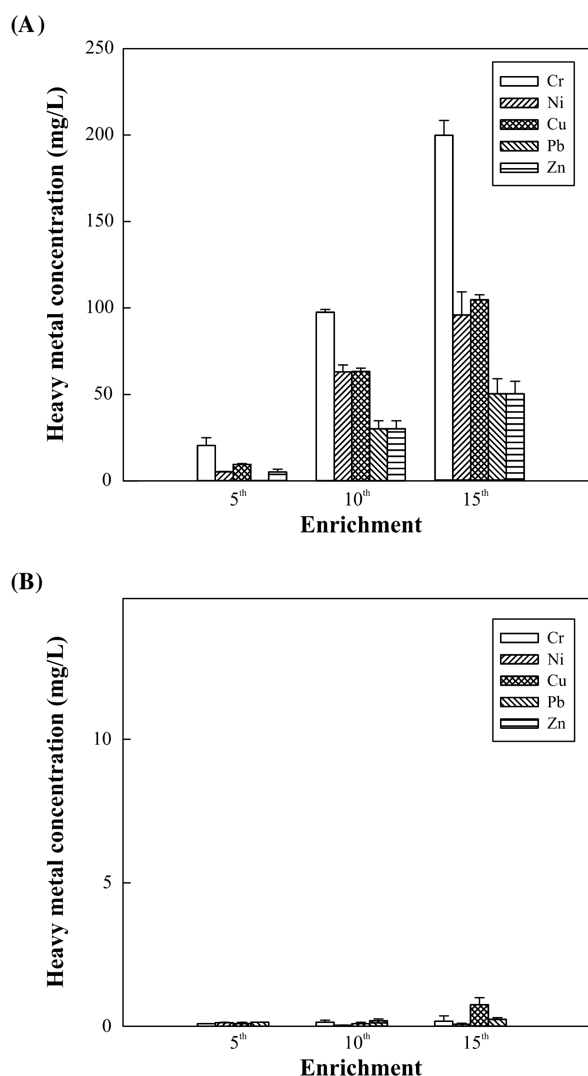

Fig. 2. Heavy metal concentrations in the medium without or with the inoculated culture on a shaker. (A) Without inoculated culture, (B) With inoculated culture.

the concentrations of Cr, Ni, Cu, Pb, and Zn after the 15th enrichment culture on a shaker was further decreased to 0.1, 0.05, 0.5, 0.2, and 0.0 mg/L, respectively (Fig. 2). All the removal efficiencies of heavy metals, except for lead (Pb) were maintained at approximately 99.0% from the 5th to 15th cultures.

These results indicated that the removal efficiencies of the heavy metals on a shaker were generally higher than those under the static condition.

Cheng *et al*. (1975) reported that the adsorption of heavy metals came about in two stages: In the first stage, a substantial portion of the heavy metals was adsorbed to the microorganisms within 30~60 min. In contrast, the process was very slow in the second stage. Cheng *et al*. (1975) supposed that the rapid initial adsorption of heavy metals to an activated sludge was attributed to the interactions between the metals and the components of the cell wall, as well as cytoplasms made up of polysaccharides, proteins, ribonucleic acids, and deoxyribonucleic acids, when low concentrations of heavy metals were present in wastewater. Nelson (1981), Huang (1982) and Khummongkoi *et al*. (1982) also provided a similar interpretation.

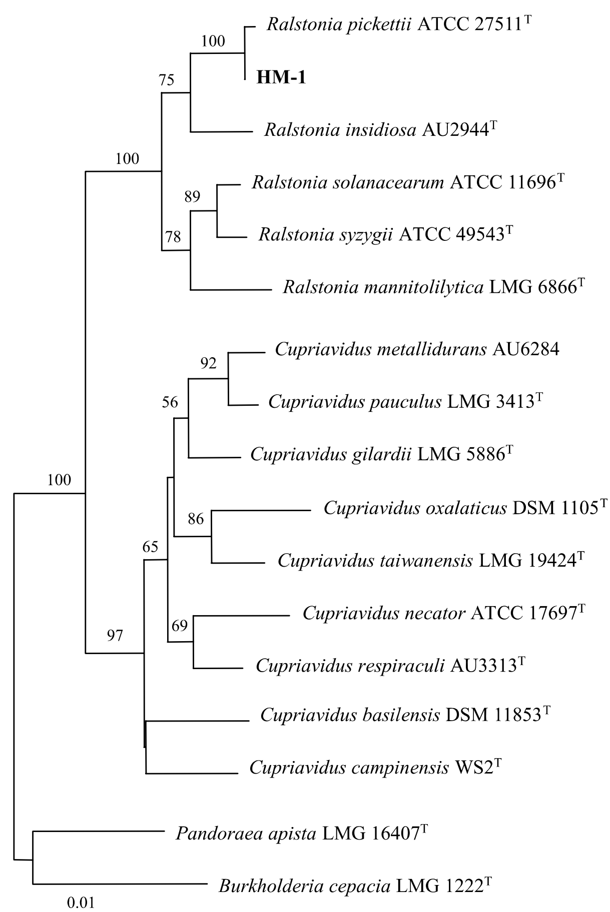

Fig. 3. Neighbour-joining tree showing the phylogenetic positions of strain HM-1 and representatives of some other related taxa based on 16S rRNA gene sequences. The bar signifies 0.01 substitutions per nucleotide position. The bootstrap values were expressed as percentages of 1,000 replicates. Percentages greater than 50 are shown at branch-points.

Identification and characterization of an isolate

Pure colonies were isolated from the 15th enriched culture in shaking condition and the isolated strain, HM-1, showed a high degree of similarity (100%) to *Ralstonia pickettii,* which belongs to β-Proteobacteria (Fig. 3). Therefore, we referred to the HM-1 isolate as *Ralstonia* sp. The activities of *Ralstonia picketti* such as poly (3-hydroxybutyrate)-degradation (Takanashi and Saito, 2006), removal of phenanthrene (Chávez-Gómez *et al*., 2003), and aromatic hydrocarbon-degradation (Leahy *et al*., 2003) have been reported.

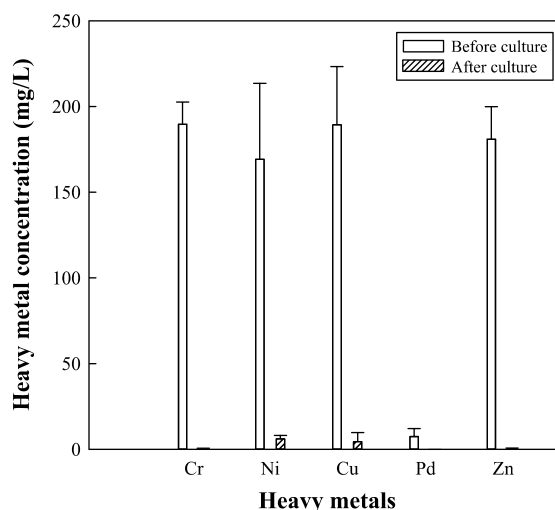

Fig. 4. Removal efficiency of heavy metals by *Ralstonia* sp. HM-1.

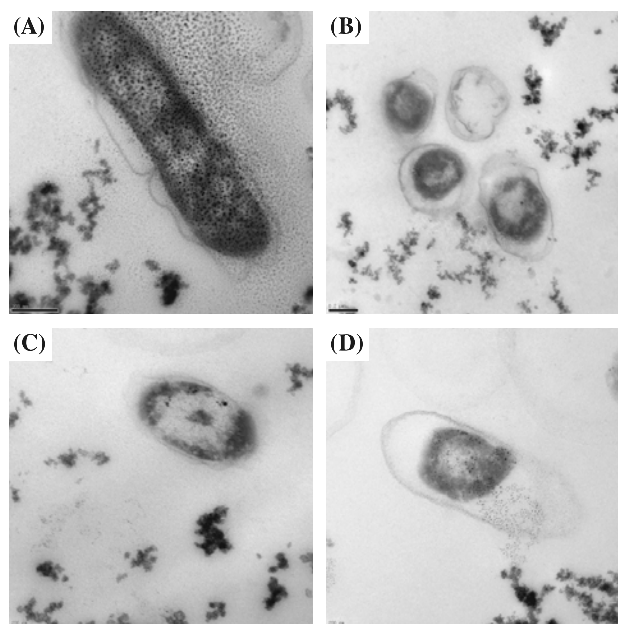

Fig. 5. The transmission electron micrograph of *Ralstonia* sp. HM-1 cultured in BS-1 medium supplemented with assorted heavy metals of Cr, Ni, Cu, Pb, and Zn at each concentration of 100 mg/L.

One of *Ralstonia* species, *R. metallidurans* was also reported as a heavy metal resistant bacterium (Roux and Covés, 2002; Mergeay *et al*., 2003).

In this study, the isolate *Ralstonia* sp. HM-1 demonstrated removal efficiencies of 99.82±0.18, 96.47±0.44, 97.85±2.42, 99.96±0.04, and 99.74±0.18% for heavy metals, when the initial concentrations of Cr, Ni, Cu, Pb, and Zn were 175, 118, 151, 10, and 162 mg/L, respectively (Fig. 4). Goyal *et al*. (2003) reported that *Streptococcus eauisimilis, Saccharomyces cerevisiae,* and *Aspergillus niger* showed a 29.4~49.2%, 7.6~17.6%, and 22.2~57.0% removal efficiency, respectively when the initial Cr(IV) concentration was 50 mg/L. The removal efficiency of lead(II), cadmium(II), copper(II), and nickel(II) by anaerobic biomass was 75% upon an initial concentration of 100 mg/L (Hawari and Mulligan, 2006). Thus, the removal efficiency of the isolated *Ralstonia* sp. HM-1 in the present study seems to demonstrate a relatively higher efficiency for metal removal. Moreover, the higher binding capacity of metal ions by microorganisms with the addition of nutrients and higher adsorption rates at higher temperatures were reported (Goyal *et al*., 2003). Therefore, if the culture conditions, including nutrient concentrations or culture temperature for the isolated *Ralstonia* sp. HM-1 are optimized, it is expected that a higher efficiency of metal removal can be achieved.

Figure 5 illustrated the transmission electron micrograph of *Ralstonia* sp. HM-1 cultured in BS-1 medium supplemented with 100 mg/L of heavy metals. Under these high concentrations of heavy metals, the microbial membrane was separated from the cytoplasm and the metals were accumulated in both the cytoplasm and cell membrane.

Microorganisms have evolved ingenious mechanisms for metal resistance and detoxification (Roane and Pepper, 2000). The surfaces of bacteria are analogous to mineral surfaces in that they possess surface functional groups that can attract cationic species such as metals (Warren and Haack, 2001). Generally, Gram-negative bacteria have thinner peptidoglycan layers and thus lower carboxylic site densities than those of Gram-positive bacteria. As a result, the results demonstrated that the least amount of metals were bound to *E. coli* cell walls (Warren and Haack, 2001). Recently, Puranik and Paknikar (1999) reported that when the Gram-negative *Citrobacter* strain, MCMB-181, was exposed to a multi-metal solution, the metals were adsorbed preferentially in the following order: $Co^{2+} < Ni^{2+} < Cd^{2+} < Cu^{2+} < Zn^{2+} < Pb^{2+}$. As for, *Ralstonia* sp. HM-1 of the present study, it is also Gram-negative and is capable of removing metals such as Cu, Ni, Cr, Pb, and Zn. According to the result illustrated in Fig. 4, the data suggests a similar sequence for the adsorbing capacity ($Ni^{2+} < Cu^{2+} < Zn^{2+}$, Cr^{2+}, Pb^{2+}) compared to *Citrobacter*. Moreover, the metal binding and deposition sequences are preferentially localized in the polar head group regions of the constituent membranes as well as along the peptidoglycan layer (Mann, 1990). For bacteria, surface reactivity is rendered more complex in that the relative importance and density of the functional groups differ for Gram-negative and Gram-positive bacteria, as well as among bacterial species. Moreover, the intracellular metal resistance mechanisms in bacteria are not clearly understood (Roane and Pepper, 2000). The likely best-known mechanisms involve metal binding or the sequestration by metallothioneins or similar proteins. Metallothioneins have a low molecular weight, cysteine-rich proteins with a high affinity for cadmium, zinc, copper, silver, and mercury metals. Metal binding by metallothioneins can result in cellular accumulations which are visible as electron dense areas within the cell matrix (Fig. 5).

Acknowledgements

This work was supported by a grant (063-051-019) from the Eco-star project of Korea Institute of Environmental Science and Technology (KIEST), for which the authors are grateful.

References

Akar, T. and S. Tunali. 2005. Biosorption performance of *Botrytis cinerea* fungal by-products for removal of Cd(II) and Cu(II) ions from aqueous solutions. *Miner. Eng.* 18, 1099-1109.

Bang, S.W., Y.K. Choi, and M.S. Han. 2001. Recent development of removal and treatment of toxic heavy metals by microorganisms. *Korean J. Environ. Biol*. 19, 93-99.

Chávez-Gómez, B., R. Quintero, F. Esparza-García, A.M. Mesta-Howard, F.J. Zavala Díaz De La Serna, C.H. Hernández-Rodríguez, T. Gillén, H.M. Poggi-Varaldo, J. Barrera-Cortés, and R. Rodríguez-Vázquez. 2003. Removal of phenanthrene from soil by co-cultures of bacteria and fungi pre-grown on sugarcane bagasse pith. *Biores. Technol.* 89, 177-183.

Cheng, M.H., J.W. Patterson, and R.A. Minear. 1975. Heavy metals uptake by activated sludge. *J. Water Pollut. Control Fed*. 47, 362-376.

Chun, J. 1995. Computer-assisted classification and identification of actinomycetes. Ph. D. thesis, University of Newcastle, Newcastle upon Tyne, UK.

Felsenstein, J. 1993. PHYLIP (Phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, WA, USA.

Goyal, N., S.C. Jain, and U.C. Banerjee. 2003. Comparative studies on the microbial adsorption of heavy metals. *Adv. Environ. Res.* 7, 311-319.

Hawari, A.H. and C.N. Mulligan. 2006. Biosorption of lead(II), cadmium(II), copper(II), and nickel(II) by anaerobic granular biomass. *Biores. Technol.* 97, 692-700.

Huang, C.P., J.F. Kao, L.P. Hsieh, and S.S. Cheng. 1982. Effect of EDTA on Cadmium in activated sludge systems. *J. Water Poll. Control Fed*. 54, 1118-1126.

Iqbal, M. and R.G.J. Edyvean. 2004. Biosorption of lead, copper, and zinc ions on loofa sponge immobilized biomass of *Phanerochaerte chryptosporium*. *Miner. Eng*. 17, 217-223.

Ismail, K., A. Tamer, and T. Siebel. 2005. Biosorption of Pb(II) and Cu(II) from aqueous solutions by pretreated biomass of *Neurospora crassa*. *Process Biochem.* 40, 3550-3558.

Khummongkoi, D., G.S. Canterford, and C. Fryer. 1982. Accumulation of heavy metals in unicellular algae. *Biotechnol. Bioeng*. 24, 2643-2660.

Leahy, J.G., K.D. Tracy, and M.H. Eley. 2003. Degradation of mixtures of aromatic and chloroaliphatic hydrocarbons by aromatic hydrocarbon-degrading bacteria. *FEMS Microbiol. Ecol*. 43, 271-276.

Maier, R.M., L.L. Pepper, and C.P. Gerba. 2000. Environmental microbiology. Academy Press, San Diego, USA.

Mann, H. 1990. Biosorption of heavy metals by bacterial biomass, p. 94-137. *In* B. Volesky (ed.), Biosorption of Heavy metals.

CRC Press, Boca Raton, FL, USA.

Mergeay, M., S. Monchy, T. Vallaeys, V. Auquier, A. Benotmane, P. Bertin, S. Taghavi, J. Dunn, D. Van Der Lelie, and R. Wattiez. 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol. Rev*. 27, 385-410.

Nelson, P.O., A.K. Chung, and M.C. Hudson. 1981. Factors affecting the fate of heavy metals in the activated sludge process. *J. Wat. Poll. Control Fed*. 53, 1323-1333.

Park, S.W. 1987. Specific chemical reactions at the cadmium sulfide-water interface. Ph. D. thesis, University of Delaware, USA.

Puranik, P.R. and K.M. Paknikar. 1999. Biosorption of lead, cadmium, and zinc by *Citrobacter* strain MCM B-181: characterization studies. *Biotechnol. Prog*. 15, 228-237.

Roane, T.M. and I.L. Pepper. 2000. Microorganisms and metal pollutants, p. 403-423. *In* R.M. Maier, I.L. Pepper, and C.P. Gerba (eds.), Environmental microbiology. Academic Press, San Diego, USA.

Roux, M. and J. Covés. 2002. The iron-containing superoxide dismutase of *Ralstonia metallidurans* CH34. *FEMS Microbiol. Lett.* 210, 129-133.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4, 406-425.

Schiewer, S. and B. Volesky. 2000. Biosorption process for heavy metal removal, p. 329-362. *In* D. Lovley (ed.), Environmental microbe metal interactions. ASM Press, Washington, D.C., USA.

Takanashi, M. and T. Saito. 2006. Characterization of two 3-hydroxybutyrate dehydrogenases in poly(3-hydroxybutyrate)-degradable bacterium, *Ralstonia pickettii* T1. *J. Biosci. Bioeng.* 101, 501-507.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 22, 4673-4680.

Veligio, F. and F. Beolchini. 1997. Removal of metals by biosorption: a review. *Hydrometallurgy* 44, 301-316.

Volesky, B. 1990. Removal and recovery of heavy metals by biosorption, p. 7-43. *In* B. Volesky (ed.), Biosorption of heavy metals. CRC Press, Boca Raton, FL, USA.

Volesky, B. 2001. Detoxification of metal bearing effluents: biosorption for the next century. *Hydrometallurgy* 59, 203-216.

Volesky, B. and Z.R. Holan. 1995. Biosorption of heavy metals. *Rev. Biotechnol. Progress* 11, 235-250.

Warren, L.A. and E.A. Haack. 2001. Biogeochemical controls on metal behaviour in freshwater environments. *Earth Sci. Rev.* 54, 261-320.