J. Microbiol. Biotechnol. (2002), **12**(2), 296–300

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Involvement of Lipopolysaccharide of *Bradyrhizobium japonicum* in Metal Binding

OH, EUN TAEX, HYUN SHIK YUN, TAE-RYEON HEO, SUNG-CHEOL KOH[1], KYE-HEON OH[2], AND JAE-SEONG SO*

Department of Biological Engineering and Center for Advanced Bioseparation Technology, Inha University, Incheon 402-751, Korea
[1]Division of Civil and Environmental Engineering, Korea Maritime University, Pusan 606-791, Korea
[2]Department of Life Science, Soonchunhyang University, Asan 336-745, Korea

Received: November 10, 2001
Accepted: March 2, 2002

Abstract Bacterial cell surface components are the major factors responsible for pathogenesis and bioremediation. In particular, the surface of a Gram-negative bacterium cell has a variety of components compared to that of a Gram-positive cell. In our previous study, we isolated an isogenic mutant of *Bradyrhizobium japonicum*, which exhibited altered cell surface characteristics, including an increased hydrophobicity. Polyacrylamide gel electrophoretic analysis of the lipopolysaccharide (LPS) in the mutant demonstrated that the O-polysaccharide part was completely absent. Meanwhile, a gel permeation chromatographic analysis of the exopolysaccharide (EPS) in the mutant demonstrated that it was unaltered. Since LPSs are known to have several anion groups that interact with various cation groups and metal ions, the mutant provided an opportunity to examine the direct role of LPS in metal binding by *B. japonicum*. Using atomic absorption spectrophotometry, it was clearly demonstrated that LPS was involved in metal binding. The binding capacity of the LPS mutant to various metal ions (Cd^{2+}, Cu^{2+}, Pb^{2+}, and Zn^{2+}) was 50–70% lower than that of the wild-type strain. Also, through an EPS analysis and desorption experiment, it was found that EPS and centrifugal force had no effect on the metal binding. Accordingly, it would appear that LPS molecules on *B. japonicum* effect the properties, which precipitate more distinctly metal-rich mineral phase.

Key words: *Bradyrhizobium japonicum*, metal binding, lipopolysaccharide (LPS), LPS mutant

Bacteria produce a wide variety of complex molecules on their surface, which contain numerous charged chemical groups (such as phosphoryl, carboxyl, and amino groups) that give the cell surface net anionic charge density. As a result, the cell surface can interact with various cations present in the environment. Since metal cations can be attracted electrostatically and bound to the cell, numerous studies have examined the metal ion-cell surface interactions of Gram-positive and Gram-negative bacteria [5, 7, 8, 10]. *B. japonicum* is a Gram-negative soil bacterium that has various cell surface molecules, including an exopolysaccharide (EPS) and lipopolysaccharide (LPS). LPSs are known to be a major factor responsible for the pathogenesis of several Gram-negative pathogenic bacteria. Also, LPSs play a crucial role in specific interactions of anionic groups in the carbohydrate portion with divalent ions, such as Cd^{2+}, Cu^{2+}, Pb^{2+}, and Zn^{2+}, in the environment [1, 3, 5, 10].

Recently, interest in the bioremediation of toxic chemicals has increased and the biomass has been found to be very useful for removing industrial pollutants, including heavy metals. In particular, a microbial biomass provides available ligand groups on which metal ions can bind. Therefore, the employment of a bacterial biomass for the treatment of metal ions in industrial effluents has already been suggested by many researchers dealing with metal-bacteria interactions [10, 14]. However, the exact mechanism of the interaction between microbial cells and metals remains unclear, although LPS is known to have several anion groups which interact with various cation groups and metal ions. In a previous study, the current authors isolated an isogenic mutant of *B. japonicum*, which had altered cell surface characteristics with an increased hydrophobicity resulting from the lack of the O-polysaccharide part of LPS [9]. Accordingly, this paper examined the involvement of LPS in the metal binding of *B. japonicum* using the LPS mutant.

*Corresponding author
Phone: 82-32-860-7516; Fax: 82-32-875-0827;
E-mail: sjaeseon@inha.ac.kr

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *B. japonicum* strains [wild-type strain 61A101c, LPS mutant JS314, and transformant JS314/pLps (JS314 transformed with pRK415/Lps)] used in this study were all described in a previous paper [9]. The cells were grown in an AMA broth (10 g of mannitol, 1 g of bacto yeast extract, 0.2 g of magnesium sulfate, and 0.2 g of sodium chloride per liter of deionized water) on a rotating shaker at 27°C [11].

Metals

The four metal salts used in this study were $CdN_2O_6 \cdot 4H_2O$, $CuN_2O_6 \cdot 3H_2O$, N_2O_6Pb (all from Fluka Chemical Co., Tokyo, Japan), and $Zn(NO_3)_2 \cdot 6H_2O$ (Sigma-Aldrich Chemical Co., St. Louis, U.S.A.). The metal solutions were prepared by dissolving the metal salts in 1% (v/v) HNO_3, except for the zinc solution, which was prepared in 1% (v/v) HCl. All materials (glassware, plastic ware, centrifuge tubes, etc.) were acid leached in 50% (v/v) HNO_3 for at least 12 h prior to use and then rinsed with ultrapure deionized water (UDW). Standard curves for the metal concentrations were prepared using an atomic adsorption standard solution of cadmium, copper, lead, and zinc (Sigma-Aldrich Chemical Co., St. Louis, U.S.A.).

Preparation of Samples for Metal-Binding Analysis

The cells were grown to the mid-exponential phase (optical density of 0.5 at 600 nm), then a 30 ml cell suspension was transferred to a sterile tube. After tubes were centrifuged at 4°C and 8,000 ×g (Eppendorf, Homburg, Germany) for 5 min, the supernatant fluid was removed and the cell pellets resuspended in 15 ml ddH_2O. One milliliter of the resuspended cell solution was transferred to 1.5-ml microcentrifuge tubes. The cells were then washed with 1 ml of ddH_2O and centrifuged at 15,000 ×g for 3 min. This procedure was repeated three times. The washed cells were resuspended in 1 ml of 0.1, 0.3, 0.5, and 0.7 g/l metal solutions. After incubation in the metal solution for 30 min at 25°C, the cells were harvested by centrifugation at 15,000 ×g for 5 min to obtain a supernatant solution containing unbound metal ions. The supernatant solution was then diluted with 1% (v/v) HNO_3 for measurement. The amount of metals bound to the cells was calculated by subtracting the remaining unbound metals from the initial amount mixed with the cells. To determine the cell weight, the same volume of cell suspension that did not contain any metal was centrifuged and then dried in a dry oven for 1 h [5].

Preparation of Metal Desorption Test

To determine the effect of centrifugal force on the binding of the metal ions, a metal desorption test was performed. After the metal adsorption experiment, the resulting cell pellets in the microtubes were washed twice with phosphate-buffered saline (PBS), separated by centrifugation at 15,000 ×g for 5 min, then the supernatant solutions were taken. The solutions were analyzed to determine the amount of metals desorbed from the bound cells.

Atomic Adsorption Spectrophotometery (AAS)

Samples containing cadmium, copper, lead, or zinc ions were analyzed to determine their metal contents using an atomic adsorption spectrophotometer (Analytical Jena 5EA, Homburg, Germany), as previously described [5].

Isolation of LPS

The LPS from the three strains was prepared as described by Hitchcock and Brown [2]. Wild-type 61A101c and two mutant strains grown on a liquid medium were harvested and suspended in 10 ml of phosphate-buffered saline (pH 7.2). A portion (15 ml) of this suspension was centrifuged at 15,000 ×g for 5 min. The pellets were solubilized in 50 µl of a lysing buffer containing 2% SDS and 4% 2-mercaptoethanol blue. The lysates were heated at 100°C for 10 min. For protein digestion, 25 µg of proteinase K (PK) solubilized in 10 µl of the lysing buffer was added to each boiled lysate and incubated at 60°C for 60 min [2].

Gel Electrophoresis and Silver Staining of LPS

The electrophoresis was performed with 4% polyacrylamide stacking gel and 12% separating gel at a constant current of 50 mA with Tris-glycine (pH 8.3) and a 0.1% SDS buffer for approximately 1.5 h. Subsequent silver staining was carried out as described previously [2, 4].

Preparation of EPS from *B. japonicum* Strains

The *B. japonicum* strains were cultured in 50 ml of AMA medium at 27°C on a rotating shaker at 150 rpm. After the cell culture reached an optical density of 1 at 600 nm, the cells were harvested and washed with 10 ml of 0.85% NaCl solution at pH 7.0, and then centrifuged at 10,000 ×g for 10 min at 4°C. The supernatants were harvested, and then the EPS was precipitated from the supernatants with 3 volumes of cold ethanol and dried. The dried EPS was resuspended in 0.1 M $NaNO_3$. Finally, the samples were filtered using a 0.20 µm syringe filter (Sartorius, Götingen, Germany) [6].

Gel Permeation Chromatography (GPC)

The EPS was analyzed using an Utrahydrogel 2000 Å column 7.8×300 mm (Waters, Milford, U.S.A.) and RI detector (Waters 410, Milford, U.S.A.). A solvent delivery module model 910 (Young in, Seoul Korea) was used to confer the EPS samples to the column by a vacuum. The mobile phase was 0.1 M $NaNO_3$, the flow rate 0.6-ml/min, and the internal and external temperatures 35°C and 50°C, respectively.

RESULTS AND DISCUSSION

Cell Surface Polysaccharide Profiling

The current authors previously reported on the isolation and characterization of an LPS-deficient mutant strain JS314, which appeared not to contain the O-polysaccharide part [12]. Subsequently, the LPS gene responsible for this mutation was cloned and the mutant successfully complemented with the cloned gene [11]. The LPS samples isolated from the two strains of *B. japonicum* [JS314 and JS314/pLps (LPS complemented strain)] were subjected to SDS-PAGE and visualized by the silver staining method of Hitchcock and Brown [2], along with the wild-type (61A101c) LPS sample. As shown in Fig. 1, the wild-type and complemented strains of *B. japonicum* displayed intact LPS profiles, as evidenced by the various bands present in the lower to upper regions of the gel. When the LPS produced by the mutant was examined, the bands corresponding to the O-polysaccharide chain were absent, demonstrating that the LPS mutant strain of *B. japonicum* was completely devoid of O-polysaccharide. To examine the possibility that the mutant had a pleotrophic phenotype, including an altered exopolysaccharide (EPS), the EPS of the three strains was examined using gel permeation chromatography (GPC). As shown in Fig. 2, the EPS samples from the three strains (wild-type, mutant, and complemented strains) exhibited almost identical patterns, indicating that the EPS samples of the three strains were the same size.

Analysis of Metal Binding

The objective of the current research was to determine contribution of the O-polysaccharide of LPS to the metal binding by *B. japonicum.* Metal binding and the subsequent fine-grained mineral development on bacterial surfaces are complex issues. Since the polysaccharide chain of LPS has anionic charges, this part interacts with cations, such as heavy metal divalent ions. It has been previously postulated that the glycosidic diphosphate moiety of LPS participates in high affinity metal binding [5, 14]. It has also been suggested that metals may form ionic interactions with LPS molecules, to stabilize the outer membrane [13]. Another factor considered in the promotion of metal precipitate formation has been the relative cell surface hydrophobicity (CSH) [5]. In a previous study, the current authors reported that the LPS mutant had an increased CSH compared with the wild-type strain [9]. Therefore, LPS would appear to govern the relative cell surface hydrophobicities of the strains and is expected to contribute to metal binding by promoting the formation of metal precipitates. Although the change in the metal binding capacity of the LPS mutant is probably due to the lack of O-polysaccharide, bacteria have many ionizable groups on their surfaces, and metal ions can have a complex reactivity, which are influenced by pH, redox potential, environmental electrolytes, and cell gradients [5]. Accordingly, to simplify the environmental factors, the bacteria were suspended in single metal solutions. Since a linear increase in metal adsorption was observed during 30 min of incubation, the metal adsorption was measured following 30 min of incubation. The concentrations of metal added ranged from 0.1 g/l to 0.7 g/l.

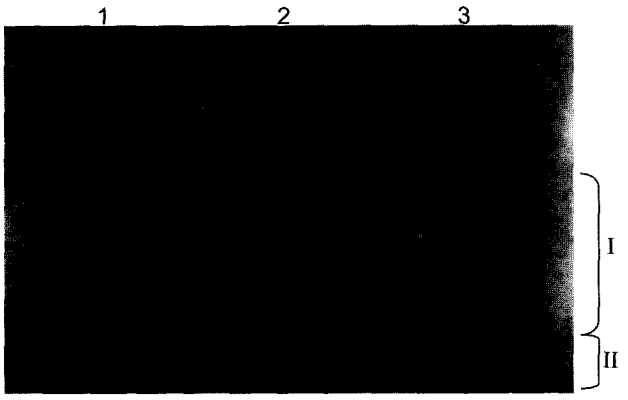

Fig. 1. LPS profile of *B. japonicum* strains.
The faster-moving (II) in each lane was identified as core LPS, while the slower-moving bands (I) were found to be LPS with O-polysaccharide. Lanes: 1=61A101c; 2=JS314/pLps; 3=JS314.

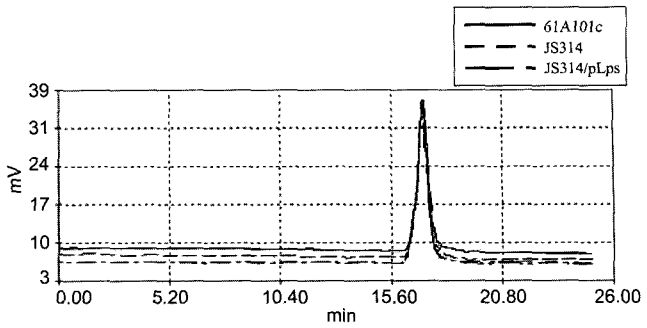

Fig. 2. Molecular weight distribution of EPS from three *B. japonicum* strains.
The cells were washed by 0.85% NaCl to remove any bound EPS from the cells. The EPS samples in the supernatants were harvested by centrifugation and precipitated by adding ethanol. The resulting precipitates were dried, resuspended in 0.1 M $NaNO_3$ and analyzed by GPC to determine their molecular weights.

The interaction of the divalent metal ions with the cells was investigated by observing the effects of the addition of Cd^{2+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} on the absorption peaks of each metal in an AAS analysis. The amount of metals bound by each of the three strains is shown in Fig. 3. There were significant differences in the amounts of metals bound by the strains. The amount of metals bound to the mutant was 50–70% lower than that bound to the other strains, whereas the wild-type and complemented strains bound almost equal amounts of metals. However, when the concentration of the four metals in the solutions increased, the amount of bound metals also increased in all three

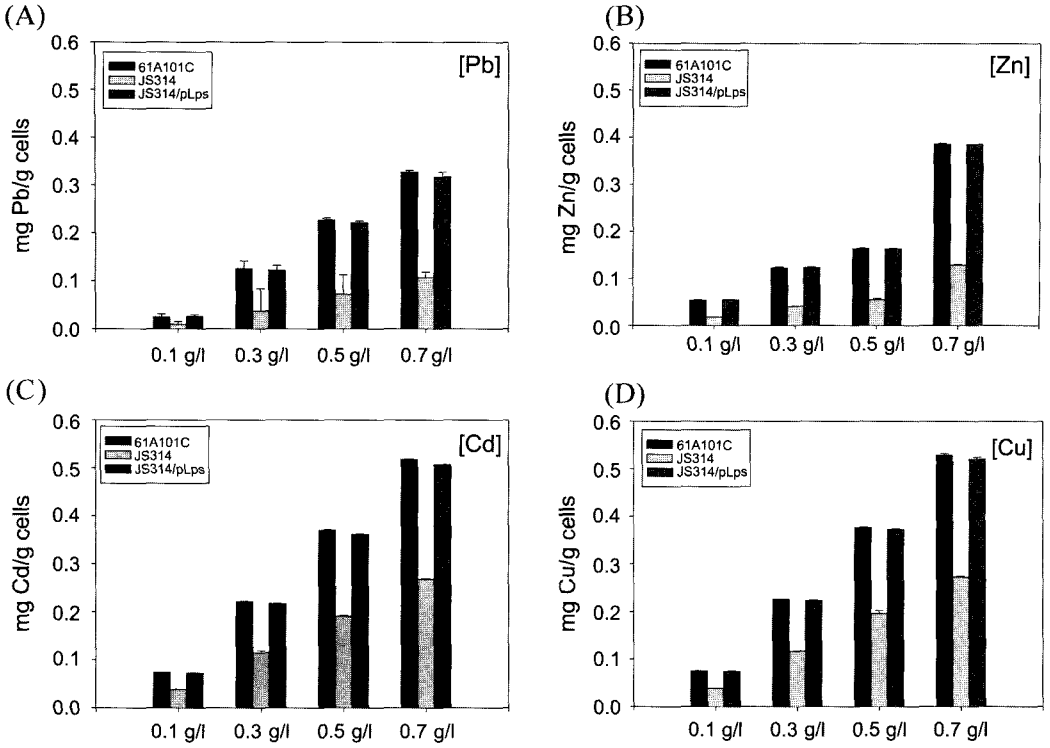

Fig. 3. Amounts of metals bound to three strains of *B. japonicum*.
(A) Amount of lead adsorbed by three strains of *B. japonicum* suspended in 0.1 g/l to 0.7 g/l Pb solutions. (B) Amount of zinc adsorbed by three strains of *B. japonicum* suspended in 0.1 g/l to 0.7 g/l Zn solutions. (C) Amount of cadmium adsorbed by three strains of *B. japonicum* suspended in 0.1 g/l to 0.7 g/l Cd solutions (D) Amount of copper adsorbed by three strains of *B. japonicum* suspended in 0.1 g/l to 0.7 g/l Cu solutions. The incubation was carried out at 25°C for 30 min.

strains. Figure 3(B) shows the amount of zinc bound by the different strains, where equal amount of zinc were bound to the wild-type and complemented strains, while less zinc was bound to the LPS mutant. Figure 3(C) shows the amount of cadmium bound by the different strains, where the amounts of cadmium and copper bound to all three strains were significantly higher than those of lead or zinc. The results of the copper binding experiment are shown in Fig. 3(D), which exhibited similar results to those obtained in the cadmium binding experiment. At higher concentrations, the wild-type strain appeared to bind more Pb, Cu, and Cd compared to the complemented strain. To determine the affinity of the metal binding to the cell surface, the amounts of metals released from the cell surface by vigorous agitation was measured as previously described [7]. Table 1 shows the amounts of desorbed metals after centrifugation of the metal binding cells. As expected, the amounts of metals desorbed from the LPS mutant strain JS314 was lower than that from the other two strains. However, all the metals tested bound tightly to the cell surface because the amounts of metals released from the bound cells was negligible (less than 10 μg) compared to the amount of bound metals (more than 300 μg). Among the four metals, cadmium was desorbed the least, suggesting that the fraction of adsorbed cadmium was much higher than the other metals. Overall, centrifugation did not affect the binding of the metals to the cells. It has been previously suggested that the cell surface charge may contribute to metal binding by promoting the formation of metal precipitates with specific physiochemical properties rather than by simply providing a reactive site to which metal ions can bind [5]. As a result, it is proposed that the O-polysaccharide of LPS is important in metal binding and affecting the cell surface charge of *B. japonicum*.

Table 1. Amounts of desorbed metals measured after desorption from cells.

	[mg metals/g cells]			
	Pb	Zn	Cd	Cu
61A101c	9.03	5.90	0.739	7.74
JS314	6.07	1.97	0.246	3.99
JS314/pLps	9.18	5.90	0.739	7.58

For adsorption, the cells were mixed with individual metals and incubated at 25°C for 30 min. Cells harvested by centrifugation were then completely resuspended in PBS. After discarding the cell pellet, the amounts of metals desorbed from the cells in the supernatant were measured. The values are averages from three experiments carried out in triplicate. Standard deviations for the data values were less than 20%.

Acknowledgment

This research was supported by funds from the Center for Advanced Bioseparation Technology, Inha University.

REFERENCES

1. Glauner, B., J. V. Holtje, and U. Schwarz. 1988. The composition of murein of *Escherichia coli*. *J. Biol. Chem.* **263:** 10088–10095.
2. Hitchcock, P. J. and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gel. *J. Bacteriol.* **154:** 269–277.
3. Jacques, M. 1996. Role of lipo-oligosaccharides and lipopolysaccharides in bacterial adherence. *Trends in Microbiol.* **4:** 410.
4. Lam, J. S., L. L. Graham, J. Lightfoot, T. Dasgupta, and T. J. Beveridge. 1992. Ultrastructural examination of the lipopolysaccharides of *Pseudomonas aeruginosa* strains and their isogenic rough mutants by freeze-substitution. *J. Bacteriol.* **174:** 7159–7167.
5. Langley, S. and T. J. Beveridge. 1999. Effect of O-side-chain-lipopolysaccharide chemistry on metal binding. *Appl. Environ. Microbiol.* **65:** 489–498.
6. Louch, H. A. and K. J. Miller. 2001. Synthesis of a low-molecular-weight form of exopolysaccharide by *Bradyrhizobium japonicum* USDA 110. *Appl. Environ. Microbiol.* **67:** 1011–1014.
7. Mullen, M. D., D. C. Wolf, F. G. Ferris, T. J. Beveridge, C. A. Flemming, and G. W. Bailey. 1989. Bacterial sorption of heavy metals. *Appl. Environ. Microbiol.* **55:** 3143–3149.
8. Park, J. Y., C. Chun, and Y. J. Yoo. 1999. Characteristics of metal biosorption of oxidized *Undria pinnatifida*. *J. Microbiol. Biotechnol.* **9:** 650–654.
9. Park, K.-M. and J.-S. So. 2000. Altered cell surface hydrophobicity of lipopolysaccharide-deficient mutant of *Bradyrhizobium japonicum*. *J. Microbiol. Methods* **41:** 219–226.
10. Pazirandeh, H., B. M. Wells, and R. L. Ryan. Development of bacterium-based heavy metal biosorbents: Enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. 1998. *Appl. Environ. Microbiol.* **64:** 4068–4072.
11. So, J.-S., W. S. Kim, and G. Stacey. 2000. Molecular characterization of a gene region involved in lipopolysaccharide synthesis in *Bradyrhizobium japonicum*: Cloning, sequencing and expression of *rfaF* gene. *FEMS Microbiol. Lett.* **190:** 109–114.
12. Stacey, G., J.-S., So, L. E. Roth, S. K. B. Lakshmi, and R. W. Carlson. 1991. A lipopolysaccharide mutant of *Bradyrhizobium japonicum* that uncouples polysaccharides from bacterial differentiation. *Mol. Plant-Microbe Interact.* **4:** 332–340.
13. Strain, S. M., S. W. Fresik, and M. Armitage. 1983. Structure and metal-binding properties of lipopolysaccharides from heptoseless mutants of *Escherichia coli* studied by ^{13}C and ^{31}P nuclear magnetic resonance. *J. Biol. Chem.* **255:** 13466–13477.
14. Vecchio, A., C. Finoli, and D. D. Simine. 1998. Heavy metal biosorption by bacterial cells. *Fresenius J. Anal. Chem.* **361:** 338–342.