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Contribution of Cell-Surface Components to Cu^{2+} Adsorption by *Pseudomonas putida* 5-x

L. Wang,* F. T. Li, and Q. Zhou

School of Environmental Science and Engineering,
State Key Laboratory of Pollution Control and Resource and Reuse,
Tongji University, Shanghai 200092, P.R. China,
E-mail: celwang@yahoo.com

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Abstract

The contribution of various cell-surface components to Cu^{2+} adsorption by a Gram-negative bacterium, *Pseudomonas putida* 5-x, that was isolated from local electroplating effluent with a high capability to accumulate heavy metal ions was studied. The cell superficial layer had a negative effect on Cu^{2+} adsorption of the bacterial cells. Cu^{2+} adsorption capacity of the separated cell envelopes was fivefold more than that of the intact cells, owing to the liberation of more and more binding sites during the separation process. Some main components in the cell envelope, such as the peptidoglycan (PEG) layer, outer membrane, and inner membrane, provide the capability for Cu^{2+} adsorption. The content of the components in the cell envelope is in the order inner membrane > outer membrane > PEG layer, and their Cu^{2+} adsorption capacity was in the order PEG layer > outer membrane > inner membrane. The total contribution of the separated PEG layer material to Cu^{2+} adsorption by the cell envelope was no more than 15%, and the outer membrane and inner membrane contributed about 30–35% and 25–30%, respectively. The relatively high phospholipid content in the outer membrane may be the major reason for the higher adsorption capacity of the outer membrane to Cu^{2+} and, hence, such a high Cu^{2+} adsorption capacity of *P. putida* 5-x cell envelope.

Index Entries: Biosorption; cell envelope; inner membrane; outer membrane; peptidoglycan layer.

Introduction

All cell surfaces are generally anionic and can interact with cationic metal ions *(1)*. Many studies have shown that soluble metal ion in

*Author to whom all correspondence and reprint requests should be addressed.

aqueous circumstance can be captured by the cell wall because of a reactive chemical site within its fabric *(2,3)*. During the last two decades, bacteria, algae, and fungi or their separated components have been used successfully as biosorbent for the removal of heavy metal ions. Among the microbes, bacteria are of particular interest because of their high cell-surface area per unit volume *(4–9)*.

Gram-positive bacterial cell wall consists of a thicker peptidoglycan (PEG) layer (constituting 40–90% of the cell wall) into which teichoic acid (TA) and teichuronic acid (TUA) are embedded *(10)*. PEG, TA, and TUA contain large amounts of electronegative groups such as carboxyl and phosphodiester *(11,12)*. Therefore, Gram-positive bacteria generally have a strong interaction with cationic ions *(13,14)*. Gram-negative bacteria exhibit weaker metal-binding capacity than Gram-positive bacteria, owing to their thinner PEG layer (only 10% of the cell envelope) and the absence of TA and TUA in the cell envelope *(10,14)*. Some researchers have preliminarily explored the roles of cell envelope and outer membrane in heavy metal binding by Gram-negative bacteria. They found that the cell envelope or outer membrane of Gram-negative bacteria such as *Pseudomonas* sp., *Escherichia coli*, and *Citrobacter* sp. provide 0.01–0.4 mmol/g of metal-binding capacities, which is only about 10–20% of the adsorption capacity by the cell wall of Gram-positive bacteria *(14–18)*. However, the role of cell-surface components, such as cell superficial layer, cell outer membrane, inner membrane, and PEG layer, in the metal adsorption by Gram-negative bacteria still has not been studied in detail, owing to their structural complexity, especially in the Gram-negative bacteria with high metal-binding capability.

Cu^{2+} widely presents in the industrial effluent of China. In our previous studies, a Gram-negative bacterium, *P. putida* 5-x, with high Cu^{2+}-binding capacity was isolated from local industrial effluent. Its adsorption conditions, adsorption properties, and the operational performance of batch and continuous biosorption reactors with the bacterial cells as biosorbent for removing Cu^{2+} were well documented *(4,19,20)*. The experimental results indicated that Cu^{2+} was accumulated mainly on the cell surface, and the binding process of the cell to Cu^{2+} obeyed the Freundlich isotherm. However, some other aspects, such as the adsorption capability of the cell envelope and the role of various cell-surface components in Cu^{2+} adsorption by the cell, have not been studied in detail. Understanding the roles of the cell-surface components in Cu^{2+} adsorption by the bacterial cell can serve as a useful aid for further enhancing metal adsorption capability of the bacterial cell by modifying its cell-surface structure and components using metabolic regulation or DNA recombination technologies.

In the present study, Cu^{2+} adsorption capacity of the separated cell envelope from *P. putida* 5-x was assessed, and the role of cell-surface components such as the cell capsule, PEG layer, and outer and inner membranes in Cu^{2+} adsorption by the cell envelope was examined.

Materials and Methods

Culture of Microorganisms

The bacterial strain used as biosorbent was *P. putida* 5-x, which was isolated from local electroplating industrial effluents. The bacterial cells were cultured in SL medium according to the method of Sze et al. *(19)*. After culturing for 34–36 h, the bacterial cells were harvested for further experiments.

Removal of Cell Superficial Layer (Capsule)

Cell capsule can be removed easily by weak eluent *(21)*. To remove the superficial layer of *P. putida* 5-x cells, 100 mg (dry wt) of the cells was washed with 25 mL of 0.1 *M* HCl with shaking at 200 rpm for 20 min at 25°C. The acid-treated cells were harvested by centrifuging at 3600*g* for 20 min and washed twice with 50 mL of 10 *M* MES buffer at pH 6.5. Transmission electron microscopy (TEM) was used to check the treatment result of the cells.

Preparation of Cell Envelopes

The intact cells (after treating with diluted HCl) were suspended in 10 *M* of pH 6.5 MES buffer solution and then broken by passing twice through a French Pressure at 1400 kg/cm. The disintegrated cell suspensions were centrifuged at 3000*g* for 10 min to remove the remaining whole cells. The supernatant was then treated with 5 mg/L of deoxyribonuclease and 10 mg/L of ribonuclease for 45 min at 25°C. The envelope pellets were separated from the supernatant by centrifuging at 15,000*g* for 60 min *(15)*. The harvested envelopes were washed five times with 0.05 *M* of pH 7.2 HEPES buffer solution and then washed twice with distilled water.

Preparation of Spheroplast Envelopes

The method of Schnaitman *(22)* with minor modification was used for separating spheroplast envelopes. The separated envelopes were resuspended in 0.1 *M* of pH 7.0 Tris buffer solution with 20,000 IU/L of lysozyme. The suspension was incubated at 25°C with intensive shaking to destroy and remove the cell PEG layer. After 60 min of incubation, the cell pellets were collected by centrifuging at 18,000*g* for 60 min, and the pellets were then washed twice with 0.05 *M* of pH 7.2 HEPES buffer, and twice with distilled water. The final pellets were judged to be spheroplast envelope material and were suspended in a small volume of distilled water and lyophilized for further experiments.

Separation of Cell Outer Membrane

The modified method of Kropinski et al. *(23)* was used for separating cell outer membranes from fresh *P. putida* 5-x cells. The separated envelopes were resuspended in 0.05 *M* of pH 7.0 Tris buffer containing 20,000 IU/L of lysozyme. The suspension was incubated at 25°C with intensive shaking to

destroy and remove the cell PEG layer. After incubating for 60 min, the cell pellets were collected by centrifuging at 18,000*g* for 60 min, and the pellets were then washed with 0.1 *M* of pH 6.5 HEPES buffer containing 0.5% Triton X-100, 20 *M* of Mg^{2+} for 30 min. The pellets obtained by centrifuging at 18,000*g* for 60 min were washed twice with 0.05 *M* of pH 7.2 HEPES buffer, and then twice with distilled water. The final pellets were judged to be the outer membrane material and were suspended in a small volume of distilled water and lyophilized for further experiments.

Preparation of PEG Layer From Cell Envelopes

The isolated envelopes were extracted with 2% sodium dodecyl sulfate (SDS) solution for 30 min at 25°C, and the suspensions were centrifuged for 60 min at 30,000*g* to separate the PEG layer materials. The separated PEG layer materials were then washed with 0.5% SDS, three times with 1 *M* of NaCl, and five times with distilled water *(3,24).*

Determination of Adsorption Capacity and Isotherm of Biosorbents

The Cu^{2+} adsorption capacity of biosorbents was tested according to the method of Wang et al. *(20)* in a pH 6.5 MES buffer solution containing 100 mg/L of biosorbent and 50 mg/L of Cu^{2+}. The contact time of biosorbent and Cu^{2+} in solution was 20 min. To determine the adsorption isotherm, a series of adsorption experiments with a constant concentration of biosorbent (100 mg/L) and different initial Cu^{2+} concentrations was carried out. After adsorption for 20 min, the Cu^{2+} adsorption isotherm of the biosorbents was determined using a concentration of Cu^{2+} in supernatant plotted against the adsorption capacity of the biosorbents.

Electron Microscopy Analysis

The fresh and HCl pretreated cells were harvested by centrifugation, dehydrated through an ethanol series, and then embedded in Spuur materials (EMS, Fort Washington, PA). Thin sections of 60 nm were prepared using a Reichert Ultracuts (Leica, Wien, Austria) equipped with a diamond knife (Diatome 45°). Sections were then examined under a transmission electron microscope (JEM-1200 EX-II TEM; JEOL, Tokyo, Japan). No fixative and stain were used in the preparation of the sections. For the cell envelope, PEG layer, and outer membrane, carbon-formvar-coated nylon grids (400 mesh) (Pelco, Redding, CA) were floated in their aqueous suspensions for 30 s, dried with blotting paper, and then examined with a scanning electron microscope (Stereoscan 440; Leica) operated at an 80-kV accelerating voltage, a 400-Pa probe current, and a 10-mm working distance.

Determination of Content of Cell-Surface Components

Protein content in the cell surface was determined by the Folin method *(25)* at 595 nm with bovine serum albumin as a standard after extraction with 90% dichloromethane.

Total lipid-phosphorous content in the cell membranes was determined by the method of ANSA *(26)*. The phospholipid content in the cell membranes could be calculated assuming an average molecular weight of 700 *(27)*.

Diaminopimelic acid (DPA) in the PEG layer materials was analyzed using the method of Work *(28)* for estimating PEG content in the PEG layer by calculating that DPA is on average 18.3% of the weight of PEG.

2-Keto-3-deoxyoctulosonic acid was determined by the method of Karkhanis et al. *(29)* for estimating the lipopolysaccharide (LPS) content in the outer membrane.

Results and Discussion

Effect of Cell Superficial Layer on Cu^{2+} Adsorption by P. putida *5-x Cells*

After treatment with 0.1 *M* of HCl, 9.11 g of treated cells was obtained from 10 g of *P. putida* 5-x fresh cells (Table 1). The Cu^{2+} adsorption capability of the treated cells was studied. The results in Table 1 show that the treatment with diluted HCl enhanced the Cu^{2+} adsorption capacity of *P. putida* 5-x cells from 67.4 to 84.3 mg/g, and that the total adsorbed Cu^{2+} increased from 674 mg by the 10 g of fresh cells to 768 mg by the 9.11 g of treated cells. This suggests that the treated *P. putida* 5-x cell is a better biosorbent for Cu^{2+} removal than the fresh cell.

A loose, superficial layer (capsule outside the fresh cell was examined by TEM, but after treating with 0.1 *M* of HCl, degradation of the loose superficial layer was observed (*see* Fig. 1A,B). This result implies that removal of the loose superficial layer (capsule) outside the fresh cell enhances Cu^{2+} adsorption capacity of the cell or, rather, that the cell superficial layer has a negative effect on Cu^{2+} adsorption of the cell.

Bacterial capsule is highly hydrated (>95%), loosely arranged homopolymers or heteropolymers of carbohydrate and protein. These polymers are very flexible and extend up to several hundred nanometers from the cell surface, are entirely bathed in water, and can be easily removed by weak eluent *(21)*. Although capsular carbohydrate and protein providing electronegative groups such as carboxyl and hydroxyl groups may contribute to heavy metal ion binding, the contribution is limited owing to the low electronegative group's density (>95% is water in capsule). In general, two electronegative groups are needed to bind a divalent Cu^{2+} through metal salt bridging *(21)*. The presence of metal salt bridging may result in a conformational change within the capsule, owing to its high hydration and flexibility and, thus, may result in other metal-binding sites on the cell outer membrane (much higher density than in capsule) becoming inaccessible to Cu^{2+} binding (*see* Fig. 2). Therefore, cell pretreatment with 0.1–0.3 *M* of HCl to remove the superficial layer outside the fresh cell (capsule) can improve its Cu^{2+} adsorption capacity. In addition, HCl pretreatment can enhance Cu^{2+} adsorption capacity of the cell through removal of the native heavy metal ions adsorbed on the cell biomass.

Table 1
Recovery of Cell-Surface Components From Fresh *P. putida* 5-x Cells
in Separation Course and Their Cu^{2+} Adsorption Capacities and Total Cu^{2+} Binding Yields[a]

	Fresh cells	Pretreated cells	Cell envelopes	Spheroplast envelopes	Outer membrane	PEG layer	Inner membrane[b]
Dried weight (g)	10	9.11	2.41	1.85	0.85	0.25	1.01
Adsorption capacity (mg/g)	67.4 ± 5.2	84.3 ± 9.6	337 ± 31	258 ± 17.6	307 ± 23	386 ± 22	217 ± 19.8
Total binding yield (mg)	674	768	812	477	258	97.3	219

[a]The pretreated cells, cell envelopes, and PEG layer materials were separated from 10 g of fresh cells, respectively. The total Cu^{2+} binding yield means the total Cu^{2+} bound by total separated components derived from 10 g of fresh cells. All data are the average value of two experiments.
[b]The data in this column are calculated values according to Eqs. 1 and 2.

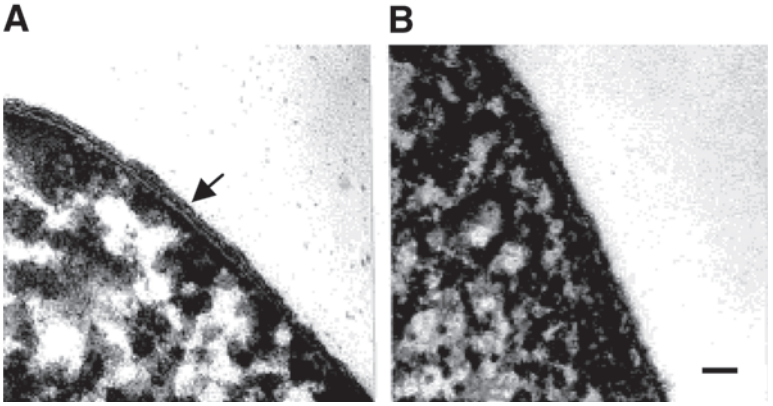

Fig. 1. TEM graphs of *P. putida* 5-x cell surface (bar = 0.1 μm): **(A)** fresh cell; **(B)** pretreated cell by 0.1 *M* HCl. A loose layer could be found on the cell surface of the fresh cell (*see* arrow).

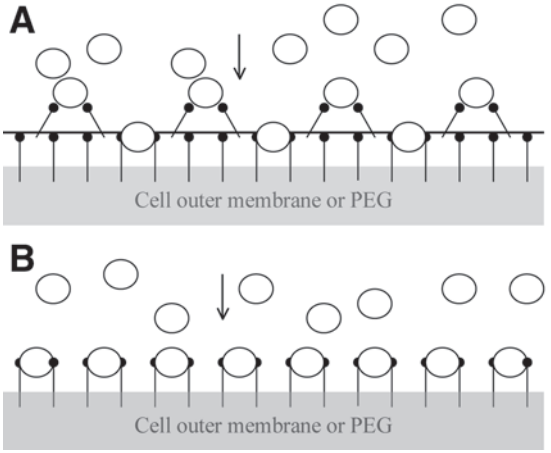

Fig. 2. Simulative Cu^{2+}-binding process by fresh cell and HCl-treated cell of *P. putida* 5-x: **(A)** fresh cell; **(B)** treated cell; horizontal lines, cell capsule; open circles, divalent Cu^{2+}; solid circles with lines, electronegative group (metal-binding site). The arrows indicate the direction of Cu^{2+} to membrane.

Cu²⁺ Adsorption on Separated Cell Envelopes of P. putida *5-x*

Many studies have indicated that isolated walls from Gram-positive bacteria have higher metal-binding capacity than isolated envelopes from Gram-negative bacteria (usually 5–10 times), owing to the difference in their cell-surface components and structure *(13,14).*

In the present study, 2.41 g (dry wt) of cell envelope materials was obtained from 10 g (dry wt) of *P. putida* 5-x fresh cells (*see* Table 1). The scanning electron microscopy (SEM) result in Fig. 3A shows that the separated cell envelope material has a typical stratified structure. This demon-

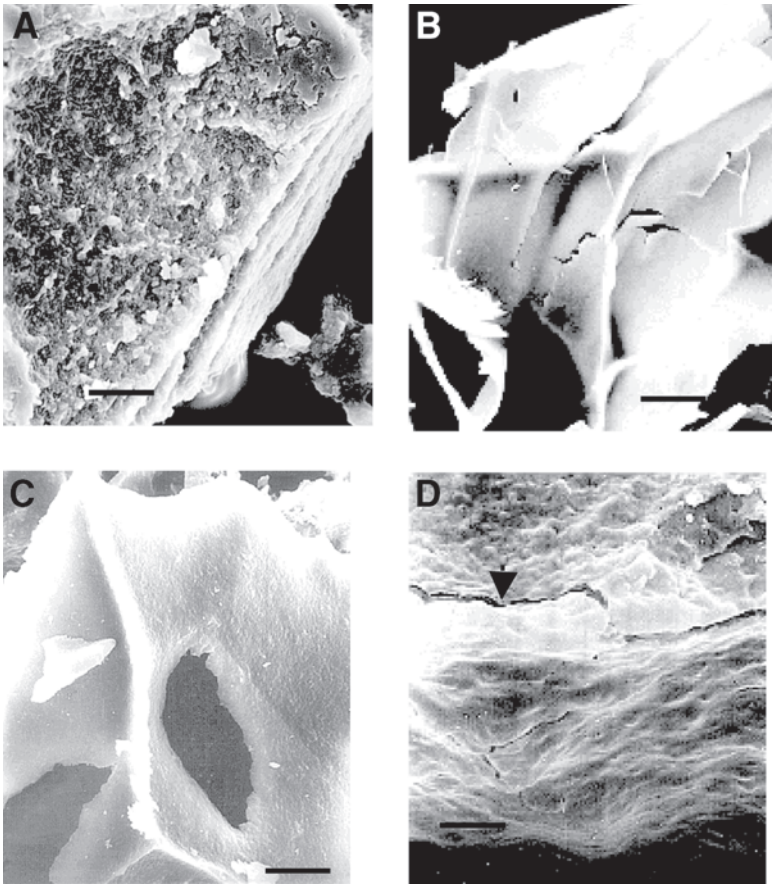

Fig. 3. SEM graph of cell envelope, PEG layer, outer membrane, and spheroplast envelope of *P. putida* 5-x: **(A)** structure-complex stratified cell envelope (bar = 1.6 μm); **(B)** structure-simple monolayer PEG layer material (bar = 3.5 μm); **(C)** thick outer membrane (bar = 1.6 μm); **(D)** bilayer spheroplast envelope with interspace (*see* arrow) (bar = 0.3 μm).

strates that the separation of the cell envelope from fresh *P. putida* 5-x cell was successful using this separation technology. Cu^{2+} adsorption capacity of the separated cell envelopes in a pH 6.5 solution containing 50 mg/L of Cu^{2+} was found to be about 337 mg/g (*see* Table 1). The adsorption capacity reached or exceeded the level of typical Gram-positive bacterial cell wall *(13,14).* In addition, the total Cu^{2+} bound by the 2.41 g of cell envelope materials was 1.2 to 1.3 times that bound by the 10 g of fresh cells (Table 1). This implies that partial metal-binding sites on the intact cell surface are inaccessible to heavy metal ions owing to spatial obstacle. However, in the separated cell envelope, more metal-binding sites on the internal part of the cell envelope become accessible to heavy metal ions. Hu et al. *(30)* also found that separated bacterial cell wall exhibits much higher heavy metal-binding capacity than the intact cell. They thought this was because the

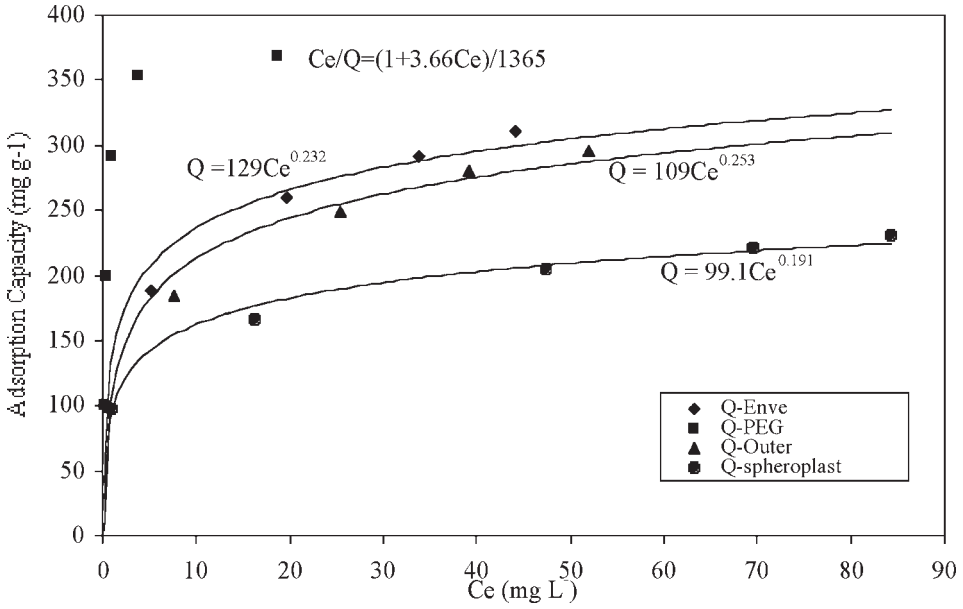

Fig. 4. Adsorption isotherms of cell-surface components isolated from *P. putida* 5-x cell.

penetration of heavy metal ion into the internal part of the cell wall is more difficult in the intact cell.

A series of adsorption experiments with a constant concentration of the cell envelope and different initial Cu^{2+} concentrations was carried out. Figure 4 presents the Cu^{2+} concentration at equilibrium and the binding capacity of the cell envelope materials. Obviously the binding process of the cell envelopes can be formulated as $Q_p = 129\ C_e^{0.232}$. This illustrates that the Cu^{2+} uptake of the cell envelope obeys the Freundlich isotherm of $Q = K_f C_e^{\ n}$ and is a physical adsorption process. The K_f value gives a measure of the adsorption capacity of the biosorbent *(31)*. The K_f value of the cell envelopes to Cu^{2+} is much higher than that of the intact cell *(20)*. This means that the cell envelope is a better adsorbent for the removal of Cu^{2+} than the fresh intact cell.

In the biosorption process, biosorbent regeneration, disposal, and transport contribute to a major cost compared with biosorbent production *(32)*. For biosorption to become a competitive method compared with existing technologies, the adsorption capacity of biosorbent should be in excess of 150 mg/g to reduce the amount of biosorbent used and thus minimize the cost of sludge regeneration, disposal, and transport *(33)*. From the total cost point of view, the cell envelope of *P. putida* 5-x is a more promising biosorbent for Cu^{2+} biosorption from industrial effluent.

The experimental results demonstrate that some bacterial cell envelopes also possess higher heavy metal adsorption capability owing to the structural complexity of Gram-negative bacteria. Yee and Fein *(34)* also

Table 2
Content of Separated Cell-Surface Components
Isolated From Fresh *P. putida* 5-x Cells Harvested in 34 h[a]

	Protein (%)	Phospholipid (%)	PEG (%)	LPS (%)
PEG layer	1.2	0.9	94.8	—
Outer membrane	31.2	39.3	—	7.8
Spheroplast envelopes	37.9	28.1	—	3.7
Inner membrane[**]	43.5	18.4	—	—

[a]The data are the average values of two tests.
[b]The data in this row are calculated values according to Eqs. 3 and 4.

argued that Gram-negative and Gram-positive bacteria had similar adsorption properties on adsorption of Cd. These results seemed to contradict traditional views. In general, Gram-positive bacterial cell walls have high metal adsorption capacity, owing to their thicker PEG layer, in which TA and TUA are embedded. PEG, TA, and TUA contain dense electronegative groups, such as carboxyl and phosphodiester, which bind heavy metals *(12).* However, the PEG layer of Gram-negative bacteria is much thinner (generally representing about 10% [w/w] of the cell envelope). This led us to determine which components in the cell envelope of *P. putida* 5-x contributed such high Cu^{2+} adsorption capacity in its Cu^{2+}-binding process.

Contribution of PEG Layer to Cu^{2+} Adsorption by P. putida *5-x Cell Envelope*

Generally, cell envelope consists of three major structural components: (1) the outer membrane, which is mostly composed of LPSs, proteins, and phospholipids; (2) the inner membrane, which is mostly composed of phospholipids and proteins; and (3) the PEG layer *(35)*. In our experiment, about 0.25 g of structure-simple monolayer PEG layer materials (Fig. 3B) (representing about 10.3% [w/w] of the cell envelope) was obtained from 10 g of *P. putida* 5-x fresh cells by SDS treatment (*see* Table 1).

The analytical results in Table 2 show that the separated PEG layer materials contained only trace proteins and phospholipids, about 95% of which was macromolecular PEG. The Cu^{2+} adsorption capacity of the PEG layer materials in a pH 6.5 solution containing 50 mg/L of Cu^{2+} was found to be approx 386 mg/g, which was not much higher than that of the whole cell envelope (Table 1). Table 1 also indicates that the total Cu^{2+} bound by the 0.25 g of PEG layer materials was only about 97.3 mg, although the loss of PEG layer materials during the separation process was inevitable. This indicates that the PEG layer only plays no more than a 15% part in Cu^{2+} binding of the cell envelope; hence, other cell-surface components, such as the outer membrane and the inner membrane, may also play an important part in Cu^{2+} binding of the cell envelope.

In addition, according to the adsorption data in Fig. 4, the binding process of the separated PEG layer material seems to be better described by the Langmuir adsorption isotherm of $Q = 1365\,C_e/(1 + 3.66\,C_e)$. This implies that the Cu^{2+} adsorption property of the PEG layer material is different from that of the intact cell and the cell envelope.

Contribution of Outer Membrane to Cu^{2+} Adsorption by P. putida *5-x Cell Envelope*

Outer membrane materials were prepared by treating cell envelope materials with lysozyme and Tween-80. About 0.84-g-thick outer membrane materials (Fig. 3C) were obtained from 10 g of fresh *P. putida* 5-x cells (Table 1). The result in Fig. 3C indicates that the separation method was efficient for separation of the outer membrane. The experimental results in Table 1 show that the outer membrane materials also exhibited a quite high Cu^{2+} adsorption capacity of about 300 mg/g, although it was still lower than that of the PEG layer materials. However, the content of the outer membrane material represented about 35% of the cell envelope, and the total Cu^{2+} bound by the 0.84 g of outer membrane material was 258 mg (Table 1), or the outer membrane material contributed more than 31% to the Cu^{2+} adsorption by the cell envelope without considering the loss of the outer membrane materials during the separation course. This indicates that the total contribution of the outer membrane exceeded the PEG layer material in the Cu^{2+} adsorption process by the cell envelope. The results of adsorption experiments shown in Fig. 4 indicate that the binding process of the outer membrane to Cu^{2+} also obeyed the Freundlich adsorption isotherm of $Q = 109\,C_e^{0.253}$ and is a physical adsorption process.

The Cu^{2+} adsorption capacity of the outer membrane is obviously higher than for those outer membranes in other Gram-negative bacteria reported in previous studies *(10,16,18)*. The analytical results in Table 2 show that the outer membrane materials contained about 39.3% phospholipids, 31.2% proteins, and 7.8% LPSs. The phospholipid content of the outer membrane material was relatively higher compared with that of the outer membranes in other Gram-negative bacteria *(36,37)*. This may be the main reason for the higher Cu^{2+} adsorption capacity of the outer membrane owing to the phospholipid containing many negatively charged groups. Hence, the cell envelopes have such a high adsorption capacity. Although the content of the LPSs is relatively lower than that of the phospholipids in the outer membrane, dense carbonyl and carboxyl groups attached to the LPSs can also contribute more negatively charged groups for heavy metal binding *(38,39)*.

Role of Inner Membrane in Cu^{2+} Adsorption of P. putida *5-x Cell Envelope*

The separated inner membrane material was not obtained owing to the difficulty of its separation, but the spheroplast envelope material was

obtained by treating the cell envelope with lysozyme. It is known that spheroplast envelope material is made up of outer and inner membrane. If the dry weight and Cu^{2+} adsorption capacity of the spheroplast envelope and outer membrane are determined, the dry weight and Cu^{2+} adsorption capacity of the inner membrane material can be calculated by the following equations:

$$W_{\text{inner}} = W_{\text{spheroplast}} - W_{\text{outer}} \tag{1}$$

$$Q_{\text{inner}} = (Q_{\text{spheroplast}} W_{\text{spheroplast}} - Q_{\text{outer}} W_{\text{outer}}) / W_{\text{inner}} \tag{2}$$

in which W_{inner}, $W_{\text{spheroplast}}$, and W_{outer} are the dry weight (g) of the inner membrane, spheroplast envelope, and outer membrane in the cell envelope, respectively; and Q_{inner}, $Q_{\text{spheroplast}}$, and Q_{outer} are the Cu^{2+} adsorption capacities (mg/g) of the inner membrane, spheroplast envelope, and outer membrane, respectively. In addition, if the content of proteins and phospholipids in the outer membrane and spheroplast envelope is determined, the content of proteins and phospholipids in the inner membrane can be calculated by the following equations:

$$C_{\text{pro-in}} = (C_{\text{pro-spheroplast}} W_{\text{spheroplast}} - C_{\text{pro-outer}} W_{\text{outer}}) / W_{\text{inner}} \tag{3}$$

$$C_{\text{P-lipid-in}} = (C_{\text{P-lipid-spheroplast}} W_{\text{spheroplast}} - C_{\text{p-lipid-outer}} W_{\text{outer}}) / W_{\text{inner}} \tag{4}$$

in which $C_{\text{pro-in}}$, $C_{\text{pro-spheroplast}}$, and $C_{\text{pro-outer}}$ are the protein content (%) in the inner membrane, spheroplast envelope, and outer membrane, respectively; and $C_{\text{P-lipid-in}}$, $C_{\text{P-lipid-spheroplast}}$, and $C_{\text{p-lipid-outer}}$ are the phospholipid content (%) in the inner membrane, spheroplast envelope, and outer membrane, respectively. Of course, these calculated data are just the theoretic values, and the error with measured value is inevitable. However, these calculated data can assist in preliminarily clarifying the role and characteristic of the inner membrane in Cu^{2+} adsorption of the cell envelope.

The spheroplast envelope materials were prepared by treating the separated cell envelope materials with lysozyme. Spheroplast envelope materials (1.85 g) were obtained from 10 g of fresh *P. putida* 5-x cells (Table 1). It is clear from Fig. 3D that the spheroplast envelope is composed of two thick membrane layers: the outer membrane and the inner membrane. An interspace is observed between the two membrane layers; this was because the PEG layer between the outer membrane and the inner membrane was degraded by lysozyme. The Cu^{2+} adsorption capacity of the spheroplast envelopes was found to be about 258 mg/g (Table 1), and the binding process obeyed the Freundlich isotherm of $Q = 99.1\, C_e 0.191$ (Fig. 4). According to the weight and Cu^{2+} adsorption capacity of the spheroplast envelope and the outer membrane, the weight and Cu^{2+} adsorption capacity of the inner membrane was determined by Eqs. 1 and 2 to be about 1.01 g and 216 mg/g, respectively (Table 1). This result indicates that the Cu^{2+} adsorption capacity of the inner membrane is lower than that of the outer

membrane, and the content of the inner membrane in the cell envelope is more than that of the outer membrane. Table 1 shows that total Cu^{2+} bound by the 1.01 g of inner membrane material was about 219 mg, or that the inner membrane materials contributed more than 25% to Cu^{2+} adsorption by the cell envelope materials. The total contribution of the inner membrane was lower than that of the outer membrane but higher than that of the PEG layer in Cu^{2+} adsorption by the cell envelope.

According to the analytical results of phospholipids and proteins in the spheroplast envelope and the outer membrane (Table 2), the content of phospholipids and proteins in the inner membrane can be determined by Eqs. 3 and 4 (about 18.7 and 43.5%, respectively). Clearly, the phospholipid content in the inner membrane is lower than that in the outer membrane, and the protein content is higher than that in the outer membrane. This result implies that, compared with the outer membrane, a lower phospholipid content and the absence of LPSs may lead to a lower Cu^{2+} adsorption capacity of the inner membrane, and the proteins seem to play a minor part in Cu^{2+} adsorption by the inner membrane or outer membrane materials.

Conclusion

The experimental results indicate that the cell superficial layer had a negative effect on Cu^{2+} adsorption by *P. putida* 5-x cells. The isolated cell envelopes possessed fivefold more Cu^{2+} adsorption capacity of the fresh intact cells owing to the liberation of more and more binding sites on the cell envelope during the separation process. All of the cell-surface components—the PEG layer, outer membrane, and inner membrane—contributed to Cu^{2+} adsorption by the cell envelopes. Their Cu^{2+} adsorption capacity was in the order PEG layer > outer membrane > inner membrane, but the content of these components in the cell envelope was in the order inner membrane > outer membrane > PEG layer. The total contribution of these cell-surface components to Cu^{2+} adsorption by the cell envelope was in the order outer membrane > inner membrane > PEG layer. The adsorption capacity of the outer membrane is obviously higher than that of those outer membranes in other Gram-negative bacteria reported previously. This may be the main reason for such a high Cu^{2+} adsorption capacity of the cell envelopes.

According to these experimental results, one can conclude that if the content of the PEG layer and the outer membrane in the cell envelope is increased, the adsorption capacity of the cell envelope will be enhanced obviously. In addition, if the content of the phospholipids and LPSs in the outer membrane is increased, the adsorption capacity of the outer membrane will be enhanced and, hence, the cell envelope.

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