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Copper biosorption by chemically treated *Micrococcus luteus* cells

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Summary

In order to clarify the binding states of copper in microbial cells, copper biosorption from aqueous systems using the chemically treated *Micrococcus luteus* IAM 1056 cells (hot water-treated, diluted NaOH-treated, chloroform–methanol-treated, and chloroform–methanol/concentrated KOH-treated cells) was examined. The intact cells of *M. luteus* adsorbed 527 μmol of copper per g cells, and its copper adsorption was very rapid and was affected by the solution pH. The chloroform–methanol/concentrated KOH-treated cells showed higher copper biosorption capacity than the intact and the other chemically treated cells. The electron paramagnetic resonance (EPR) parameters, $g_{\|}$ and $|A_{\|}|$, of Cu(II) ion in microbial cells indicate that Cu(II) ion in the intact and all the chemically treated cells have coordination environments with nitrogen and oxygen as donor atoms, being similar to those of type II proteins. The parameter $g_{\|}$ also indicated that the coupling between Cu(II) ion and the cell materials in the $CHCl_3$–MeOH/concentrated KOH-treated cells is rather more stable than those between Cu(II) ion and the cell materials in the other treated cells.

Introduction

Biosorption of heavy metals has received much attention from the standpoints of recovery of useful metals and of removal of toxic metals. Microorganisms are the most potentially useful materials for heavy metal biosorption, because of their abilities to take up metal ions, suitability for natural circumstances and low cost. Recently, much research has been concentrated on the study of copper biosorption from aqueous systems using bacteria (Mattuschka *et al.* 1994; Macaskie 1995; Philip *et al.* 1995; Chang *et al.* 1997; Karna *et al.* 1999), actinomycetes (Mattuschka *et al.* 1994), fungi (Huang & Huang 1996; Kapoor *et al.* 1999), yeasts (Brady & Duncan 1994), and algae (Nakajima *et al.* 1979; Matheickal & Yu 1999). These works have shed light on our understanding of the mechanisms of copper biosorption. In a previous paper, one of the authors screened 32 bacteria for selective biosorption of heavy metal ions, and found that *Bacillus subtilis* IAM 1026, *Micrococcus luteus* IAM 1056 and *Pseudomonas stutzeri* IAM 12097 have excellent abilities to adsorb copper selectively from metal mixed solution (Nakajima & Sakaguchi 1986). Further screening of these bacteria for copper biosorption showed, that *M. luteus* IAM 1056 had the highest ability to adsorb copper. Previous results showed that heavy metal biosorption using chemically treated microbial cells gave important information on the states of heavy metals in the cells (Nakajima *et al.* 1981). In this paper, in order to clarify the binding states of copper in microbial cells, copper biosorption by chemically treated cells of *M. luteus* IAM 1056 was examined using electron paramagnetic resonance (EPR) spectrometry.

Materials and Methods

Strains, medium and growth conditions

Strains used in this study were generously donated by IAM Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, The University of Tokyo. Chemicals (guaranteed reagents) used in this study were obtained from Nacarai Tesque, Inc. and Wako Pure Chemical Industries, Ltd. Media for growing bacteria used in this study were: 3 g of meat extract, 5 g of polypepton, 5 g sodium chloride in 1 l of deionized water, pH 6.5. Microbial cells were grown in 300 ml medium in a 500 ml culture flask with continuous shaking (130 rev/min) at 30 °C.

Cells in linearly growing phase were collected by centrifugation (18,000 ×*g*), washed thoroughly with isotonic sodium chloride solution, and then used for adsorption experiments.

Preparation of chemically treated cells

Chemically treated cells were prepared as follows (Nakajima *et al.* 1981): (1) Hot water-treated cells. One gram (dry weight basis) of fresh cells of *M. luteus* IAM 1056 was treated with 100 ml of deionized water at 100 °C under reflux for 2 h. The cells were then collected by centrifugation, washed thoroughly with deionized water, and used for the subsequent experiments. The yield was 801 mg dry weight. (2) Dilute sodium hydroxide (NaOH)-treated cells. One gram (dry weight basis) of fresh *Micrococcus* cells was treated with 100 ml of 0.2% NaOH solution and stirred continuously at room temperature for 20 h. The cells were then collected by centrifugation, washed thoroughly with isotonic sodium chloride solution until the pH of the wash solution was in neutral range around 7, and then used for subsequent experiments. The yield was 763 mg dry weight. (3) $CHCl_3$–MeOH-treated cells. Two grams (dry weight - basis) of fresh *Micrococcus* cells were treated with 100 ml of 96% (w/v) methanol at 65 °C under reflux for 10 min. A 200 ml of chloroform were added to the suspension, the mixture was stirred for 20 min, and the cells were then collected by centrifugation (18,000 ×*g*). The cells were suspended again in 100 ml $CHCl_3$–MeOH (2:1) solution, and the suspension was stirred for 20 min. The cells were again collected by centrifugation (18,000 ×*g*), and used for subsequent experiments. The yield was 1516 mg dry weight. (4) $CHCl_3$–MeOH/concentrated potassium hydroxide (KOH)-treated cells. Half of the chloroform–methanol-treated cells (758 mg dry weight basis) were suspended in 100 ml of 24% (w/v) KOH solution, and the suspension was left to stand for 2 h under N_2 flow, being stirred occasionally. The cells were collected by centrifugation (18,000 × *g*), and suspended in 10 ml of 24% (w/v) KOH solution again for 2 h. The cells were collected by centrifugation (18,000 ×*g*), washed thoroughly with isotonic sodium chloride solution until the pH of the wash solution was in neutral range around seven, and then used for subsequent experiments. The yield was 169 mg dry weight.

Copper biosorption experiments

The copper adsorption experiments were conducted as follows: precultured fresh cells or chemically treated cells (20 mg dry weight basis) were suspended in 40 ml of a solution containing the desired amounts of copper. Copper was supplied as copper(II) nitrate. The solution pH was adjusted to desired values with 0.1 N HCl and 0.1 N NaOH solutions. After each suspension had been shaken (130 rev/min) at 25 °C, the cells were collected by centrifugation (18,000 ×*g*) and freeze-dried. Amounts of copper adsorbed were determined by measuring copper contents in the supernatant with an inductively coupled plasma quantometer (Shimadzu ICPQ-1000II). The experiments were conducted three times and averaged.

Determination of copper contents in microbial cells

The amounts of copper in the cells were determined by neutron activation analysis. Freeze-dried cells were irradiated at the Japan Atomic Energy Research Institute, in JRR-4 reactor for 1 min at a thermal neutron flux of 5×10^{13} n/cm^2 · s. The 1039.0 keV of γ-ray from ^{66}Cu (the half life 5.10 min) and 1345.5 keV of γ-ray from ^{64}Cu (the half life 12.8 h) were used for analysis.

Electron paramagnetic resonance measurements

Five milligrams of the freeze-dried powder samples were put into a quartz sample tube of 5 mm diameter, and then used for EPR analysis. Electron paramagnetic resonance spectra were measured using X-band ESR spectrometer (JEOL JES RE-1X and JES TE-100) under the conditions of microwave frequency, 9.44 GHz; magnetic field, 310 mT; field amplitude, 75 mT; field modulation, 100 kHz; modulation width, 0.32 mT; microwave power 5 mW, and the time constant, 0.3 s.

Results and Discussion

Copper biosorption abilities of bacteria

In a previous paper, 32 bacteria were screened for selective biosorption of heavy metal ions, and it was found that *B. subtilis* IAM 1026, *M. luteus* IAM 1056 and *P. stutzeri* IAM 12097 had excellent abilities to adsorb copper selectively from mixed metal solutions (Nakajima & Sakaguchi 1986). Thus, further screening for the maximal copper adsorption was conducted using these three species of bacteria in solutions containing $4 \sim 80 \times 10^{-5}$ M copper (pH 5). Copper adsorption by each bacterium obeys the Langmuir adsorption isotherm, $Q = k \cdot Q_m \cdot C_e/(1 + k \cdot C_e)$, where Q_m is the maximum adsorption capacity (μmol/g dry cells), C_e, the equilibrium copper concentration (M), and k, the adsorption–desorption equilibrium constant (Figure 1). The Langmuir parameters of each bacterium are summarized in Table 1. *Micrococcus luteus* has the highest Q_m value, 527 μmol/g, among the bacteria tested. The k values of these bacteria were about $1.10–2.14 \times 10^4$ l/mol. Chang *et al.* (1997) estimated the copper biosorption capacity of *P. aeruginosa* PU21 as 22.1–23.1 mg/g dry cells (348–364 μmol/g dry cells), and k as 4.43–6.65 mg/l ($1.50–2.25 \times 10^4$ l/mol). Their results were almost in the same range as those of our results. Philip *et al.* (1995) obtained the higher copper biosorption capacity, 50 mg/g dry cells (786 μmol/g dry cells), for *P. aeruginosa*. However, they did not give the k value.

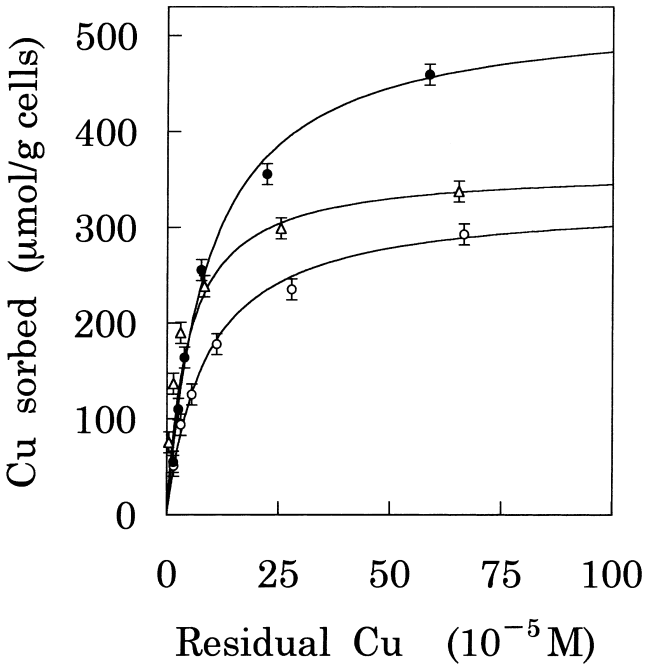

Figure 1. Isotherm of copper adsorption at pH 5 by bacteria. ○: *B. subtilis* IAM 1026, ●: *M. luteus* IAM 1056, △: *P. stutzeri* IAM 12097. Twenty milligrams (dry weight basis) of microbial cells were suspended in 40 ml of a solution (pH 5) containing 4–80 × 10^{-5} M copper for 1 h. Each point represents mean ± standard deviation of triplicates.

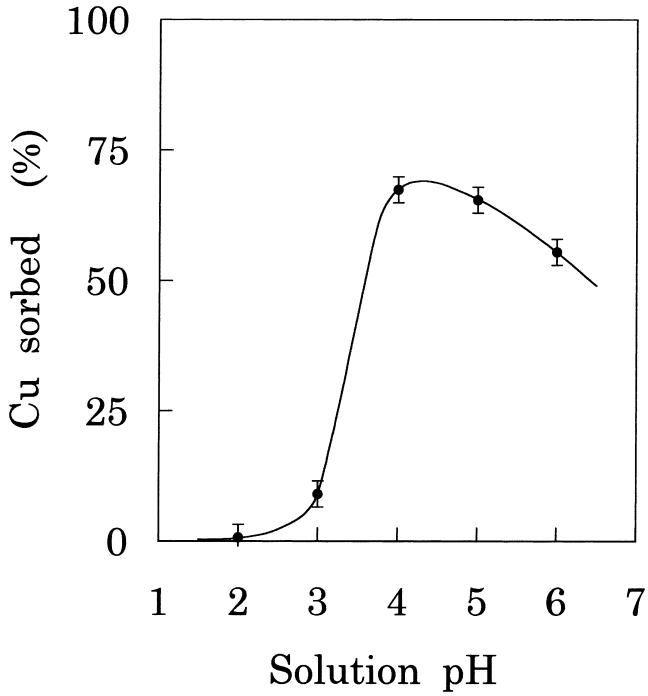

Figure 2. Effect of solution pH on the copper adsorption by *M. luteus* IAM 1056. Microbial cells (20 mg dry weight basis) were suspended in 40 ml of a solution (pH 2–6) containing 4 × 10^{-4} M copper for 1 h. Each point represents mean ± standard deviation of triplicates.

Table 1. Parameters of Langmuir isotherm for Cu biosorption by bacteria.

Species	Q_m (μmol/g cells)	k (10^4 l/mol)
B. subtilis IAM 1026	327 ± 7	1.14 ± 0.03
M. luteus IAM 1056	527 ± 12	1.10 ± 0.06
P. stutzeri IAM 12097	361 ± 20	2.14 ± 0.12

Parameters represent mean ± standard deviation of estimation.

Kapoor *et al.* (1999) examined both the Langmuir and Freundlich models for heavy metal biosorption of *Aspergillus niger*. In our case, as a matter of course, both models were examined. As $\log(Q) - \log(C_e)$ plots showed non-linear curves, further discussion on the Freundlich model was omitted.

As *M. luteus* IAM 1056 has an excellent ability to adsorb copper, the binding states of copper in *M. luteus* cells were examined.

Aspects of copper biosorption by the intact M. luteus *cells*

The adsorption of copper by intact *Micrococcus* cells was markedly affected by the solution pH. The maximum copper adsorption was observed at around pH 4 (Figure 2). The amounts of copper adsorbed by the cells rapidly decreased below pH 4, and gradually decreased above pH 5. Chang *et al.* (1997) showed the increase of copper biosorption by *P. aeruginosa* PU21 with the increase of the solution pH up to pH 6, and found no maximum peak. These results indicated that the pH profile of copper biosorption by bacteria will differ with different species of bacteria.

The adsorption of copper by intact *Micrococcus* cells was very rapid. The adsorption reached equilibrium within 10 min after contact with copper solution. Chang *et al.* (1997) also showed that the biosorption of copper by *P. aeruginosa* PU21 reached equilibrium within 30 min after contact with copper solution. The rapid adsorption by bacteria was also observed in uranium biosorption (Cotoras *et al.* 1992). Thus, heavy metal biosorption by bacterial cells is rapid.

Copper biosorption by chemically treated M. luteus *cells*

In order to clarify the binding states of copper in microbial cells, *M. luteus* cells were treated with hot water, diluted NaOH, $CHCl_3$–MeOH, and $CHCl_3$–MeOH/concentrated KOH, and then the copper adsorption by the chemically treated cells was examined from a solution (pH 5) containing 8 × 10^{-4} M of copper (Table 2). The amounts of copper adsorbed by the hot water-treated, diluted NaOH-treated, and $CHCl_3$–MeOH-treated cells (mg Cu per g dry cells) are almost same as those by the intact cells. The ratios of copper adsorption to that of the intact cells were nearly in proportion to the yields. These results indicated that the copper adsorbing abilities of residual cell components in these treated cells were almost same as those of the extracted components (20–25% of the intact cells), such

as oligo- and polysaccharides, proteins and many low molecular weight substances for hot-water treatment, lipids for $CHCl_3$–MeOH treatment, and proteins and polysaccharides for diluted NaOH treatment. On the other hand, the amounts of copper adsorbed by the $CHCl_3$–MeOH/concentrated KOH-treated cells were much larger than those of other cells that the ratio of copper adsorption to that of the intact cells went up to 24%. The residual components of the treated cells (about 17% of the intact cells) have a higher ability to adsorb copper than the eluted components (lipids, proteins and polysaccharides).

EPR of Cu(II) ion adsorbed in the chemically treated Micrococcus *cells*

The EPR spectrum of Cu(II) ion in the chemically treated *Micrococcus* cells was measured. Since ordinal divalent copper complexes are in a tetragonally distorted octahedral environment, the EPR spectrum of a powder sample is of the axial type, showing a major absorption to higher field at $g_\perp$ and lesser absorption to lower field at $g_\parallel$. As the nuclear spin of each naturally occurring isotope, ^{63}Cu and ^{65}Cu, is 3/2, the EPR spectrum of Cu(II) will show a hyperfine splitting of four features. The EPR spectrum of Cu(II) ion is described by the following spin Hamiltonian;

$$\mathscr{H} = \mu_B[g_\perp(HxSx + HySy) + g_\parallel HzSz] + A_\perp(SxIx + SyIy) + A_\parallel SzIz, \quad (1)$$

where μ_B is the Bohr magneton, $g_\perp = (gxx + gyy)/2$ and $g_\parallel = gzz$, the principle axis components of g-values, $A_\perp = (Axx + Ayy)/2$ and $A_\parallel = Azz$, the principle axis components of hyperfine coupling constant, Hx, Hy, Hz, the components of outer magnetic field, Sx, Sy, Sz, the components of electron spin, Ix, Iy, Iz, the components of copper nuclear spin, $S = 1/2$ and I(Cu) = 3/2.

Table 2. Copper biosorption by chemically-treated *M. luteus* cells.

Microbial cells	Yield (mg)	Cu adsorbed (μmol/g)
Intact cells	1000	427.6 ± 3.3 (100.0)
Chemically treated cells		
Hot water-treated	801	437.7 ± 3.8 (82.0)
Diluted NaOH-treated	763	434.8 ± 2.5 (77.5)
$CHCl_3$–MeOH-treated	758	424.2 ± 4.0 (75.2)
$CHCl_3$–MeOH/KOH-treated	169	609.7 ± 2.8 (24.1)

The chemically treated cells (20 mg dry weight basis) were suspended in 40 ml of a solution (pH 5) containing 8×10^{-4} M of copper for 1 h. Values in parentheses indicate the ratios of copper adsorption expressed as percentage of that of intact cells. Sorption amounts (μmol/g) represent mean ± standard deviation of triplicates.

The g-values are given as follows (modified from Abragam & Pryce 1951; Gersmann & Swalen 1962);

$$g_\parallel = \mathrm{ge}(1 - 4\lambda/\Delta_1) \quad (2)$$

$$g_\perp = \mathrm{ge}(1 - \lambda/\Delta_2) \quad (3)$$

$$A_\parallel = P[-\alpha^2(4/7 + \kappa) + (g_\parallel - \mathrm{ge}) + (3/7)(g_\perp - \mathrm{ge})] \quad (4)$$

$$A_\perp = P[\alpha^2(2/7 - \kappa) + (11/14)(g_\perp - \mathrm{ge})], \quad (5)$$

where ge = 2.0023; the g-value of free electron, λ; the spin-orbit coupling constant (−828 cm^{-1} for free Cu(II) ion), $\Delta_1 = E(dxy) - E(dx^2 - y^2)$, $\Delta_2 = E(dxz) - E(dx^2 - y^2)$; the ligand field constants, P; the dipole term (0.036 cm^{-1} for free Cu(II) ion), α^2; the d-electron density of Cu(II) ion, and κ; the correction for Fermi contact term. Thus, $g_\parallel$ and $|A_\parallel|$, being associated with the ligand field constants and the d-electron density of Cu(II) ion, can often be used to assign structures of copper complexes (Peisach & Blumberg 1974). A part of the EPR spectrum of Cu(II) ion in chemically treated *Micrococcus* cells is shown in Figure 3, and the parameters obtained from these spectra, $g_\parallel$, $g_\perp$, and $|A_\parallel|$ are listed in Table 3. The EPR spectra indicate the typical powder pattern of Cu(II) ion in a tetragonally distorted

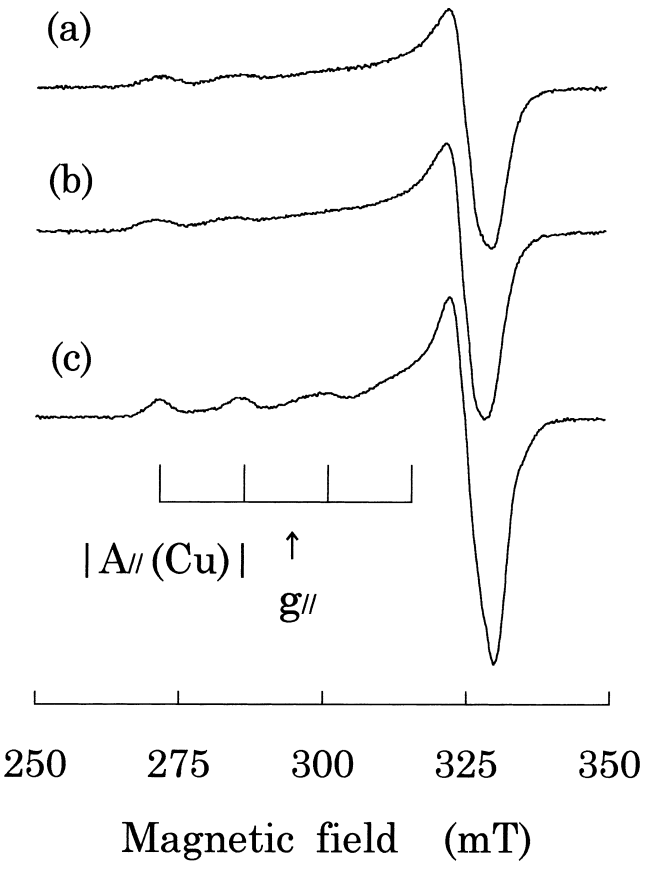

Figure 3. EPR spectra of Cu(II) in the intact and chemically treated cells of *M. luteus* IAM 1056. (a) Intact cells, (b) hot water-treated cells, (c) $CHCl_3$–MeOH/concentrated KOH-treated cells.

Table 3. ESR parameters of Cu(II) ion in the *M. luteus* cells.

Microbial cells	$g_{\parallel}$	$g_{\perp}$	$\lvert A_{\parallel}\rvert$ (mcm^{-1})
Intact cells	2.260 ± 0.004	2.040 ± 0.004	18.5 ± 0.5
Chemically treated cells			
Hot water-treated	2.280 ± 0.004	2.043 ± 0.004	17.6 ± 0.5
Diluted NaOH-treated	2.260 ± 0.004	2.040 ± 0.004	18.9 ± 0.5
$CHCl_3$–MeOH-treated	2.262 ± 0.004	2.042 ± 0.004	18.2 ± 0.5
$CHCl_3$–MeOH/KOH-treated	2.247 ± 0.004	2.037 ± 0.004	19.1 ± 0.6

Parameters represent mean ± standard deviation of estimation.

octahedral environment. As $g_{\parallel}$ and $|A_{\parallel}|$ in Table 3 are in the range of 2.247–2.280 and 17.6–19.1 mcm^{-1}, Cu(II) in each cells, having ligands with nitrogen and oxygen donor atoms, will be in a similar coordination environment as those of type II copper proteins (Peisach & Blumberg 1974). As the Cu(II) ion is an intermediate acid, it can combine well with functional groups such as carboxylic acids and amines in extended materials in the cells. Thus, oxygen and nitrogen donor atoms should originate from carboxyl and amino groups. Kapoor *et al.* (1997) suggested that both carboxyl and amino groups play an important role in copper biosorption of *A. niger*. Similar results were also found in copper adsorption by *B. subtilis* cell walls (Beveridge & Murray 1980). The $g_{\parallel}$ value of Cu(II) ion in the $CHCl_3$–MeOH/concentrated KOH-treated cells is smaller to those of other cells, while that of Cu(II) ion in the hot water-treated cells is larger. From Equation (2), the smaller $g_{\parallel}$ value leads to the larger Δ_1 value, and the larger $g_{\parallel}$ value to the smaller Δ_1 value. As the larger Δ_1 value mean a more stable coupling between metal ions and ligands (Martell & Hancock 1996), the coupling between Cu(II) ion and the cell materials in the $CHCl_3$–MeOH/concentrated KOH-treated cells is somewhat more stable than those between Cu(II) ion and the cell materials in the other treated cells. As the elution by the diluted NaOH and $CHCl_3$–MeOH treatments was so small, the copper-binding environments were almost the same as those of the intact cells. Thus, $g_{\parallel}$ and $|A_{\parallel}|$ of Cu(II) ion in the diluted NaOH treated cells and the treated cells were almost same as those of the intact cells.

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