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Copper-Binding Characteristics of Exopolymers from a Freshwater-Sediment Bacterium†

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Copper-binding activity by exopolymers from adherent cells of a freshwater-sediment bacterium was demonstrated by a combination of equilibrium dialysis and flameless atomic absorption spectrometry. Crude, cell-free exopolymer preparations containing protein and polysaccharide components bound up to 37 nmol of Cu per mg (dry weight). A highly purified exopolysaccharide preparation bound up to 253 nmol of Cu per mg of carbohydrate. The conditional stability constant for the crude exopolymer-Cu complex was 7.3×10^8. This value was similar to those obtained for Cu complexes formed with humic acids and xanthan, an exopolysaccharide produced by *Xanthomonas campestris*. Studies conducted at copper concentrations, pHs, and temperatures found in sediments from which the bacterium was isolated indicated that the exopolymers were capable of binding copper under natural conditions.

The distribution of heavy metals and their resultant bioavailability and geochemistry in aquatic habitats are to a great extent controlled by organic and inorganic substances. Humic and fulvic acids (2, 25), oxides (10), and biota (4, 13, 16) all contribute to the partitioning of metals in sediments. Recently, attention has been directed to the role of particle-associated bacteria in metal distribution (15).

Bacteria are commonly associated with surfaces in aquatic environments. Attachment to sediment particles and other surfaces is mediated by bacterial cell surface polymers (5). These exopolymers have also been shown to complex various metals (6). Titus and Pfister (26) demonstrated that cadmium uptake by a *Pseudomonas* species was greater than that of an artificial sediment.

However, few studies of metal binding by bacterial polymers have been conducted under in situ conditions, nor have direct comparisons been made between bacterial products and other metal-binding substances in the environment. Furthermore, most exopolymer-metal binding studies conducted in the past have not taken into consideration the contribution of intracellular or cell wall polymers that commonly contaminate exopolymer preparations (24). The recent development of a procedure for the isolation of firmly bound exopolymers from adherent cells of a freshwater-sediment bacterium (R. M. Platt, G. G. Geesey, J. D. Davis, and D. C. White, submitted for publication) and the availability of metal chemistry data for sediments from which the bacterium was isolated (8) provided the opportunity to define exopolymer-metal interactions under conditions that approached those in the natural environment.

Of the metals of toxicological concern in aquatic environments, copper exhibits the greatest tendency to associate with organic matter (3). Microbial exopolymers could therefore chelate copper in sediments. Since microorganisms and their associated exopolymers are known to be ingested by a variety of benthic detritivores (21–23), those polymers which interact with copper could serve as a route of entry of the metal into benthic food webs.

The copper-binding characteristics of various exopolymer fractions from a sediment bacterium are described below and compared with those of other bacterial exopolymers and organic substances thought to be important in controlling copper distribution in freshwater sediments. A method is described by which a large number of binding assays can be carried out simultaneously and economically under conditions approximating those that exist in the sediment environment.

MATERIALS AND METHODS

Organism. The bacterium used in this study was isolated from metal-laden sediments in the lower Fraser River in British Columbia, Canada. The isolate was a nonfermentative, oxidase-positive rod possessing a firmly bound microcapsule which mediated attachment of cells to surfaces (Platt et al., submitted for publication). Although a number of the biochemical characteristics of the organism were defined (Table 1), the bacterium has not yet been positively identified.

Exopolymer isolation. Cells were cultured in cell aggregation medium under conditions which encouraged capsule production and cell adhesion as described previously (Platt et al., submitted for publication). Exopolymer was released from the cells by a combination of blending and centrifugation in the presence of EDTA (Platt et al., submitted for publication). Exopolysaccharide purification followed the procedure of Platt et al. (submitted for publication), using phenol extraction to remove protein. The three exopolymer preparations used for the metal binding studies were obtained from material at different stages of purification of the exopolysaccharide component. Each preparation was dialyzed against double-distilled water and lyophilized before reconstitution for binding studies. The contribution of various components to the exopolymer material is presented in Table 2. Chemical determinations were carried out by methods described previously (Platt et al., submitted for publication).

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TABLE 1. Biochemical characteristics of freshwater-sediment bacterium[a]

Assay	Reaction
Motility (25°C)	+
Glucose oxidation	+
Arabinose oxidation	+
Lactose oxidation	+
N_2 gas	−
TSI medium (25°C)	Growth; no reaction
Indole	−
2-Keto-D-gluconate (tests for presence of 2-β-gluconic acid)	+
NO_3^- reduction (to NO_2^-)	+
Lysine decarboxylase	−
Fluorescence (25°C)	−
Oxidase	+
Pigment	−
Odor	Not atypical

[a] All tests were run at 25°C, read daily for 4 days, and then repeated.

Metal binding assay. Exopolymer fractions, xanthan, and humic acids were dissolved in glass-distilled double-distilled water, and the pH was adjusted to 6.0 unless otherwise indicated. Copper solutions (1,000 mg/liter) were prepared by dissolving electroanalytical-grade copper metal in Ultrex-grade nitric acid (J. T. Baker Chemical Co., Phillipsburg, N.J.). Dilutions were made in glassware cleaned thoroughly in detergent, soaked in a 5% nitric acid solution for 24 h, and then rinsed in double-distilled water before air drying in a metal-free area.

The binding assay was carried out with a multichamber equilibrium dialysis apparatus similar to that described by Furlong et al. (7), except that the wells were constructed of Teflon to minimize metal contamination. Before use, the dialysis apparatus was cleaned by the method described above for glassware. Test samples (100 μl) were added to one well, and copper solution (100 μl) was added to a well separated from the sample well by a membrane (Spectrapor 4; molecular weight cutoff, 12,000 to 14,000; Spectrum Medical Industries, Inc., Los Angeles, Calif.) which had been rinsed and equilibrated in double-distilled water. As many as 36 assays were run simultaneously with one apparatus. After it was loaded, the apparatus was rotated at 3.5 rpm at 4°C until copper equilibrium was achieved across the membrane (typically, 36 h). The solution in each well was then transferred to metal-free microcentrifuge tubes, and the copper concentration was determined with a Perkin-Elmer 5000 atomic absorption spectrometer equipped with a Perkin-Elmer 500 heated graphite analyzer.

Data analysis. Copper binding by each sample was determined by subtracting the copper concentration in the well to which the metal solution was initially added (free copper) from the copper concentration in the sample well (free copper plus that complexed by the sample material). Binding constants were obtained graphically from the Langmuir linear expression $c/x = 1/k_1k_c + c/k_1$ (11), where c is the free copper concentration, x is moles of copper bound per unit weight of metal-binding component, k_1 is the maximum binding ability (MBA), and k_c is the conditional stability constant. The data were fit to a straight line using a linear least-squares regression equation.

Potentiometric titration. Titrations were adapted from the method of Lasik and Gordiyenko (12). Highly purified exopolysaccharide was diluted to achieve a concentration of 23 μg of carbohydrate per ml in double-distilled water, and the pH was adjusted to 3.80. The solution was titrated with 0.005 N sodium hydroxide under a stream of N_2 gas. The pH was monitored with a Corning model 125 pH meter equipped with a Corning Ag-AgCl combination electrode. A subsample of the exopolysaccharide solution was mixed with copper solution to achieve a final copper concentration of 1 μg/ml and a final carbohydrate concentration of 23 μg/ml. The pH was adjusted to 3.80, and the mixture was titrated as above. Ionic strength was maintained at 0.10 M in all titrants by the addition of potassium nitrate.

Multielement analysis of exopolysaccharide. Highly purified exopolysaccharide (23 to 35 μg/ml) was dialyzed against double-distilled water and aspirated into an inductively coupled plasma atomic emission spectrometer programmed for simultaneous analysis of up to 29 elements (1). Nebulizer dimension, sample flow rate, and computer-controlled signal analysis were coordinated to accommodate 70-μl sample volumes. Background and interferences were determined for each element according to the method of Alexander and McAnulty (1).

Chemicals. Technical-grade humic acid (HI, 675-2) was obtained from Aldrich Chemical Co., Milwaukee, Wis. Purified xanthan (powder) produced by Kelco (San Diego, Calif.) was obtained from J. Kim, California State University, Long Beach.

TABLE 2. Exopolymer composition at various stages of purification of polysaccharide component

Preparation	Carbohydrate (μg/ml)	Protein (μg/ml)	DNA (μg/ml)
Whole cells (44.5 mg [dry wt]/ml)	3,250	16,900	1,398
Crude exopolymer (after alcohol precipitation; 0.83 mg [dry wt]/ml)	77	100	13
Partially purified exopolymer (after DNA and lipid removal; 2.9 mg/ml)	200	170	ND[a]
Highly purified exopolysaccharide (after DEAE chromatography; 0.5 mg/ml)	42	6	ND

[a] ND, Not detected.

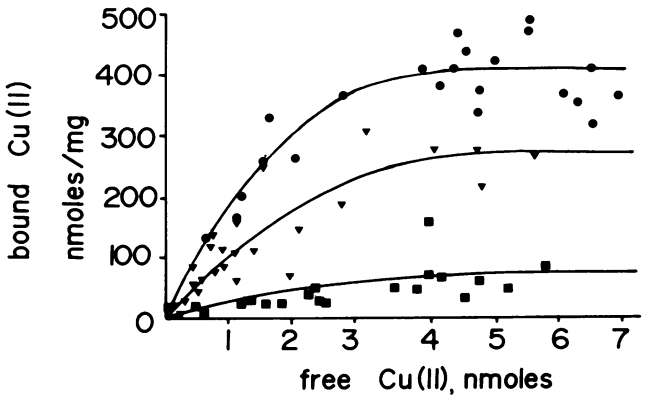

FIG. 1. Freundlich isotherms of Cu^{2+} binding to crude, cell-free exopolymer (●), partially purified exopolymer (▼), and highly purified exopolymer (■) fractions.

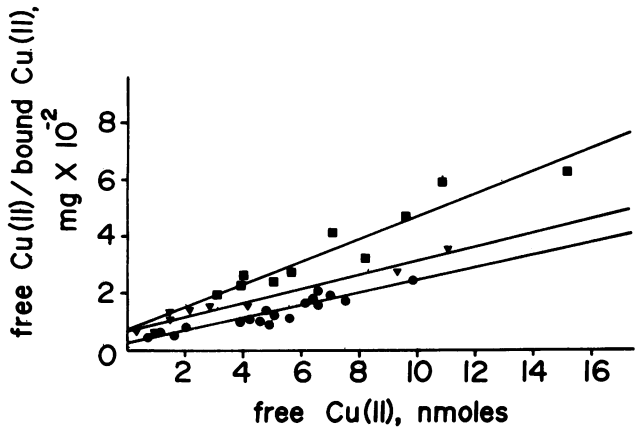

FIG. 2. Langmuir plot of Cu^{2+} binding to crude, cell-free exopolymer (●), partially purified exopolymer (▼), and highly purified exopolymer (■) fractions.

RESULTS

Equilibrium dialysis studies demonstrated copper binding by capsular material isolated from a freshwater-sediment bacterium. Saturation of copper-binding sites occurred at lower dissolved copper concentrations after removal of DNA and protein from the capsule matrix (Fig. 1). A linear relationship was obtained from a Langmuir plot of the copper-binding capacity of crude, partially purified, and highly purified exopolysaccharide preparations at copper concentrations ranging from 25 to 4,000 ng/ml (Fig. 2). The MBA derived from the Langmuir isotherms decreased from 489 nmol of Cu per mg of carbohydrate for the crude capsular material to 253 nmol of Cu per mg of carbohydrate for the highly purified exopolysaccharide preparation (Table 3).

A plot of MBA versus protein concentration of preparations at different stages of exopolymer purification yielded a straight line, which when extrapolated to zero protein concentration provided an MBA value of 200 nmol of Cu per mg of carbohydrate (Fig. 3). Based on the assumption that the protein and polysaccharide components contributed all of the copper-binding sites on the crude exopolymer, the MBA of the protein component was estimated to be 193 nmol of Cu per mg of protein.

The conditional stability constants (k_c) for the copper-binding sites contained within the various exopolymer preparations were fairly similar, ranging from 4.4×10^8 to 7.3×10^8 (Table 3).

Although an effort was made to conduct these copper binding studies in the absence of competing ions, multielement analysis of preparations of highly purified exopolysaccharide revealed that a variety of cations remained associated with the polymer even after dialysis against three changes of double-distilled water (Table 4). The concentration of some metals such as Fe and Pb varied among exopolymer preparations.

TABLE 3. Cu^{2+}-binding characteristics of cell surface preparations

Cell surface prepn	MBA (nmol/mg of carbohydrate)	k_c ($\times 10^8$)
Cell-free exopolymer	489	7.3
Partially purified exopolysaccharide	401	4.4
Highly purified exopolysaccharide	253	5.7

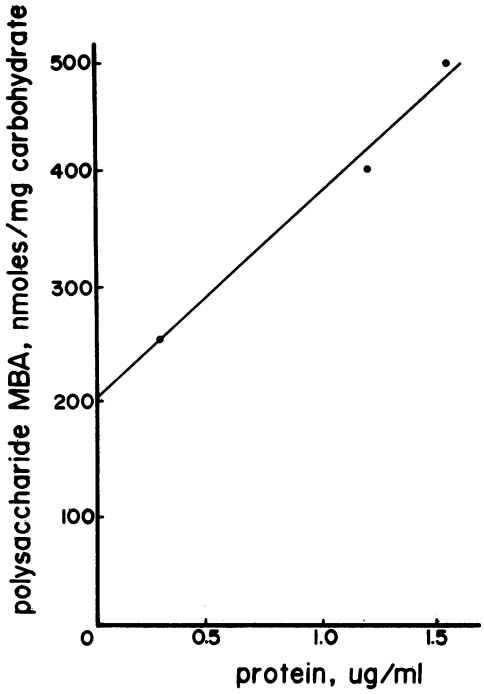

FIG. 3. Effect of protein removal on MBA of exopolymer to copper.

The effect of pH on copper binding to the highly purified exopolysaccharide preparation was determined over a copper concentration range of 50 to 4,000 ng/ml. Langmuir plots demonstrated that the MBA increased and the k_c decreased with increasing hydrogen ion concentration over the pH range 5.0 to 7.0 (Fig. 4; Table 5). Potentiometric titration of the highly purified exopolysaccharide before and after addition of 1 μg of copper per ml produced a shift in the pK_a from 4.90 to 4.05 (Fig. 5). These results indicate that at least some copper binding by the capsular polysaccharide occurred at protonated sites.

The copper-binding characteristics of the crude capsular material were compared with that of other polymeric mate-

TABLE 4. Cations associated with preparation of highly purified exopolysaccharide[a]

Element	ng/μg of carbohydrate	
	Prepn 1	Prepn 2
Ca	34	29
Mg	16	15
Pb	30	9.0
Fe	12	1.2
Zn	14	12
Cu	4.6	3.6
Ni	4.2	1.9
Cd	1.9	0.39
Cr	1.5	0.67

[a] Analyses were conducted on 100-μl volumes of highly purified preparation of exopolysaccharide after dialysis against double-distilled water. The carbohydrate concentrations of preparations 1 and 2 were 33 and 24 μg/ml, respectively.

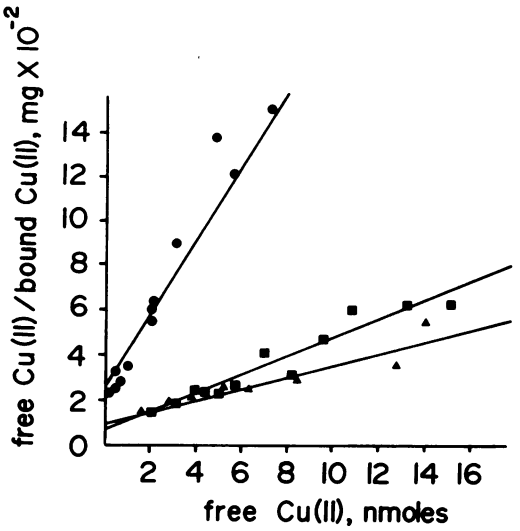

FIG. 4. Langmuir plots of Cu^{2+} binding to the highly purified exopolymer at various hydrogen ion concentrations. Symbols: ●, pH 7.00; ■, pH 6.00; ▲, pH 5.00.

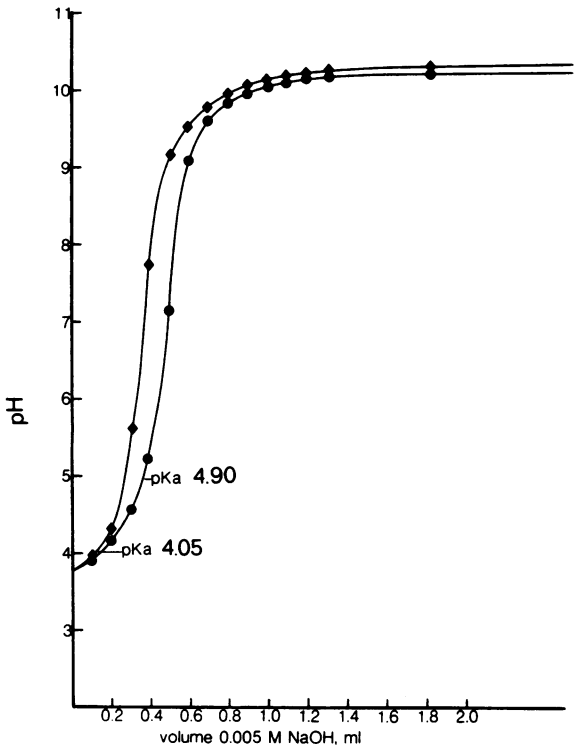

FIG. 5. Potentiometric titration of the highly purified exopolymer in the presence (◆) and absence (●) of 1 μg of copper per ml.

rials. Xanthan, an exopolysaccharide produced by *Xanthomonas campestris*, and a commercial preparation of humic acids were assayed for copper binding by the same procedure used for the capsule preparations described above and yielded MBA values of 123 and 807 nmol of Cu per mg (dry weight) and k_c values of 1.8×10^8 and 6.6×10^8, respectively. The MBA values of these polymers were, therefore, considerably higher than that exhibited by the crude exopolymer preparation when compared on a dry weight basis (Table 6). The k_c value of the crude exopolymer, however, was similar to that of the humic acids and higher than that of xanthan (Table 6).

DISCUSSION

Firmly bound exopolymers produced by adherent cells of a freshwater-sediment bacterium were evaluated by a combination of equilibrium dialysis and atomic absorption spectrometry and found to bind copper. Equilibrium dialysis was selected over other techniques for this study because it allowed assay conditions which approached those of the natural environment. The lengthy equilibration times and large volume requirements normally associated with this technique (17) were minimized in this study by the use of a multichamber equilibrium dialysis apparatus.

Previous studies showed that exopolymers extracted from cells of the sediment bacterium by the method used in this study are contaminated by only small quantities of intracellular (3%) or cell wall (10 to 13%) polymers (Platt et al., submitted for publication). It is unlikely, therefore, that the bulk of the copper binding demonstrated in each of the exopolymer preparations was contributed by cell material other than that which was bound to the outside of the bacterium at the time of extraction.

Copper binding by the highly purified exopolysaccharide component was detected at free copper concentrations as low as 25 ng/ml, which was the lower limit of sensitivity of the technique. Previous studies indicated that sediment pore water in the area from which the bacterium was isolated contained approximately 4 ng of Cu per ml (8). These data suggest that the exopolysaccharides elaborated by this bacterium are capable of complexing free copper at concentrations normally found in surface sediments of the river.

Reactions between heavy metals and organic material are influenced by pH and hardness ions (20). The copper MBA variations caused by pH variations that were demonstrated by the exopolysaccharide in this study were similar to those reported for copper-humic acids interactions (9). The decrease in MBA with increased pH may have been due to a reduction in the concentration of free copper since copper hydroxide formation is favored under these conditions (14). The pH of pore water in surface sediments of the lower Fraser River varies from 6.0 to 7.0 (unpublished data). Under these conditions, the copper MBA of the highly

TABLE 5. Effect of pH on copper-binding characteristics of highly purified exopolysaccharide

pH	MBA (nmol/mg of carbohydrate)	k_c ($\times 10^8$)
5.00	395	2.6
6.00	253	5.7
7.00	62	6.1

TABLE 6. Cu^{2+}-binding characteristics of crude, cell-free exopolymer, humic acids, and xanthan on a dry weight basis

Fraction	MBA (nmol/mg [dry wt])	k_c ($\times 10^8$)
Cell-free exopolymer	37	7.3
Humic acids	807	6.6
Xanthan	123	1.8

purified exopolysaccharide would be expected to range from 62 to 253 nmol of Cu per mg of carbohydrate.

The increase with higher pHs of the k_c value of the copper-exopolysaccharide complex may have been due to a reduction in competition at copper-binding sites by H^+ ions. Displacement of H^+ ions by copper was supported by potentiometric titrations of the exopolysaccharide in the presence and absence of the metal. Correspondingly, Zunino and Martin (28) found that H^+ ions were released from humic acids, karaya gum, and *Hansenula holstii* exopolysaccharide during exposure to increased concentrations of copper. Thus, while an increase in pH apparently reduced the concentration of copper species capable of binding to exopolymer, the concomitant decrease in concentration of H^+ ions competing with copper at the binding site resulted in a more stable interaction (higher affinity) between the reactive copper species and the exopolymers.

Previous studies indicated that reactive copper concentrations in surface sediments of the lower Fraser River and its tributaries fluctuate in response to flow-related events in the overlying water (8). In view of the apparent competition between copper and H^+ for sites on the bacterial exopolymers, it is likely that the fluctuations in copper concentration result in pH changes in the pore water surrounding these exopolymer-encapsulated bacteria in the river sediments.

Analysis with an inductively coupled plasma atomic emission spectrometer demonstrated that polyvalent elements other than copper also bind to the expolysaccharide. The extent to which they compete with copper has not yet been investigated. However, they are present in sediment pore water and undoubtedly have some effect on copper binding by the bacterial exopolymers.

Of the naturally occurring organic material in sediments, humic substances are believed to be among the most important in binding metals such as copper (18, 19). The similar stability constants obtained for humic acids and exopolymers from the sediment bacterial isolate and *X. campestris* in this study suggest that a variety of naturally occurring organic compounds possess a common class of copper-binding sites which contribute a large fraction of their total copper complexation capacity. The MBA values indicate, however, that humic acids and *X. campestris* exopolymers possessed more binding sites per unit (dry weight) than the exopolymers of the sediment bacterial isolate.

The absolute contribution of bacterial exopolymers to total sediment copper-binding capacity is a function of the amount of exopolymer associated with each cell and the total sediment bacterial density. It is conceivable that adherent bacteria such as the one used in this study bind a significant fraction of the reactive particle-associated copper which exists in river beds (8). Unfortunately, no technique has been shown to provide accurate quantitative estimates of bacterial exopolymers in sediments, although the extracellular polysaccharide determination described recently by Uhlinger and White (27) may prove useful in this regard. As estimates of exopolymer content of sediment bacterial populations become available, they can be combined with the stability constants and maximum binding values obtained in this study to compute the equilibrium distribution of copper between these and other metal-binding components in aquatic environments.

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