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Metal-Binding Characteristics of the Gamma-Glutamyl Capsular Polymer of *Bacillus licheniformis* ATCC 9945†

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The metal-binding affinity of the anionic poly-γ-D-glutamyl capsule of *Bacillus licheniformis* was investigated by using Na^+, Mg^{2+}, Al^{3+}, Ca^{2+}, Cr^{3+}, Mn^{2+}, Fe^{3+}, Ni^{2+}, and Cu^{2+}. Purified capsule was suspended in various concentrations of the chloride salts of the various metals, and after dialysis the bound metals were analyzed either by graphite furnace atomic absorption spectroscopy or by inductively coupled plasma-mass spectrometry. Exposure of purified capsule to excess concentrations of Na^+ revealed it to contain 8.2 μmol of anionic sites per mg on the basis of Na binding. This was confirmed by titration of the capsule with HCl and NaOH. Other metal ions were then added in ionic concentrations equivalent to 25, 50, 75, 100, 200, and 400% of the available anionic sites. The binding characteristics varied with the metal being investigated. Addition of Cu^{2+}, Al^{3+}, Cr^{3+}, or Fe^{3+} induced flocculation. These metal ions showed the greatest affinity for *B. licheniformis* capsule in competitive-binding experiments. Flocculation was not seen with the addition of other metal ions. With the exception of Ni^{2+} and Fe^{3+} all capsule-metal-binding sites readily saturated. Ni^{2+} had low affinity for the polymer, and its binding was increased at high metal concentrations. Fe^{3+} binding resulted in the development of rust-colored ferrihydrite which itself could bind additional metal. Metal-binding characteristics of *B. licheniformis* capsule appear to be influenced by the chemical and physical properties of both the capsule and the metal ions.

Metal binding has been demonstrated by a wide variety of structures on and within the surface of bacteria (7, 9, 21, 25). These structures include peptidoglycan (Pg) (4, 10, 11), teichoic and teichuronic acids (8), lipopolysaccharides (LPS) (17), surface protein arrays (6), and extracellular polymers or capsules (7, 18, 21, 26, 28, 29). Several studies have investigated metal binding by well-characterized bacterial polymers. In this manner the identification of the primary metal-binding determinates, such as the carboxylate groups in the Pg of *Bacillus subtilis* 168 (11) and the phosphoryl groups of LPS in *Escherichia coli* K-12 (17), has been made possible.

Many bacteria in nature produce an amorphous polymer or capsule which extends from the cell surface. Bacterial capsules are long polymers of sugars or amino acids which may extend 0.1 to 10 μm or more from the cell surface (5). These outermost structures of bacteria have been referred to as capsules, slimes, extracellular polymers, and glycocalyces (7, 21, 25). Although in a strict structural sense they can be differentiated into distinct entities (5), for the purposes of this paper they will be referred to as capsule. With few exceptions, the capsules of most organisms are anionic because of the presence of carboxylate or phosphate groups (7). The anionic characteristics of these polymers would be expected to attract metal cations through electrostatic interactions. It is therefore not surprising that metal binding by bacterial capsules and encapsulated organisms has been well documented (for a review, see reference 21). Although metal-binding specificities and selectivities for several metals have been shown in most of these investigations, few descriptive studies have been carried out using purified capsule of known chemical structure. Here we report the metal-binding characteristic of the highly anionic poly-γ-glutamic acid capsule polymer of *Bacillus licheniformis* ATCC 9945, the structure of which was first described by Troy (31).

(A preliminary report of this work has been presented [R. J. C. McLean and T. J. Beveridge, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, Q-146, p. 307].)

MATERIALS AND METHODS

Organism and culture conditions. *B. licheniformis* ATCC 9945 was originally obtained from F. A. Troy, Department of Biological Chemistry, University of California, Davis, and stored in the culture collection, Department of Microbiology, University of Guelph, on slants of tryptic soy agar (Difco Laboratories, Detroit, Mich.) at 30°C. For capsule production, the organism was subcultured for 24 h on petri plates of medium E (23) supplemented with 1.5% agar. Medium E contained the following (in grams per liter): L-glutamic acid, 20.0; citric acid, 12.0; glycerol, 80.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_2 \cdot 6H_2O$, 0.04; $CaCl_2 \cdot 2H_2O$, 0.15; $MnSO_4 \cdot H_2O$, 0.42; and Bacto Agar (Difco), 15. The pH was adjusted to 7.4, and the medium was sterilized by autoclaving. The use of medium E supplemented with 1.5% agar was found to greatly enhance capsule production by *B. licheniformis* and also to greatly ease its purification.

Capsule production and isolation. Growth of *B. licheniformis* and capsule isolation were essentially variations of the protocol used by Troy (31). Pure colonies of *B. licheniformis* grown on medium E were selected on the basis of maximum capsule production (mucoid appearance) and subcultured an additional 24 h at 30°C on petri plates of medium E. Loopfuls of mucoid *B. licheniformis* cells were then suspended in phosphate-buffered saline (5 mM K_2HPO_4, 4.5

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† R.J.C.M. dedicates this paper to the memory of J. C. McLean and A. D. Law.

mM KH_2PO_4, 150 mM NaCl, pH 7.4), mixed by vortexing, and then used to inoculate plates of medium E. Cell suspension (100 μl [ca. 10^8 cells/ml]) was spread evenly over the plates as a lawn. After 48 h of growth at 30°C, the cells were harvested from the growth medium by scraping with a glass microscope slide into a beaker containing distilled water. The cells were then centrifuged at 16,000 × *g* for 10 min (saving the supernatant [Su 1] which contained loosely bound capsule), resuspended into an equal volume of buffer containing 0.01 M Tris (pH 7.1)–0.03 M NaCl, and blended for 2 min at room temperature to shear capsule from the cells. On blending, the cells were then removed by centrifugation at 16,000 × *g*, and the supernatant (Su 2) containing most of the capsule was saved. The fractions containing the capsule (Su 1 and Su 2) were then combined with 4 volumes of cold ethanol and left overnight at −20°C. Precipitated capsule was concentrated by centrifugation at 48,000 × *g* for 20 min at −20°C, resuspended in a minimal volume of H_2O, and dialyzed against distilled water for 24 to 48 h at 4°C to remove ethanol. The dialyzed capsule was then lyophilized to remove water, resuspended in a minimal volume of water to which were added ribonuclease (20 μg ml^{-1}), deoxyribonuclease (20 μg ml^{-1}), and 10 mM $MgCl_2$ to remove contaminating nucleic acids. In addition, 0.02% (wt/vol) sodium azide was added to inhibit bacterial growth. After a 1-h incubation at 37°C, additional RNase and DNase were added and the mixture was left for an additional hour. Proteins were then removed by two successive 1-h incubations of the capsule with pronase (5 μg ml^{-1}) at 37°C. All enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. After dialysis against deionized water, the capsule was then further purified by suspension in 0.25 M NaCl and precipitation with ethanol at −20°C as described above. After further dialysis and lyophilization the purified capsule was checked for protein, teichoic acid, teichuronic acid, nucleic acid contamination, and background metal content (see below) before being stored at room temperature for use.

Protein determination. Protein content of the purified capsule was determined according to the method of Lowry et al. (24) by using lysozyme (Sigma) as the standard. Since low levels of protein were determined by this procedure, the possibility of interference with this assay by glutamic acid polymers was investigated by using commercially available poly-L-glutamic acid (Sigma). In addition the sample was hydrolyzed overnight at 60°C in 1 M HCl and analyzed for amino acid content with the amino acid analyzer at the Department of Animal Science and Nutrition, University of Guelph.

P_i assay. Purified capsule was assayed for phosphate content indicative of phospholipid (i.e., membrane), nucleic acid, and teichoic acid contamination by using the protocol of Ames and Dubin (1).

Uronic acid assay. Purified capsule was assayed for teichuronic acid contamination with the uronic acid colorimetric assay of Davidson (14) by using the potassium salt of D-glucuronic acid (Sigma) as the standard.

Precautions against metal contamination. All chemicals used were analytical grade and were purchased from local suppliers. All water used was high-resistance (18 MΩ cm^{-1}) water (HRW) (pH 3.8 to 4.5) produced from a Milli-Q water system (Millipore Canada Ltd., Mississauga, Ontario, Canada) fitted with an ion-exchange cartridge kit (part no. CDOF 01205) to remove trace metal and organic contamination. Polyethylene and polycarbonate plasticware was used in all metal-binding experiments. To avoid metal contamination (principally due to Na, K, Mg, Ca, Al, and Zn), the plasticware was first leached overnight in 7.5 M nitric acid, rinsed overnight in HRW, and finally air dried. Metal-free dialysis tubing (12 to 14 kDa exclusion) used in metal-binding experiments was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Prior to use, it was rinsed five times in HRW and left overnight at 4°C. Plastic talcum powder-free gloves rinsed in HRW were worn for all manipulations of dialysis tubing. In addition, background metal contamination was monitored in all experiments as described below.

Quantification of available anionic sites. Three replicates containing 10 mg of purified capsule were suspended for 1 h at room temperature in 10 mM NaCl (1 mg ml^{-1}). After dialysis against HRW to remove unbound metal, the samples were lyophilized and analyzed for Na by atomic absorption spectrometry (AA) as described below. Since Na^+ is a small monovalent cation, each anionic site on the *B. licheniformis* capsule should theoretically bind one Na ion. Measurement of the bound sodium would therefore give an indication of the number of anionic metal-binding sites available. In addition, the number of anionic sites on sodium-free capsule was verified by titration with 0.01 M HCl and back-titration with 0.01 M NaOH. The pH of the starting capsule suspension before titration (1 mg ml of HRW^{-1}) was 6.04.

Metal-binding protocol. Na binding revealed that there was 8.2 μmol of anionic sites per mg of capsule. Consequently, for metal-binding experiments, metal concentrations were initially set so as to be the ionic equivalent of 50, 100, or 200% of the available sites. Metals used were the chloride salts of Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Al^{3+}, Cr^{3+}, and Fe^{3+}. For divalent metals such as Ca^{2+}, the relevant concentrations used were 2.05, 4.1, and 8.2 μmol mg^{-1}, respectively. For trivalent metals such as Fe^{3+}, concentrations were 1.37, 2.73, and 5.47 μmol mg^{-1}, respectively. Additional datum points were collected for Mn^{2+} and Fe^{3+} (ionically equivalent to 25, 75, and 400% of the available sites) and for Ni^{2+} at 400%, since these metals did not appear to saturate the capsule when added at 100%. Metal binding involved suspending ca. 10 mg of capsule directly into a freshly prepared solution of the chloride salt of the metal so that the final concentration of capsule was 1 mg ml^{-1}. After 1 h of equilibration at room temperature, the capsule-metal mixture was placed into metal-free dialysis tubing, and unbound metal was removed by dialysis using trace metal-free, sulfur-free dialysis tubing for 5 h at room temperature against HRW. Samples were then placed into tared plastic containers, frozen, lyophilized, weighed to 0.01 mg, and suspended in 7.5 M HNO_3 (1 mg ml^{-1}) to totally dissolve the polymer and release all bound metal prior to metal analysis by inductively coupled plasma-mass spectrometry (ICP-MS) or AA. Prior to ICP-MS analysis, an additional 500-fold dilution was made to keep the measured-count rates low enough to prevent any damage that might reduce the lifetime of the detector. pH measurements of metal solutions at an ionic concentrations equivalent to 100% of the capsule-binding sites and metal-capsule mixtures were also taken before and after metal binding (Table 1).

Metal contamination was monitored by the use of a process control. This consisted of suspending ca. 10 mg of capsule in HRW to a concentration of 1 mg ml^{-1}, and after 1 h of incubation at room temperature, dialyzing and lyophilizing it as described above. Metal content of the capsule was determined by ICP-MS and compared with that of untreated capsule. In addition, the metal content of the

TABLE 1. pHs of metal-capsule mixtures[a]

Metal added[b]	pH	
	Without capsule	With capsule
None[c]	3.81	6.26
Na^+	5.29	5.51
Mg^{2+}	5.38	5.49
Ca^{2+}	5.20	5.26
Mn^{2+}	5.09	4.95
Ni^{2+}	5.27	5.16
Cu^{2+}	4.50	4.20
Al^{3+}	4.45	4.44
Cr^{3+}	3.46	4.07
Fe^{3+}	2.89	2.92

[a] No buffers were added to solutions. The pH value of capsule suspended in HRW fluctuated within 0.2 pH units. All other values are within 0.02 pH units.

[b] Concentrations of the chloride salts of the above metals were added to the ionic equivalent of 100% of the anionic sites on *B. licheniformis* capsule as follows: 8.2, 4.1, and 2.73 μmol mg^{-1} for monovalent, divalent, and trivalent metals, respectively.

[c] Suspended in HRW (18 MΩ cm^{-1}).

HNO_3 used to suspend all capsule for ICP-MS or AA was determined.

AA. Quantitative metal analysis was performed by flameless atomic absorption with a Perkin-Elmer model 2380 atomic absorption spectrometer equipped with an HGA-400 graphite furnace. Operating conditions were as outlined in the manufacturer's operating manual. Standard solutions for metal analysis were prepared by dissolving the chloride salt of each metal (analytical grade purchased from Fisher Scientific Company, Don Mills, Ontario, Canada) in HRW to an initial stock solution concentration of 0.100 M. The capsule was initially suspended in 50% (vol/vol) HNO_3 overnight at room temperature to totally dissolve the polymer and release all bound metal. Subsequent dilutions of the standard metal solution and capsule to the working range of the instrument were performed with either HRW or 0.1% (vol/vol) HNO_3, depending on the metal being analyzed, in accordance with the manufacturer's operating manual. Triplicate samples were analyzed, and each sample value is a mean derived from three to five separate analyses.

ICP-MS. The ICP-MS instrument used was a Perkin-Elmer SCIEX ELAN 500 (SCIEX Canada, Thornhill, Ontario, Canada). Modifications made to the original instrument included the use of a conventional ICP-atomic emission spectroscopy torch. A mass flow controller (model 1259B; MKS Instruments, Andover, Mass.) was installed on the nebulizer gas line. A peristaltic pump (Miniplus II; Gilson Medical Electronics Inc., Middleton, Wis.) was put on the sample delivery tube in order to ensure a constant sample introduction rate of 1.0 ml min^{-1}.

The plasma conditions for this work were as follows: torch, low flow (PlasmaTherm); radiofrequency power, 1.2 kW; reflected power, ≤5 W; plasma gas flow, 9.0 liter min^{-1}; auxiliary gas flow, 2.0 liter min^{-1}; nebulizer gas flow, 0.7 liter min^{-1}; sample delivery rate, 1.0 ml min^{-1}. The MS settings for this work were as follows: Bessel box stop, −6.1 V; Bessel box barrel, 2.1 to 7.5 V; Einzel lenses 1 and 3, −12.8 to −18.0 V; Bessel box end lenses, −6.2 to −16.4 V; Pt sampler orifice diameter, 1.14 mm; Pt skimmer orifice diameter, 0.89 mm; interface pressure, ca. 1 Torr; MS pressure, 0.8×10^{-5} to 3.1×10^{-5} Torr.

Measurements were made with the multiple-elements software (provided with the instrument) by "peak hopping" rapidly from one mass to the other, staying only a dwell time of 20 ms at each mass, until the total measuring time (per measurement) of 0.5 s was reached. Three measurements were made per peak; one measurement was done at the central mass while the other two were done at ± 0.1 atomic mass unit from the assumed peak center. A resolution of 1.1 atomic mass unit (peak width) at 10% peak height was maintained throughout the studies. The species monitored included two to five of the following: $^{23}Na^+$, $^{24}Mg^+$ or $^{25}Mg^+$ $^{44}Ca^+$, $^{52}Cr^+$, $^{55}Mn^+$, $^{54}Fe^+$ or $^{57}Fe^+$, $^{60}Ni^+$, $^{63}Cu^+$, and $^{76}Cr_2{}^+$. $^{76}Ar_2{}^+$ is a background ion which originates from the plasma; it was always monitored as an internal standard in parallel with the analyte(s) to ensure against drift (resulting from the effect of concomitant elements [2]). This approach has the advantage of not requiring any sample treatment which could increase contamination.

Standard solutions were prepared from 1,000-mg $liter^{-1}$ stock monoelemental solutions (Spex Industries, Edison, N.J.) for ICP spectrometry. Dilutions were done with HRW and with the appropriate amount of high-purity nitric acid (Seastar, Sidney, British Columbia, Canada) to reach a final concentration of 1% HNO_3. Alignment of the plasma and sensitivity of the instrument were optimized daily by using $^7Li^+$, $^{103}Rh^+$, and $^{208}Pb^+$ (3). Calibrations were carried out by using two to five standard solutions. All standards and sample intensities were blank subtracted by using 1% (vol/vol) HNO_3 as the blank. The stability was such that no internal standardization was required, except for the determination of Ni, in which the background line $^{76}Ar_2{}^+$ was used as the internal standard to improve precision. In this latter case, each intensity (whether from a standard or a sample) was compared with the corresponding one of $^{76}Ar_2{}^+$ (i.e., measured at the same time), and the resulting ratios were used to perform the calibration. Metal binding by *B. licheniformis* capsule was reported as micromoles of metal bound per milligram (dry weight) of material, where the material consisted of capsule, bound metal, and any other complexed species such as OH, Cl, O, etc.

X-ray diffraction. Powder X-ray diffraction analysis of precipitates was carried out with a Rigaku miniflex X-ray diffractometer. Copper Kα radiation was used with a wavelength of 0.15405 nm. The scan rate of the goniometer was variable between 2° and 0.5° min^{-1}; most scans were taken at the former setting. The diffractometer was interfaced with a personal computer such that data could be stored and later recalled for further analysis.

TABLE 2. Contamination present in *B. licheniformis* capsule preparation

Compund	Quantity present[a]	Likely contaminant
Phosphate	0.10 μmol	Membrane phospholipids, nucleic acids, or teichoic acids
Protein	14.97 μg	Interference of glutamic acid polymer with assay
Uronic acids	8.37 μg	Teichuronic acids

[a] Per milligram of capsule.

RESULTS

Growth of *B. licheniformis* on medium E (23) induced such large quantities of capsule in the bacterial colonies that the colonies could often be poured from their petri plates. Trace levels of contamination due to proteins, phospholipids, nucleic acids, teichoic acids, and teichuronic acids were

TABLE 3. Background metal content of *B. licheniformis* capsule[a]

Metal	Quantity present (μmol mg of capsule^{-1})
Na	2.980
Mg	0.620
Ca	0.0855
Mn	0.00624
Ni	0.001
Cu	0.00053
Al	ND[b]
Cr	<0.001
Fe	0.00134

[a] Determined by AA.
[b] ND, Not done.

seen in the purified polymer (Table 2). This could not be alleviated through repeated ethanol precipitations (data not shown). Protein contamination (14.97 μg mg^{-1}) according to the procedure of Lowry et al. (24) was likely due to an interference of the glutamic acid capsule polymer with the assay, since similar analysis of commercially available polyglutamic acid (Sigma) gave a protein concentration of 12 μg mg of polyglutamic acid^{-1}. In addition, amino acid analysis of the purified capsule polymer showed it to contain only glutamic acid (data not shown).

Investigation of metal binding by bacterial capsules presents several logistical problems. The greatest difficulty lies in separating the unbound metal from the capsule-metal complex. If the capsule is immobilized tightly onto either a bacterial or an artificial surface or the capsule-metal mixture forms as insoluble floc or precipitate, then separation can be easily accomplished by techniques such as centrifugation. With the exception of Cu^{2+}, Cr^{3+}, and Fe^{3+} no precipitates formed, and the capsule could not be sedimented even by ultracentrifugation (100,000 × *g*). Ultrafiltration using filters with a 10,000-molecular-weight exclusion gave us inconsistent results. Our most satisfactory and reproducible results arose from dialysis of the metal-capsule mixture against HRW (18 MΩ cm^{-1}) with trace metal-free, sulfur-free, dialysis tubing (Bethesda Research Laboratories, Gaithersburg, Md.). ICP-MS was employed for the bulk of the metal analysis, since it was similar to graphite furnace AA in its detection limits (0.01 to 0.1 μg liter^{-1}) (15) while giving a much higher degree of precision and rapidity of measurement.

Purified capsule contained small quantities of metals (Table 3), likely arising from background metal sorption from the growth medium or the purification protocol. A small quantity of Mg contamination was present during Ca^{2+} binding (Fig. 1). When suspended in NaCl the capsule was able to bind 8.2 μmol of Na^+ mg^{-1}. In all subsequent metal-binding experiments, the number of available electronegative binding sites (i.e., COO^-) of the capsule was therefore assumed to be 8.2 μmol of COO^- mg^{-1}. This was confirmed by acid-base titration. pH readings before and after addition of a number of different metals revealed that the reactive groups of the capsule had a natural buffering capacity and could sorb metallic ions (Table 1). This buffering was most evident at pH values between 3 and 4.5. Most metals appeared to saturate these sites when added at concentrations of 4.1 μmol mg^{-1} (divalent cations) (Fig. 1 and 2) or 2.73 μmol mg^{-1} (trivalent cations) (Fig. 3) (100% saturation). In the case of Mg^{2+}, Ca^{2+}, and Cr^{3+}, this saturation was reached at lower concentrations. The exceptions to this saturation were Mn^{2+}, Ni^{2+} (Fig. 2), and Fe^{3+}

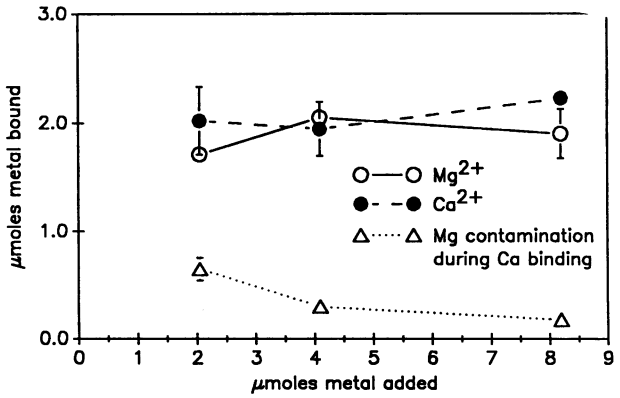

FIG. 1. Mg^{2+} and Ca^{2+} binding by *B. licheniformis* capsule. There was a small quantity of Mg contamination present during Ca^{2+} binding.

(Fig. 4). Because of this, additional datum points were collected. Binding of most metals tested could be approximated according to the Langmuir adsorption isotherm (16), $U/B = 1/KN + U/N$, where U represents unbound metal, B represents bound metal, N represents the number of binding sites, and K represents the average binding constant (μmol mg of capsule^{-1}, when the metal is added at a concentration of 1 μmol mg of capsule^{-1}) for the metal-capsule interaction. N and K were calculated by conducting a least squares linear regression analysis on a plot of U/B versus U. The resulting values for each metal are listed in Table 4. In this case the slope represents $1/N$ and the Y intercept represents $1/KN$ (16).

On the basis of Langmuir adsorption isotherms (Table 4), the number of binding sites per mg of capsule ranged from 1.60 for Cr^{3+} to 5.70 for Fe^{3+}, with the majority of metals exhibiting values between 2 and 3. Binding constants varied considerably, ranging from negative values for Mg^{2+} and Cr^{3+} to 10.63 for Mn^{2+}. Since Cr^{3+} and Mg^{2+} were bound readily by this polymer, we would suggest that Langmuir adsorption isotherms do not adequately describe the metal-binding characteristics of this polymer. All other mathematical models tested, including Hill plots, reciprocal-binding curves (analogous to Lineweaver-Burke plots), and Scatchard plots (16), showed even less correlation with correlation coefficient (r^2) values ranging from 0.3 to 0 (data not shown).

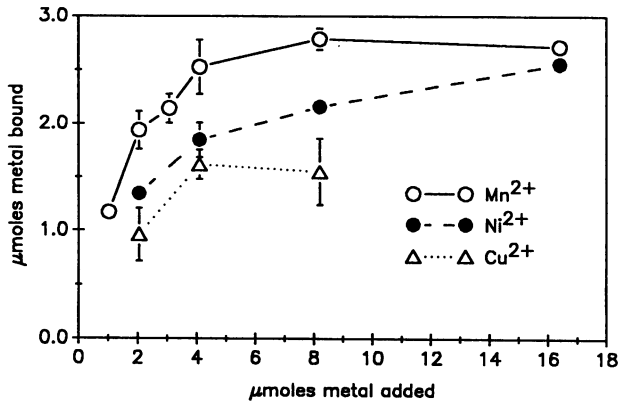

FIG. 2. Cu^{2+}, Mn^{2+}, and Ni^{2+} binding by *B. licheniformis* capsule.

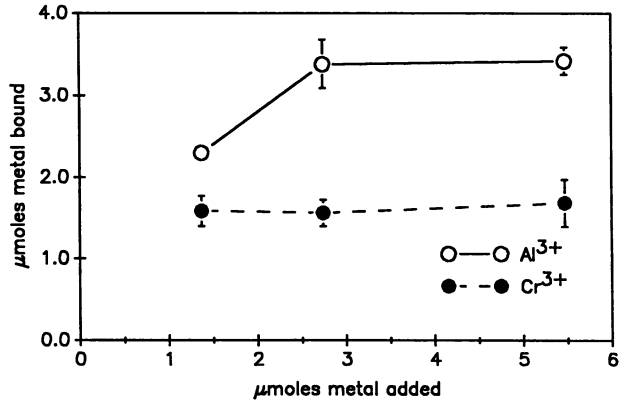

FIG. 3. Al^{3+} and Cr^{3+} binding by *B. licheniformis* capsule.

Formation of precipitates occurred when *B. licheniformis* capsule was mixed with chloride salts of Al^{3+}, Cu^{2+}, Cr^{3+}, and Fe^{3+}. Flocculation and precipitation occurred rapidly (within 0 to 5 min) and were observed for all metal concentrations tested. Powder X-ray diffraction of precipitates formed from suspending capsule (1 mg ml^{-1}) in 2.73 mM Al^{3+}, Cr^{3+}, and Fe^{3+} or 4.1 mM Cu^{2+} showed the precipitates to possess nonordered amorphous structures (data not shown) indicated by a lack of crystal peaks and a broad "amorphous hump" at diffraction angles (2Θ) between 10° and 30° in all cases. On the basis of visual appearance and the ionic composition of the solutions it is probable that these mineral substances consist of the hydroxides or hydrated oxides of the various metals [i.e., white $Al(OH)_3$ (aluminum hydroxide); blue $Cu(OH)_2$ (copper hydroxide), $CuCl_2 \cdot 3Cu(OH)_2$ (γ: paratacamite or δ: atacamite), or $Cu_2(OH)_3Cl$ (atacamite); dark green $Cr_2O_3 \cdot xH_2O$ (chromium oxide); and rust-colored $Fe(OH)_3$ (ferric hydroxide) (33)]. In previous competition experiments when *B. licheniformis* capsule was suspended (1 mg ml^{-1}) in a solution containing each of the chloride salts of 12 different metals at 1 mM, Cr, Cu, and Fe(III) demonstrated relatively high binding affinities (Table 5) (25). The formation of these precipitates is therefore likely due in part to this binding affinity.

DISCUSSION

Capsules can represent an indispensable component of most bacteria in their natural environment in that they facilitate adhesion to surfaces, promote biofilm formation (13), and offer protection against a wide variety of harmful substances such as antibiotics, biocides, and adverse conditions such as predation and the immune response (12). The capsules of most bacteria are anionic, so it is not surprising that a number of studies have shown these extracellular polymers to bind cationic substances such as metal ions (7, 18, 21, 25, 26, 28, 29). Geesey and Jang (21) have described the general interaction between metal ions and organic molecules as an acid-base reaction involving the interaction of a metal ion (M^{n+}), a proton (H^+), and an organic ligand (L) as $M^{n+} + LH \rightarrow M^{n+}\text{-}L + H^+$. We observed a decrease in pH values when *B. licheniformis* capsule was exposed to the various metals (Table 1), likely due in part to the liberation of protons from the carboxylate metal-binding sites. Since our capsule consists of a homopolymer of poly-γ-glutamic acid, these sites must be carboxylates and the metal-binding reaction could therefore be described as $COOH + M^+ \rightarrow COOM + H^+$ for monovalent metal ions (M^+). As might be expected, electrostatic attractions between the anionic polymers and cationic metal ions represent the initial chemical forces involved in metal binding (21). Indeed, we have seen that iron and calcium binding by a neutral capsular polysaccharide such as curdlan (a β-1,3 glucan produced by a strain of *Alcaligenes faecalis* [22]) is virtually nonexistent, especially when compared with that of

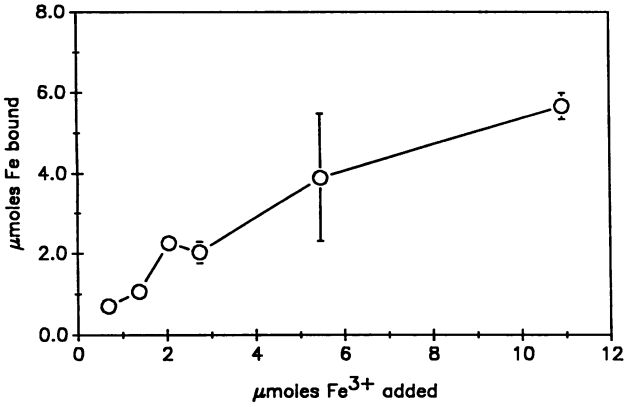

FIG. 4. Fe^{3+} binding by *B. licheniformis* capsule.

TABLE 4. Langmuir adsorption isotherms for metal binding by *B. licheniformis* capsule[a]

Metal	Slope (SE)	*y* intercept (SE)	r^2	*N*	K^b
Mg^{2+}	0.535 (0.034)	−0.035 (0.207)	0.984	1.87	−15.37
Ca^{2+}	0.444 (0.021)	0.073 (0.123)	0.991	2.25	6.11
Mn^{2+}	0.366 (0.004)	0.034 (0.076)	0.998	2.73	10.63
Ni^{2+}	0.370 (0.009)	0.385 (0.150)	0.995	2.71	0.96
Cu^{2+}	0.621 (0.082)	0.284 (0.586)	0.890	1.61	2.19
Al^{3+}	0.425 (0.022)	0.077 (0.091)	0.981	2.35	5.49
Cr^{3+}	0.623 (0.052)	−0.001 (0.249)	0.953	1.60	−504.6
Fe^{3+}	0.175 (0.022)	0.129 (0.182)	0.793	5.70	1.35

[a] All values were calculated as described in the text by using linear regression analysis program from Lotus 123 software release 2.01 (Lotus Development Corporation, Cambridge, Mass.).
[b] Expressed as micromoles per milligram (dry weight).

TABLE 5. Relative affinity for metals by *B. licheniformis* capsule[a]

Metal	Bound metal[b]
Na^+	0.259
K^+	0.223
Mg^{2+}	0.073
Ca^{2+}	1.044
Mn^{2+}	0.071
Co^{2+}	0.100
Ni^{2+}	0.080
Cu^{2+}	0.890
Zn^{2+}	0.149
Al^{3+}	ND[c]
Cr^{3+}	0.940
Fe^{3+}	1.340

[a] Relative metal binding was measured by suspending lyophilized capsule (1 mg ml^{-1}) into a trace metal solution containing each of the chloride salts of the above metals at 1 mM (25). Unbound metals were removed through dialysis as described in the text.
[b] Determined by AA and expressed as micromoles of metal bound per milligram (dry weight).
[c] ND, Not done.

the highly anionic capsule of *B. licheniformis* (R. J. C. McLean and T. J. Beveridge, unpublished data). For highly charged capsules such as that of *B. licheniformis*, salt bridging by multivalent metal species might be a major factor in polymer conformation and produce distinct, so-called salt forms of the capsule.

Highly stable aquo ions such as Na^+ and Mg^{2+} usually establish proper binding stoichiometries between available polymer sites and bound ions. This was the primary reason why Na^+ was used to establish the number of available sites in the capsule (8.2 μmol mg [dry weight] of capsule^{-1}). Multivalent ions such as Mn^{2+}, Al^{3+}, Cr^{3+}, and Fe^{3+} can form redox couples which are highly interactive with soluble anions such as OH^- and consequently form insoluble precipitates. These multivalent ions frequently interact strongly with biological surfaces which, depending on the ionic character of the local environment, can nucleate a diverse array of metallic aggregates (7). For this reason, an accurate description of metal ion binding of these less stable aquo ions to capsule is difficult and may involve a range of binding kinetics (i.e., metal to polymer, metal to metal, dehydration, etc.), ionic-path tortuosities, hydrolysis products, and ill-defined interfacial effects. A broader discussion of this issue can be found in references 32 and 19.

As mentioned before, the metal binding of *B. licheniformis* capsule could be approximated by using Langmuir binding isotherms (Table 4). The anomalies that arose (notably, negative binding constants for Mg^{2+} and Cr^{3+} and an elevated binding constant for Mn^{2+}) suggest that this represents an imperfect model. A major requirement of the Langmuir binding equation is that all binding sites are equal and that binding by one site does not influence the binding by any other site. This is an improper assumption for capsule-metal binding. Any conformational change (e.g., salt bridging) induced by metal binding would necessarily block some potential sites from a metal ion. In addition, a bound metal such as Fe^{3+} can form a metal-binding complex such as $Fe(OH)_3$.

The most noticeable aspect of metal binding by *B. licheniformis* capsule is the difference in binding character seen with each metal (Fig. 1 through 3). Metals such as Mg^{2+}, Mn^{2+}, Cu^{2+}, and Al^{3+} appeared to readily saturate the available metal-binding sites at the concentrations used. The subtle differences among these metals are likely due to the character of the aquo ion (hydrated radius, heat of hydration, and valency) and the ion's effects on the polymeric matrix of the capsule. Capsules by their nature are very hydrated and consist of highly flexible polymers (5). Consequently, penetration of Mg^{2+}, Mn^{2+}, Cu^{2+}, and Al^{3+} by free diffusion throughout the capsule should be rapid and not restricted, but their binding and neutralization by the polymer could be distinctly different from one another; it would depend on the spacing between COO^- groups and the ability of the polymer or adjacent polymers to fold to accommodate intra- and intermolecular salt bridging. In salt bridging, the presence of the metal may induce a conformational change within the capsule such that its physical and chemical properties may be altered (21). As a consequence of conformational changes, other metal-binding sites may become inaccessible, and the overall result will be a low saturation point. In the case of Ca^{2+} and Cr^{3+} (Fig. 1 and 3), it is likely that the capsule is not amenable to these ions in that only a limited number of sites can be filled.

The behavior of iron with bacterial capsule is further complicated by the fact that ferric hydroxide spontaneously forms unless the solution redox potential and pH are kept low (20). It is possible that these iron precipitates could have formed in the fluid phase and then annealed to the capsule polymers, but noncapsule controls showed the rate of their formation to be extremely slow (1 to 6 h), and our experience with other bacterial polymers (8, 10, 17, 18) suggests the precipitates were capsule mediated. In addition, all Fe solutions used in this study were freshly prepared and used within 10 min of preparation. The consequence of ferrihydrite formation was an overall elevation of total metal binding. This explains the results seen with Fe^{3+} binding by *B. licheniformis* capsule (Fig. 4).

At the beginning of this study we anticipated that the metal-binding characteristics of bacterial capsules would resemble those of other bacterial structures, notably Pg (4, 10, 11), within gram-positive walls. In these cases, metal binding was partially attributed to the chemical properties of the metallic aquo ions involved, since in the case of *B. subtilis* walls, Mg^{2+} was bound at a much higher degree than was Ca^{2+} and since low concentrations of Ni^{2+} were bound (10). The major structures responsible for metal binding by gram-positive walls are Pg and, to a lesser extent, teichoic acids in *B. subtilis* (11) and teichuronic acids in *B. licheniformis* (8). In comparison, the metal-binding characteristics of *B. licheniformis* capsule are different. Of note, roughly equal quantities of Ca^{2+} and Mg^{2+} are bound, significant quantities of Mn^{2+} and Ni^{2+} can be bound, and Cu^{2+} binding results in the formation of a precipitate. For this reason, the binding differences must be due to the chemical and permeability differences between the wall and the capsule.

The major functional groups involved in metal binding by Pg within *B. subtilis* walls and the poly-γ-glutamic acid capsule of *B. licheniformis* are carboxylate residues, so on this basis alone one would not expect any differences in metal binding. Several major chemical and structural differences exist between capsules and Pg. The charge density of *B. licheniformis* capsule (expressed as COO^- per unit area) is very much higher than that in Pg. Pg, especially in living cells, is highly stressed and inflexible because of turgor. This stress, coupled with extensive cross-linking between Pg strands, results in the anionic carboxylate residues being held in a more or less rigid orientation (7). In addition, secondary wall polymers, notably teichoic and/or teichuronic acids, are present. These may also increase the rigidity of the gram-positive wall, and in the case of *B. licheniformis* walls, make a significant contribution to the overall metal-binding capacity of the cell wall (8).

Capsule polymers are much more flexible in that they generally are not cross-linked by covalent bonds. They are highly hydrated thrixotrophic structures in that they can exist in either a gel or a fluid phase with salt bridging of strands by multivalent metallic aquo ions stabilizing the gel phase (5). Conceivably, they should be better able to adapt to the coordination sphere of a particular metal through folding into an induced fit-binding conformation. X-ray diffraction analysis of capsule from *E. coli* M41 (27) has shown that cations do not appreciably affect the crystallographic structure; however, other physical properties of capsules are affected (30), most notably, gelling properties. Future studies will investigate this phenomenon.

From a cellular perspective, differences in metal-binding characteristics between cell walls and capsules would benefit the organism. Some metals, notably Mg^{2+}, are required by autolysins within the cell wall, whereas others, such as Cr^{3+}, can be toxic to a variety of enzymes including membrane- and possibly wall-associated enzymes; it is therefore not

surprising that these metals are preferentially bound in the wall and capsule, respectively.

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