APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4531–4536
0099-2240/94/$04.00+0

Vol. 60, No. 12

Precipitation of Metallic Cations by the Acidic Exopolysaccharides from *Bradyrhizobium japonicum* and *Bradyrhizobium* (*Chamaecytisus*) Strain BGA-1

J. CORZO,[1]* M. LEÓN-BARRIOS,[2] V. HERNANDO-RICO,[1] AND A. M. GUTIERREZ-NAVARRO[2]

Departamento de Bioquímica y Biología Molecular[1] and Departamento de Microbiología y Biología Celular,[2] Universidad de La Laguna, 38071 Tenerife, Spain

Received 21 June 1994/Accepted 23 September 1994

The interaction between the acidic exopolysaccharides produced by two *Bradyrhizobium* strains and several metal cations has been studied. Aqueous solutions in the millimolar range of Fe^{3+} but not of Fe^{2+} precipitated the exopolysaccharides from *Bradyrhizobium* (*Chamaecytisus*) strain BGA-1 and, to a lesser extent, *Bradyrhizobium japonicum* USDA 110. The precipitation was pH dependent, with a maximum around pH 3. The precipitate was redissolved by changing the pH and by Fe^{3+} reduction or chelation. Deacetylation of *B. japonicum* polysaccharide increased its precipitation by Fe^{3+}. At pH near neutrality, the polysaccharide from *Bradyrhizobium* (*Chamaecytisus*) strain BGA-1 stabilized Fe^{3+} solutions, despite the insolubility of $Fe(OH)_3$. Aluminum precipitated *Bradyrhizobium* (*Chamaecytisus*) polysaccharide but not the polysaccharide produced by *B. japonicum*. The precipitation showed a maximum at about pH 4.8, and the precipitate was redissolved after Al^{3+} chelation with EDTA. Precipitation was inhibited by increases in the ionic strength over 10 mM. *Bradyrhizobium* (*Chamaecytisus*) polysaccharide was also precipitated by Th^{4+}, Sn^{2+}, Mn^{2+}, and Co^{2+}. The presence of Fe^{3+} increased the exopolysaccharide precipitation by aluminum. No precipitation, gelation, or increase in turbidity of polysaccharide solutions occurred when K^+, Na^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, or U^{6+} was added at several pH values. The results suggest that the precipitation is based on the interaction between carboxylate groups from different polysaccharide chains and the partially hydrolyzed aquoions of Fe^{3+}, Al^{3+}, Th^{4+}, and Sn^{2+}.

The interaction between microbial anionic polymers and heavy metals has important ecological and practical implications. It can be useful for removing toxic heavy metals from solutions (5, 23). It can mediate metal fixation on clay particles in soils and can also play an important role in some steps of the geochemical cycles of some elements (2, 3). Among the molecules able to bind heavy metals are bacterial polysaccharides, linked to the cell surface and excreted to the medium. There are several examples of this interaction (15, 18, 22). In some cases (10, 18, 22) the complex between the metal and the polysaccharide remains soluble, but in other cases it precipitates (15). The binding of metals to microbial exopolymers is not restricted to polysaccharides: the γ-glutamyl capsule of *Bacillus licheniformis*, as well as cell wall polymers of some gram-positive bacteria, can also bind various metal cations (3, 16, 17).

Bacteria belonging to the genera *Rhizobium* and *Bradyrhizobium* secrete different types of extracellular polysaccharides (EPS), some of which are acidic. Since the EPS from *Rhizobium* species were thought to be the determinants of symbiosis specificity, much work has been done on their structure and properties, and therefore they are well-known molecules. The best characterized are the acidic polysaccharides secreted by diverse *Rhizobium* strains. These EPS can selectively bind monovalent and divalent metals, depending on the degree of acetylation of the molecule (10). The *Bradyrhizobium* EPS have been less well studied. *B. japonicum* produces an acidic EPS composed of glucose, mannose, galactose, and galacturonic acid (7), while the EPS from *B. elkanii* (formerly *B. japonicum* DNA complementation group II) (12) is a polymer of a repeating unit composed of three rhamnoses and one 4-O-methylglucuronic acid (8). The composition of the acidic polysaccharide produced by *Bradyrhizobium* (*Chamaecytisus*) strain BGA-1 is similar to that produced by *B. japonicum* (14). These molecules are rather different from the EPS produced by *Rhizobium* species. As far as we know, there is no information about the interaction of the bradyrhizobial polysaccharides with metals.

In this paper we describe the precipitation of the EPS from two *Bradyrhizobium* strains by trivalent metal cations. Furthermore, we have studied the precipitation as a function of physical and chemical conditions and the degree of acetylation of the molecule.

MATERIALS AND METHODS

Bacteria and culture conditions. *Bradyrhizobium* (*Chamaecytisus*) strain BGA-1 was isolated from nodules of *Teline stenopetala* (13). *B. japonicum* USDA 110 was provided by Ramón Bellogín, Universidad de Sevilla, Seville, Spain. Bacteria were grown at 28°C under aeration in 4-liter batches of a mannitol-yeast extract-salts medium (25).

Isolation and purification of the acidic polysaccharides. Seven-day-old cultures were centrifuged at 10,000 × *g*, and the supernatants were concentrated to one-third of their original volume by rotary evaporation under reduced pressure at 45°C. Cold ethanol (3 volumes) was added, and the precipitated polysaccharide was collected by centrifugation, dialyzed against deionized water, and lyophilized. The crude polysaccharide was stored at −20°C until use.

The acidic polysaccharide was purified by taking 0.5 g from the crude polysaccharide and stirred for 3 h in 25 ml of 0.1 M EDTA–0.05 M KCl in 0.1 M Tris buffer (pH 8.0). The

* Corresponding author. Phone: 34-22-602726. Fax: 34-22-602724.

undissolved residue was eliminated by centrifugation, and the supernatant was dialyzed against 20 mM KCl in 20 mM Tris (pH 8.0). The dialyzate was applied to a column (2.5 by 40 cm) of DEAE-Sephacel anion exchanger (Pharmacia) equilibrated with the dialysis buffer and eluted with a linear gradient of KCl ranging from 20 to 300 mM in 20 mM Tris buffer (pH 8.0); the total volume of eluate was 500 ml, and the gradient was monitored by conductivity. The flow rate was 25 ml h^{-1}. Fractions (5 ml) were collected, and the polysaccharide was detected as described below. Fractions eluting between 170 and 230 mM KCl containing the main polysaccharide peak were pooled, dialyzed against deionized water, and lyophilized. Bacterial growth was inhibited by adding 0.02% chlorhexidine (Hibitane) to all the dialysis and chomatography solutions except for the last dialysis step.

Protein and nucleic acid contamination of polysaccharide preparations was monitored by recording the absorption spectrum of polysaccharide solutions (5 mg ml^{-1}) in water between 205 and 300 nm.

Deacetylation of polysaccharide. Deacetylation was performed by hydrolysis in KOH as described by Marqués (15). Polysaccharide (30 mg) was dissolved in 100 ml of 10 mM KOH and incubated for 4 h at 25°C. The solution was neutralized with HCl, dialyzed against distilled water, and lyophilized.

Analysis of carbohydrate. Carbohydrate in the samples was measured by a modification of the method of Dubois et al. (6): 0.2 ml of the sample solution was diluted to 1 ml with water, 50 μl of 80% (wt/vol) phenol in water was added, and the samples were incubated for 30 min in a water bath at 60°C. Then 3 ml of H_2SO_4 was added, and the samples were left to stand at room temperature for 30 min. Their A_{480} was read. Glucose was used as a standard, and the polysaccharide was expressed as milligram of glucose equivalent.

Iron and aluminum determination. Ferric ions were colorimetrically measured by the sulfosalicylic acid method (9) modified for small sample volumes. To 0.5 ml of sample, 0.3 ml of 10% sulfosalicylic acid in water was added, followed by 0.3 ml of 20% ammonium hydroxide and then by 2.1 ml of water; the A_{420} was recorded with water as a reference sample. Ferric chloride solutions in 10 mM HCl were used as a standard. This method allowed us to measure ferric ion concentrations from 50 μM to 1.5 mM, encompassing the concentration range used in this study.

Aluminum and iron contents in 10 mM EDTA-containing samples were determined by flame-induced atomic absorption in a type 400 spectrometer (Perkin-Elmer, Norwalk, Conn.).

Precipitation studies. The precipitation was studied by mixing appropiate volumes of polysaccharide and cation solutions in Eppendorf tubes. After the incubation time, the samples were centrifuged at 6,500 × *g* for 30 s. Supernatant samples were picked up in duplicate to measure both polysaccharide and metal remaining in solution. The precipitates were washed twice with water and redissolved in 1 ml of 10 mM EDTA to measure the polysaccharide and metal that precipitated. In other sets of experiments, the formation of polysaccharide-metal complexes was monitored by turbidimetry at 480 nm after polysaccharide and cation solutions had been mixed. Since the pH changed during the interaction between cation and polysaccharide, the study of the pH dependence of the precipitation was performed by addition of variable volumes (from 5 to 100 μl) of 10 mM NaOH to the EPS solution in order to adjust the pH up to the stated value 10 min after the addition of the cation.

NMR. ^1H nuclear magnetic resonance (NMR) spectra were recorded on a WP-200SY NMR spectrometer (200 MHz) (Bruker, Billerica, Mass.) at 24°C. Polysaccharide samples (5 to 20 mg) were dissolved in D_2O, freeze-dried, and redissolved in 0.5 ml of D_2O.

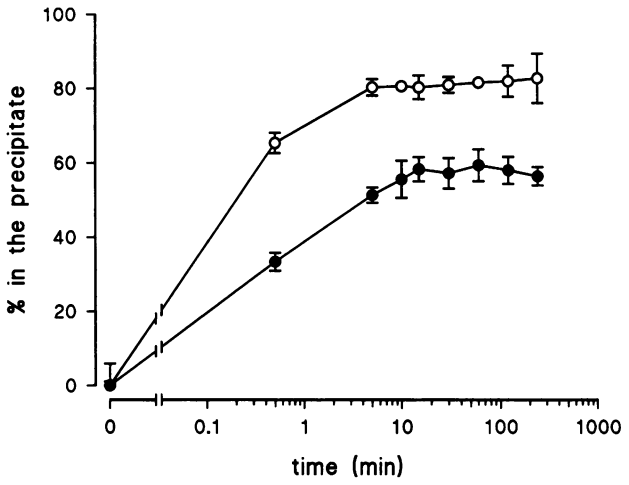

FIG. 1. Time course of precipitate formation after mixing acidic EPS from *Bradyrhizobium* BGA-1 with $FeCl_3$ (final concentrations, 1 mg of EPS per ml and 1 mM $FeCl_3$). Symbols: ●, iron; ○, polysaccharide. Results are means of three determinations ± standard deviations (SD).

Chemicals. All reagents were analytical grade. Na^+, K^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}, Sn^{2+}, Al^{3+}, Fe^{2+}, and Fe^{3+} were used as chlorides; Pb^{2+} and Th^{4+} were used as nitrates; Cu^{2+}, Zn^{2+}, and Mn^{2+} were used as sulfates; and U^{6+} was used as uranyl nitrate. All solutions and procedures used deionized water (18.3 MΩ) from a NanoPure system (Barnstead, Newton, Mass.).

RESULTS

Characteristics of the polysaccharides. Polysaccharide samples did not contain protein and showed little (less than 0.5%) nucleic acid contamination. EPS from strain BGA-1 was readily dissolved (up to 15 mg ml^{-1}) in water and formed clear and stable solutions. The solubility of EPS increased to 80 mg ml^{-1} when it was dissolved in 0.25 M NaCl. EPS from strain USDA 110 was slightly less soluble in water or salt solutions than was that from BGA-1. Solutions of both polysaccharides (1 mg ml^{-1}) in water were acidic (pH 4.8 for USDA 110 and 4.6 for BGA-1). Fe^{3+} or Al^{3+} concentrations in 5-mg ml^{-1} solutions of both polysaccharides in water were under the detection limit.

NMR spectra were similar to those previously reported (14, 21). The ^1H signal attributable to acetyl groups was greater (48 versus 2% of total ^1H) in the EPS from USDA 110 than in the EPS from *Bradyrhizobium* strain BGA-1. After deacetylation, this signal was reduced but did not disappear (it decreased to 2% of total ^1H in USDA 110 and 0.5% in BGA-1).

EPS precipitation with iron. Addition of 100 μl of 20 mM Fe^{3+} (pH 1.9) to 1.9 ml of a 1.5-mg ml^{-1} solution of EPS from BGA-1 (pH 4.6) resulted in a decrease (about 2 units) in the pH, and the solution became cloudy. Both EPS and Fe^{3+} were partially removed as a precipitate by centrifugation, and the supernatant was clear. All the reactions were fast, and most of the precipitate was produced in less than 10 min; thereafter, almost no change in precipitation or pH occurred (Fig. 1). The precipitation or pH change required both EPS and Fe^{3+}

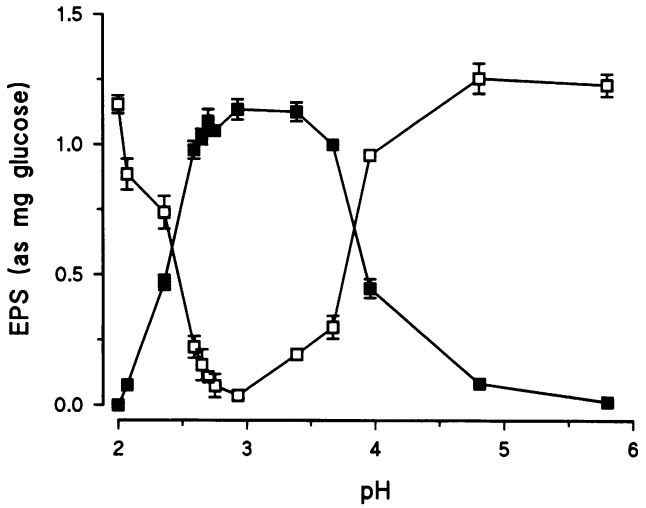

FIG. 2. Effect of pH on the precipitation of EPS from *Bradyrhizobium* strain BGA-1 (1 mg/ml) by 1 mM $FeCl_3$. The pH was recorded 10 min after mixing, and the samples were centrifugated 4 h after. Data are expressed as milligrams of glucose equivalents recovered in the sediment (solid symbols) and supernatant (open symbols). Results are the means of three determinations ± SD. Error bars not shown are within the symbol.

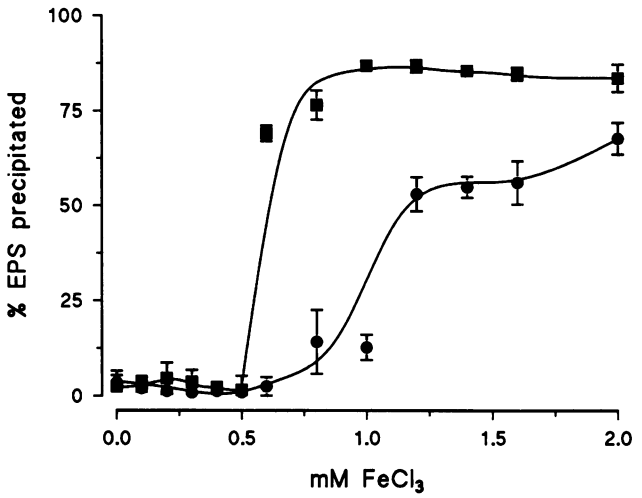

FIG. 3. Effect of Fe^{3+} concentration on the solubility of polysaccharides from *Bradyrhizobium* species. Polysaccharides (1 mg/ml) were dissolved in 10 mM sodium acetate buffer (pH 3.6). Data refer to the percentage of polysaccharide precipitated after 4 h of mixing EPS and $FeCl_3$ solutions. Symbols: ■, BGA-1 polysaccharide; ●, USDA 110 polysaccharide.

because pure solutions at pH values between 2.0 and 3.0 remained unchanged during the time of the experiment.

The precipitation of strain BGA-1 EPS by Fe^{3+} showed a pH dependence (Fig. 2). The solution remained slightly turbid at pH between 5.5 and 6, but no precipitate was recovered by centrifugation, probably because of the small particle size of the iron-EPS complex. The Fe^{3+}-EPS precipitate was redissolved by changing the pH out of the range 2.2 to 5.5. It was then reprecipitated by changing the pH back to a value within the above range. It was also redissolved by 5 min of incubation in 10 mM EDTA, ascorbic acid, or sodium dithionite at pH 3.0. After EDTA or reductants were eliminated by dialysis, the EPS was reprecipitated by further Fe^{3+} addition. Below pH 2.8 the Fe^{3+}-EPS precipitate was white, but at higher pH values it was increasingly rust colored. The proportion of iron contained in the precipitate increased from pH 2.5 to 5 regardless of the polysaccharide studied (Table 1).

EPS produced by strain USDA 110 showed a similar behavior, but its precipitation required higher Fe^{3+} concentrations than did precipitation of EPS from BGA-1 (Fig. 3). However, after the EPS was deacetylated, it precipitated with 1 mM Fe^{3+} (Fig. 4).

As controls of precipitation specificity, 1 or 10 mM (final concentrations) Fe^{3+} was added to 1- or 10-mg ml^{-1} solutions of soluble starch (Sigma), dextran 50000 (Fluka), or colominic acid (Sigma) in water, and the pH was adjusted to within pH 3 to 6 with NaOH. In all cases the solutions remained unchanged (without precipitation or turbidity formation).

TABLE 1. Effect of pH on the Fe^{3+}/polysaccharide ratio[a] in the precipitates obtained after mixing solutions of $FeCl_3$ with native or deacetylated EPS from *Bradyrhizobium* strain BGA-1 and *B. japonicum* USDA110

pH	Fe^{3+}/polysaccharide for:			
	BGA-1		USDA110	
	Native	Deacetylated	Native	Deacetylated
2.5	7.8	7.1	NP[b]	7.6
2.95	20.9	16.5	24.0	17.9
3.9	35.1	27.7	NP	30.5
5.0	59.2	46.8	NP	37.2

[a] Fe^{3+} is expressed as micrograms of iron; polysaccharide is expressed as milligrams of EPS. We used 0.5 mg of polysaccharide ml^{-1} and 1 mM Fe^{3+}. The pH of the samples was adjusted with NaOH after 10 min from mixing EPS and cation solutions. Samples were stirred, left to stand for 4 h, and centrifuged. The iron and EPS present were measured as stated in Materials and Methods.

[b] NP, no precipitate was found.

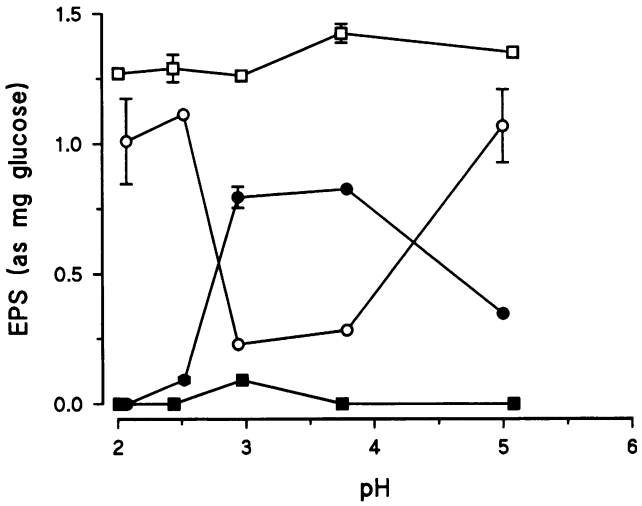

FIG. 4. Effect of pH on the precipitation of EPS from *Bradyrhizobium* strain USDA 110 (1 mg/ml) by 1 mM $FeCl_3$. The pH was recorded 10 min after mixing, and the samples were centrifugated 4 h later. Symbols: ■, □, native polysaccharides; ●, ○, deacylated polysaccharides. Data are expressed as milligrams of glucose equivalents recovered in the sediment (solid symbols) and supernatant (open symbols). Results are the means of three determinations ± SD. Error bars not shown are within the symbol.

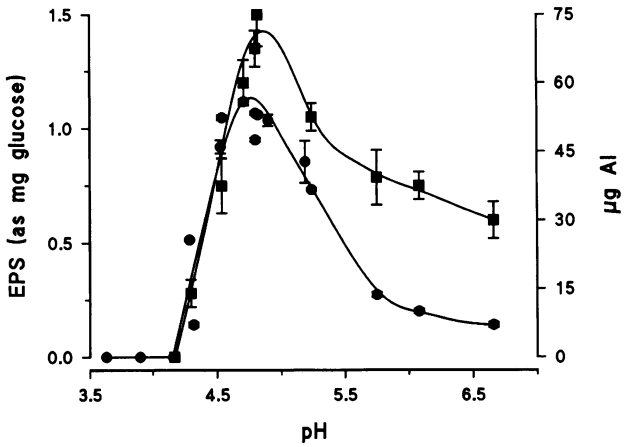

FIG. 5. Effect of pH on the precipitation of EPS from *Bradyrhizobium* strain BGA-1 (1 mg/ml) by 1 mM $AlCl_3$. The pH was recorded 10 min after mixing, and the samples were centrifuged after 4 h. Polysaccharide and Al present in the precipitate were measured. Symbols: ●, polysaccharide; ■, aluminum. Results are the means of three determinations ± SD.

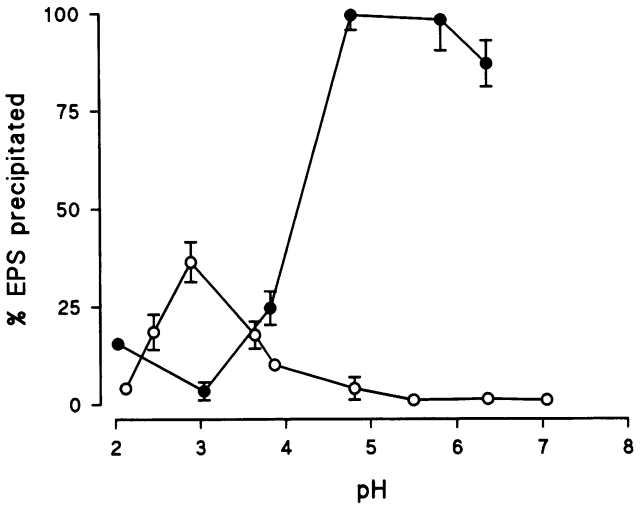

FIG. 6. pH dependence of precipitation of EPS from *Bradyrhizobium* strain BGA-1 by Sn^{2+} (○) and Th^{4+} (●) (1 mg of EPS per ml and 1 mM cation). Results are expressed as the percentage of polysaccharide present in the precipitate 4 h after mixing EPS and cation solutions.

Although Fe^{3+} is known to be insoluble at neutral pH values, the solutions remained stable for several weeks when the pH of 1 mM Fe^{3+} and 1-mg · ml^{-1} solutions of EPS from BGA-1 were adjusted between pH 6.5 and 7.5. However, neutral EPS solutions were unable to redissolve ferric hydroxyoxide when they were added after the precipitation of the cation. Other polysaccharides (starch, dextran, or colominic acid) also stabilized Fe^{3+} in neutral solution.

In contrast to the results obtained in the presence of Fe^{3+}, solutions of EPS from USDA 110 or BGA-1 were not precipitated and did not become turbid when Fe^{2+} was added at concentrations ranging from 0.1 to 10 mM.

EPS precipitation with Al^{3+}. EPS solutions from BGA-1 became cloudy in the presence of 1 mM Al^{3+} (with a marked pH dependence), and a white precipitate was recovered between pH 4.2 and 6.5 (Fig. 5). The precipitate was redissolved after treatment with 10 mM EDTA. It also redissolved after treatment at increasing pH, but, unlike the Fe-EPS precipitate, the Al-EPS precipitate required a pH above 9.0 to be redissolved. The Al/EPS ratio in the precipitate increased when the pH was increased from 4.5 to 6 (Fig. 5). No precipitate was formed with native or deacetylated EPS from USDA 110, although their solutions became cloudy in the presence of Al^{3+} at pH values between 4.8 and 6.5. There was neither turbidity nor detectable precipitation of aluminum in the absence of EPS during a 4-h incubation in the range of pH tested.

Effect of other metals on polysaccharide solutions. There was neither precipitation, gelation, nor turbidity with K^+, Na^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, and U^{6+} at 1 mM cation and 1 mg of EPS ml^{-1} from BGA-1 at pH values ranging from 2.0 to 8.0. Solutions of EPS remained stable in the presence of 500 mM K^+, 100 mM Ca^{2+}, or 100 mM Mg^{2+} at pH 3.5 or 8.0. EPS solutions with 10 mM Ca^{2+} remained unchanged after boiling or freezing. The behavior of the EPS in Sephacryl HR200 or Sephacryl HR400 columns was the same when chromatographed in water or in the presence of 10 or 100 mM K^+, Ca^{2+}, or Mg^{2+}, thus indicating that there was no change in the hydrodynamic properties of the polysaccharide in the presence of these cations. EPS from BGA-1 were precipitated by 1 mM Sn^{2+} and Th^{4+}, with a pH dependence shown in Fig. 6. Millimolar solutions of Mn^{2+} and Co^{2+} also precipitated EPS (5 and 10% of EPS, respectively), with maximal precipitation at pH values around neutrality. Neither native nor deacetylated EPS from USDA 110 were precipitated by any of the tested cations.

Effect of ionic strength on the EPS precipitation. An increase in the salt concentration in solutions of EPS produced by BGA-1 decreased their precipitation by Fe^{3+} and by Al^{3+}. This effect was larger for $CaCl_2$ than for NaCl and depended on the ionic strength of the solutions (Fig. 7) rather than being a specific effect for Ca^{2+}. A difference in the precipitation of EPS by each cation was that low-salt addition (below 10 mM NaCl or 5 mM $CaCl_2$) increased precipitation by Al^{3+} but inhibited precipitation by Fe^{3+}. The inhibitory effect of the increase in ionic strength was also observed with EPS from

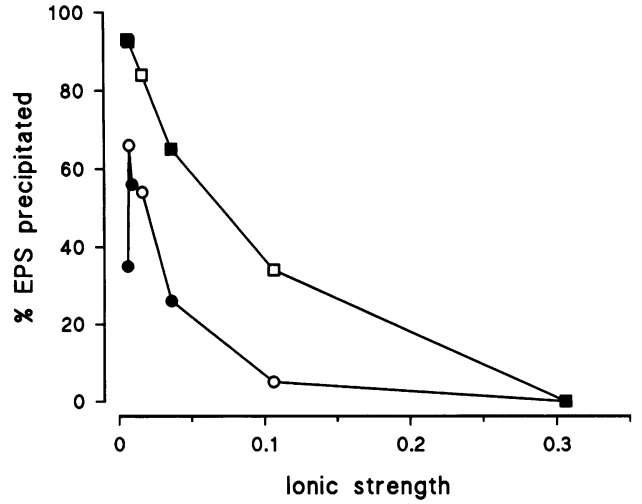

FIG. 7. Effect of ionic strength on precipitation of EPS from *Bradyrhizobium* strain BGA-1 by Al^{3+} and Fe^{3+}. The pH values were adjusted to 3.0 for Fe^{3+} precipitation (□, ■) and 4.7 for Al^{3+} precipitation (●, ○). Ionic strength was adjusted by adding NaCl (open symbols) or $CaCl_2$ (solid symbols) and was calculated as $\frac{1}{2}\Sigma M_i Z_i^2$.

TABLE 2. Effect of $AlCl_3$ on the precipitation of EPS-iron complex[a]

$AlCl_3$ concn (mM)	% Recovered in precipitate[b]:		
	EPS[c]	Fe[d]	Al[d]
0	87.5 ± 3.5	40.9 ± 1.3	
0.75	89.9 ± 5.3	30.7 ± 5.9	0
1.0	81.9 ± 4.9	30.6 ± 3.2	0
2.0	78.8 ± 6.1	26.4 ± 6.3	5.2 ± 0.9
5.0	44.4 ± 4.3	10.9 ± 2.7	3.1 ± 1.5

[a] Reactant concentrations were 1 mg of EPS per ml and 1 mM Fe^{3+}. The pH was 3.1 after incubation for 10 min. The total incubation time was 4 h.
[b] Data are the mean of three determinations and are expressed as a percentage of the total EPS or cation recovered in the precipitate.
[c] Determined by the phenol-sulfuric acid method.
[d] Determined by atomic absorption spectroscopy.

USDA 110: 1.5 mM Fe^{3+} did not precipitate EPS from USDA 110 in 100 mM sodium acetate (pH 3.6), but the same iron concentration precipitated about half of the EPS when the salt concentration was lowered to 10 mM.

$AlCl_3$ exerted an inhibition on the precipitation of EPS from BGA-1 by Fe^{3+} similar to that produced by $CaCl_2$ or NaCl. At optimum pH for iron precipitation, the addition of increasing amounts of $AlCl_3$ resulted in a decrease of the precipitation (Table 2). However, under optimum conditions for precipitation by Al^{3+}, $FeCl_3$ did not exhibit similar inhibition. In fact, under these conditions, precipitation was greatly enhanced and iron was completely removed (Table 3), even though it was not precipitated at that pH in the presence of EPS alone. This agrees with the increased precipitation by Al^{3+} found at low salt concentration, but $FeCl_3$ increased EPS precipitation at ionic strengths (over 0.02) at which Ca^{2+} and Na^+ were inhibitory.

DISCUSSION

Binding of heavy metals to microbial extracellular polymers is a well-known process, but relatively few attempts have been made to determine the environmental dependence of the interaction. A well-studied polymer is the capsular poly-γ-glutamate of *Bacillus licheniformis* (17), which, although different from the bradyrhizobial EPS, also precipitates Al^{3+} and Fe^{3+} but not Fe^{2+}. McLean et al. (16, 17) proposed a mechanism of precipitation based on the binding of Fe^{3+} to poly-γ-glutamate that results in the development of rust-colored ferrihydrite, which itself binds additional ferric cations. However, the precipitation of polysaccharides from *Bradyrhizobium* species and iron could be due to a specific interaction between the soluble cation and the polysaccharide independent of ferrihydrite formation, as suggested by the facts that the precipitate was white and could be redissolved at pH values at which ferrihydrite remained precipitated. This explanation is also valid for the interaction of polysaccharide and Al^{3+}, which results in precipitate formation at pH values at which the aluminum remains soluble. Furthermore, the fact that EPS from BGA-1 was not precipitated by Cu^{2+} at neutral pH, at which insoluble copper hydroxyoxides are formed, also supports a mechanism different from the coprecipitation between polysaccharide and insoluble neutral hydroxyoxides. Certainly, it is possible that some of the iron precipitate was bound in a nonspecific way, perhaps as the hydroxyoxide, that could explain the increase of the Fe/EPS ratio in the precipitate when the pH was raised from 2.5 to 5.0.

TABLE 3. Effect of $FeCl_3$ on the precipitation of EPS-aluminum complex[a]

$FeCl_3$ concn (mM)	% Recovered in the precipitate[b]		
	EPS[c]	Fe[d]	Al[d]
0	76.6 ± 5.1		40.3 ± 3.7
0.75	96.4 ± 4.3	98.1 ± 2.2	33.3 ± 4.6
1.0	97.0 ± 1.9	99.3 ± 0.9	45.5 ± 6.5
2.0	100	100	70.1 ± 3.1
5.0	100	99.1 ± 1.3	72.7 ± 4.3

[a] Reactant concentrations were 1 mg of EPS per ml and 1 mM Fe^{3+}. The pH was 4.8 after incubation for 10 min. The total incubation time was 4 h.
[b] Data are the means of three determinations and are expressed as percentages of the total EPS or cation recovered in the precipitate.
[c] Determined by the phenol-sulfuric acid method.
[d] Determined by atomic absorption spectroscopy.

Since both Fe^{3+} and Al^{3+} are present in aqueous solution as hexa-aquo complexes that are readily hydrolyzed, several molecular species could be responsible for the precipitation. Because precipitation is fast and the cation solutions were prepared immediately before use, it is unlikely that hydrolyzed polymeric species were participating in the precipitation process, because they are formed rather slowly (1). The pH dependence of precipitation suggests that the precipitating species is the first one hydrolyzed, $M(H_2O)_5(OH)^{2+}$, which interacts with the ionized carboxyl groups in the EPS. In fact, the pH dependence curves of the precipitation reaction agree well with the curves for the presence of the first hydrolyzed species for Fe^{3+} and Al^{3+} (4). This is also valid for precipitation by Sn^{2+} and Th^{4+}, because the precipitation is maximum at the pH at which the carboxyl groups are ionized, and the metal is present as a divalent cation complex (1, 24). Binding of the positively charged iron-hydroxide species to anionic polymers has been proposed by Beveridge (2) and Ghiorse (11) as the first step in iron deposition by microorganisms. This mechanism could be of general importance in the formation of metallic deposits by microorganisms. An example of a well-known interaction between a cation and polysaccharide is the gelling of alginates by Ca^{2+}. This requires the ionic binding between the cation and two carboxylates from guluronate residues from two polysaccharide chains (19). In a similar way, precipitation of EPS from *Bradyrhizobium* species could be due to linking polysaccaride chains by a divalent cationic bridge. When the hydrolyzed cation has a net charge different from 2+, bridge formation between two chains is not possible and the EPS-cation complex does not precipitate. Our data do not preclude the existence of interactions between polysaccharide and other cationic species that may not result in precipitation. The stabilization of Fe^{3+} by solutions of EPS at neutral pH could be explained by such nonprecipitating interactions.

Acetyl groups in EPS from *B. japonicum* USDA 110 inhibited the precipitation with iron. The interference of acetyl groups in the interaction between other bacterial EPS and cations has been reported (10, 15). Since the degree of acetylation of the polysaccharides from *B. japonicum* changes with the age of the culture (20), the potential of coprecipitation with metal cations could be a variable property of these molecules. However, acetylation was not the only factor involved in the precipitation with cations, because aluminum, thorium, and tin did not precipitate native or deacylated EPS from USDA 110.

We have not attempted to look for a biological function of the coprecipitation of EPS with some cations. In this regard, the most important cations would be Al^{3+} and Fe^{3+}, because they are very common in soils. Because aluminum has been reported to be toxic for both the members of the family *Rhizo-*

biaceae and the plants, the complexation and precipitation of Al^{3+} by polysaccharides could be a detoxifyng mechanism, because in this case the precipitation occurs at pH values compatible with the growth of *Bradyrhizobium* strain BGA-1 (13). Although the metal concentrations used in this work are higher than those typical of soil, we could have amplified a reaction that may occur on a smaller scale. The study of this possibility requires a careful selection of experimental conditions, because our data showed that rather similar polysaccharides are clearly distinct in their behavior and that pH and ionic strength are critical.

ACKNOWLEDGMENTS

This work was supported by a grant from the Canary Island Government.

We are indebted to A. G. Ravelo for his assistance in interpretation of NMR spectra and to C. Arvelo for atomic absorption spectroscopy determination.

REFERENCES

1. **Baes, C. F., and R. E. Mesmer.** 1976. The hydrolysis of cations. John Wiley & Sons, Inc., New York.
2. **Beveridge, T. J.** 1989. Role of cellular design in bacterial metal accumulation and mineralization. Annu. Rev. Microbiol. **43:**147–171.
3. **Beveridge, T. J., and R. J. Doyle.** 1989. Metal ions and bacteria. John Wiley & Sons, Inc., New York.
4. **Bohn, H., B. McNeal, and G. O'Connor.** 1985. Soil chemistry, 2nd ed. John Wiley & Sons, Inc., New York.
5. **Brown, M. J., and J. N. Lester.** 1979. Metal removal in activated sludge: the role of bacterial extracellular polymers. Water Res. **13:** 817–837.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, A. Rebers, and F. Smith.** 1956. Colorimetric methods for determination of sugars and related substances. Anal. Biochem. **28:**350–356.
7. **Dudman, W. F.** 1976. The extracellular polysaccharides of *Rhizobium japonicum*: compositional studies. Carbohydr. Res. **46:**97–110.
8. **Dudman, W. F.** 1978. Structural studies of the extracellular polysaccharides of *Rhizobium japonicum* strains 71A, CC708, and CB1795. Carbohydr. Res. **66:**9–23.
9. **Fries, J.** 1971. Análisis de Trazas. Métodos fotométricos comprobados. E. Merck AG, Darmstadt, Germany.
10. **Geddie, J. L., and I. L. Sutherland.** 1993. Uptake of metals by bacterial polysaccharides. J. Appl. Bacteriol. **74:**467–472.
11. **Ghiorse, W. C.** 1984. Biology of iron and manganese depositing bacteria. Annu. Rev. Microbiol. **38:**515–550.
12. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. **38:**501–505.
13. **León-Barrios, M., A. M. Gutiérrez-Navarro, R. Pérez-Galdona, and J. Corzo.** 1991. Characterization of Canary island isolates of *Bradyrhizobium* sp. (*Chamaecytisus proliferus*). Soil Biol. Biochem. **23:**487–489.
14. **León-Barrios, M., A. M. Gutiérrez-Navarro, R. Pérez-Galdona, J. Díaz-Siverio, J. Trujillo, and J. Corzo.** 1992. Acidic exopolysaccharide from *Bradyrhizobium* (*Chamaecytisus proliferus*). J. Appl. Bacteriol. **72:**91–96.
15. **Marqués, A. M., R. Bonet, M. D. Simon-Pujol, M. C. Fusté, and F. Congregado.** 1990. Removal of uranium by an exopolysaccharide from *Pseudomonas* sp. Appl. Microbiol. Biotechnol. **34:**429–431.
16. **McLean, R. J. C., D. Beauchemin, and T. J. Beveridge.** 1992. Influence of oxidation state on iron binding by *Bacillus licheniformis* capsule. Appl. Environ. Microbiol. **58:**405–408.
17. **McLean, R. J. C., D. Beauchemin, L. Clapham, and T. J. Beveridge.** 1990. Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. Appl. Environ. Microbiol. **56:**3671–3677.
18. **Mittelman, M. W., and G. G. Geesey.** 1985. Copper-binding characteristics of exopolymers from a freshwater sediment bacterium. Appl. Environ. Microbiol. **49:**846–851.
19. **Morris, E. R., D. A. Rees, D. Thom, and E. J. Welsh.** 1977. Conformation and intermolecular interactions of carbohydrate chains. J. Supramol. Struct. **6:**259–274.
20. **Mort, A. J., and W. D. Bauer.** 1980. Composition of the capsular and extracellular polysaccharides of *Rhizobium japonicum*. Changes with culture age and correlations with binding of soybean seed lectin to the bacteria. Plant Physiol. **66:**158–163.
21. **Mort, A. J., and W. D. Bauer.** 1982. Application of two new methods for cleavage of polysaccharides into specific oligosaccharide fragments. Structure of the capsular and extracellular polysaccharides of *Rhizobium japonicum* that bind soybean lectin. J. Biol. Chem. **257:**1870–1875.
22. **Plude, J. L., D. L. Parker, O. J. Schommer, R. J. Timmerman, S. A. Hagstrom, J. J. Joers, and R. Hnasko.** 1991. Chemical characterization of polysaccharide from the slime layer of the cyanobacterium *Microcystis flos-aquae* C3-40. Appl. Environ. Microbiol. **57:** 1696–1700.
23. **Rudd, T., R. M. Sterritt, and J. M. Lester.** 1984. Complexation of heavy metals by extracellular polymers in the activated sludge process. J. Water Pollut. Control Fed. **56:**1260–1268.
24. **Smith, R. M., and A. M. Martell.** 1989. Critical stability constants. Second supplement, vol. 6. Plenum Press, New York.
25. **Vincent, J. M.** 1970. A manual for the practical study of the root nodule bacteria. Blackwell Scientific Publications, Ltd., Oxford.