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Localization and Partial Characterization of Soybean Lectin-Binding Polysaccharide of *Rhizobium japonicum*

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Immunoelectron microscopy was combined with partial characterization of isolated exopolysaccharide to study binding of soybean lectin by *Rhizobium japonicum* strain USDA 138. Lectin-binding activity resided in two forms of exopolysaccharide produced during growth: an apparently very high-molecular-weight capsular form and a lower-molecular-weight diffusible form. At low-speed centrifugation, the capsular form cosedimented with cells to form a viscous, white, cell-gel complex which was not diffusible in 1% agar, and the diffusible form remained in the cell-free supernatant. Electron microscopic observation of the cell-gel complex after labeling with soybean lectin-ferritin conjugate revealed that capsular polysaccharides, frequently attached to one end of the cells, were receptors for lectin. The outer membrane of the cell bound no lectin. Various preparations of exopolysaccharide isolated from the culture supernatant were tested for lectin binding, interaction with homologous somatic antigen, and the presence of 2-keto-3-deoxyoctonate and were chromatographed in Sepharose 4B and 6B gel beds. Lectin binding was restricted to a polysaccharide component designated as lectin-binding polysaccharide. This polysaccharide, as present in the cell-free culture supernatant, was a diffusible acidic polysaccharide devoid of 2-keto-3-deoxyoctonate, with a molecular weight of 2×10^6 to 5×10^6. It was concluded that the soybean lectin-binding component of *R. japonicum* is an extracellular polysaccharide and not a lipopolysaccharide and that the diffusible lectin-binding polysaccharide probably differs from the very high-molecular-weight lectin-binding polysaccharide of the loose capsule (slime) only in the degree of polymerization.

Lectins obtained from soybean plants react specifically with *Rhizobium japonicum*, the bacterium which nodulates soybeans in a nitrogen-fixing symbiosis. This reaction provides the basis for the lectin recognition hypothesis (7) that was proposed to account for the specificity expressed between legume-*R. japonicum* pairs. The component of *R. japonicum* involved in the specific binding of soybean lectin (soybean agglutinin [SBA]) has been considered to be exopolysaccharide (EPS) (2, 5, 7-9, 11, 16, 20) or lipopolysaccharide (12, 26). Evidence is clearly in favor of EPS as the lectin-binding material.

Calvert et al. (9) presented electron microscope data to demonstrate the capsular nature of the lectin-binding material, and Bhuvaneswari et al. (5) used a radioassay to estimate the number of binding sites on the cell surface. Lectin-binding polysaccharide (LBPS), however, is not limited to the capsule since lectin-binding activity was also found as diffusible polysaccharide in the low-speed centrifuge fraction of the culture (21).

In this report, the results of further study of the nature and localization of LBPS of *R. japonicum* are summarized. Electron microscopic methods were used to characterize the LBPS associated with the cell surface. LBPS isolated from the culture supernatant was purified and partially characterized.

MATERIALS AND METHODS

Cultures. *R. japonicum* strain USDA 138 cultures were grown either in a yeast extract-mannitol-salt medium (21) or in a chemically defined medium (4) on a rotary shaker at 25°C and were carried on yeast extract-mannitol-salt slants.

EPS. EPS was prepared from the culture supernatant after low-speed centrifugation (3,000 × *g*, 30 min; for details, see below). Clear, cell-free supernatant was concentrated and desalted by membrane ultrafiltration (Diaflo membrane ultrafiltration system, Amicon Corp.). The membrane used was XM300, which has a molecular weight cut-off at 300,000. EPS so prepared was designated as semipurified EPS. EPS was also prepared by precipitation from the culture supernatant with 3 volumes of acetone. The precipitate collected by centrifugation was dissolved in distilled water and reprecipitated once with acetone. Water-soluble EPS was then lyophilized.

Acidic EPS was prepared by Cetavlon (cetyltrimethylammonium bromide) precipitation (15). Cetavlon solution (20%) was added to a semipurified EPS solution (10 mg/ml) to a final concentration of 2%. The precipitate was collected by centrifugation and solubilized overnight in water. Acidic EPS was precipitated with 3 to 4 volumes of absolute ethanol and dissolved in water. Alcohol precipitation was repeated 3 times. The final acidic EPS was dissolved in 1% NaCl solution, dialyzed against distilled water, and lyophilized.

Isolation of EPS by phenol extraction was performed by the procedure of Westphal and Jann (25). A phenol solution (95%) and a cell-gel complex (for details, see below and the legend to Fig. 1) were mixed in equal volumes at 68°C and incubated at that temperature for 5 min. After cooling in an ice bath, the aqueous and phenol phases were separated by centrifugation. Both fractions were collected, dialyzed exhaustively against distilled water, and lyophilized.

Enzyme treatment. EPS solution (10 mg/ml) in phosphate buffer (50 mM, pH 7) was treated with DNase, RNase (100 μg/ml each, Miles Laboratories, Inc., Elkhart, Ind.), and pronase (1 mg/ml, B grade, Calbiochem-Behring Corp., La Jolla, Calif.), respectively, for 60 min at room temperature. Pronase-treated EPS was extracted by phenol-water (25) or treated with 8 M urea, dialyzed against water, and lyophilized.

Column chromatography of EPS. The semipurified EPS (10 mg/ml) in distilled water was chromatographed on a column (2.6 by 32 cm) with either a Sepharose 6B gel bed or a Sepharose 4B gel bed. A 1- to 2-ml amount of the EPS solution was layered on the gel. Elution was with 10 mM phosphate buffer, pH 7, at the rate of 1 0.05-ml drop every 20 to 30 s. Fractions (40 drops, 2.15-ml each) were collected.

Preparation and purification of SBA. SBA was prepared from soybean seeds by the method of Liener and Pallansch (13). Crude SBA from the 30 to 70% ammonium sulfate-precipitated fraction was purified by affinity chromatography on an acid-treated Sepharose 6B column as described by Allen and Johnson (1). SBA desorbed with a 0.2 M galactose solution as a single peak detectable by a UV monitor. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis yielded a single protein band. The SBA was identical in electrophoretic behavior to that obtained by affinity chromatography with a covalently linked carbohydrate ligand on a gel matrix (22).

Preparation and purification of anti-*R. japonicum* USDA 138 antibody. Heat-killed *R. japonicum* cells were used to immunize rabbits as described previously (6). The antiserum was fractionated by ammonium sulfate precipitation, and immunoglobulin G was purified by affinity chromatography on a protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

Chemical determinations. Carbohydrate was determined by the method of Toennies and Kolb (19) modified as follows: a test tube containing 2.5 ml of 0.1% anthrone solution in 72% H_2SO_4 was held in an ice bath, and a 0.5-ml sample was layered on the reagent. The contents were mixed and incubated in a boiling water bath for 10 min. Absorbance at 620 nm was determined in the Beckman model B spectrophotometer after the sample was cooled. Glucose solutions containing 1 to 100 μg/0.5 ml were used as a standard.

2-Keto-3-deoxyoctonate was determined by the thiobarbituric acid test as described by Weissbach and Hurwitz (24), using a 250-μl sample.

Fluorescence microscopy. SBA and anti-*R. japonicum* antibody were conjugated with fluorescein isothiocyanate and used in fluorescence microscopy as reported previously (8).

Ouchterlony gel diffusion. Ouchterlony gel diffusion was performed as previously described (20). Plates containing EPS and SBA were incubated at room temperature in a moist chamber for 48 h.

Hemagglutination inhibition assay. The hemagglutination inhibition assay was performed as described previously (21), with a serial twofold-diluted SBA solution (50 μl), starting at 100 μg/ml, and glutaraldehyde-treated, trypsinized rabbit erythrocytes (100 μl). The assay was performed in a microtiter U plate (Dynatech Laboratories, Inc., Alexandria, Va.). Fifty-microliter sample solutions were used.

Conjugation of ferritin to SBA. The method described by Nicolson and Singer (14) to conjugate ferritin and plant agglutinin with glutaraldehyde as the coupling agent was generally followed. Glutaraldehyde (0.2 ml of a 0.5% solution, prepared from an 8% electron microscopy grade solution [Polyscience Inc., Warrington, Pa.]) was added with stirring to 3.0 ml of 50 mM phosphate buffer at pH 7 containing 10 mg of SBA, 54 mg of galactose, and 45 mg of ferritin (Miles Laboratories). After incubation at room temperature for 1 h with continuous stirring, 4 drops of NH_4Cl (0.1 M) were added. The mixture was dialyzed thoroughly against phosphate buffer (50 mM), pH 7. Dialyzed SBA-ferritin conjugate was centrifuged at 10,000 × *g* for 30 min. The resulting clear supernatant containing SBA-ferritin was purified on an acid-treated Sepharose 6B affinity column (1) and eluted specifically with 0.2 M galactose in phosphate buffer solution (50 mM, pH 7).

Electron microscopy. A 2-ml portion of a late-exponential-phase culture was added to 0.5 ml of SBA-ferritin solution or SBA solution (5 mg/ml). The mixture was incubated at room temperature for 1 h, during which time noticeable agglutination occurred. The mixture was then centrifuged. The resulting pellet (see below and the legend to Fig. 1B) was washed three times (10 ml each) with phosphate buffer (50 mM, pH 7) by inverting the tube gently without breaking the pellet. After 30 min at room temperature, the washing solution was removed by suction and replaced. Cells labeled with SBA-ferritin were suspended mechanically in 2 ml of phosphate buffer, mounted on a Formvar-carbon-coated electron microscopic grid, and examined directly in an electron microscope without further staining. For thin sectioning, the pellet was fixed with 2% glutaraldehyde (electron microscopy grade, 8% in distilled water in a sealed vial under nitrogen [Polyscience]), washed, fixed by osmium tetroxide, dehydrated, and finally embedded in epoxy resin as described previously (20). Sections were cut on an LKB ultramicrotome with a diamond knife and stained with uranyl acetate and lead citrate.

Controls included the addition of galactose to SBA-

ferritin solution to a final concentration of 0.2 M before adding the mixture to the culture portion or the blocking SBA-ferritin binding sites by pretreatment of cells with SBA solution. *R. leguminosarum*, a SBA-nonbinding *Rhizobium* species, was used as a control for nonspecific binding.

Procedures for ruthenium red staining were essentially as described by Springer and Roth (17).

EPS was prepared for the electron microscope by placing 1 drop of purified EPS (100 μg/ml of distilled water) on a Formvar-carbon-coated grid. After 1 min, free liquid was removed by capillarity, using a piece of filter paper touched to the edge of the grid. The specimen was shadow-cast with platinum-carbon at an angle of 45°. All specimens were examined in a Siemens-Elmiskop I electron microscope operated at 80 kV.

RESULTS

Distribution of LBPS in *R. japonicum* culture. Figure 1A shows the typical separation of a 14-day culture of strain USDA 138 into three fractions after low-speed centrifugation (3,000 × *g*, 30 min). The whitish cell-gel complex comprised up to one-third of the culture volume and consisted of viscous EPS and embedded cells. The cells were easily visualized within the complex by immunofluorescence microscopy. Centrifugation at 100,000 × *g* for 120 min reduced only slightly the volume of the cell-gel complex, and repeated washing had no effect on the gel volume. With each wash, however, additional cells were separated from the complex and pelleted.

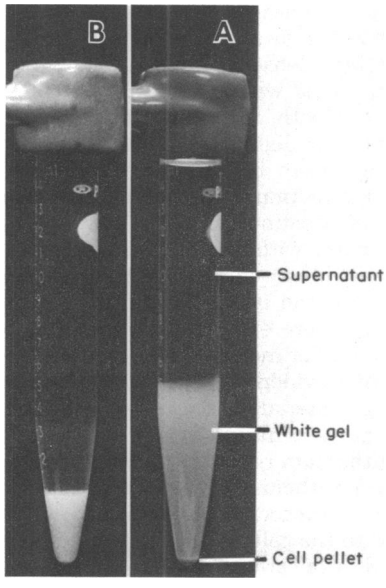

FIG. 1. *(A) Typical appearance of a 2-week-old culture of R. japonicum after centrifugation at 3,000 × g for 30 min. The culture was separated into three fractions: a cell pellet, a white gel containing a cell-gel complex, and a cell-free supernatant. (B) Agglutination of cell-gel complex by adding soybean lectin. The volume of agglutinated cell-gel complex shrank to about 20% of the original volume.*

Addition of SBA to the cell-gel complex caused visible aggregation of the complex, followed by substantial reduction in its volume. The same result was observed when SBA solution was added into the culture portion in that a visible agglutination occurred after incubation for 1 h. The observation of agglutination could be hastened by centrifugation. The resulting volume of the cell-gel complex was only about 20% of the unagglutinated cell-gel volume (Fig. 1B). In addition, the pelleted cell-gel-SBA complex was stable during subsequent washing, whereas the untreated cell-gel complex was readily dispersed to form a homogeneous suspension when either a washing solution was added or the centrifuge tube was shaken gently.

Separation of the whole culture by the scheme shown in Fig. 2 yielded fractions which were analyzed for the presence of LBPS (Table 1). Ouchterlony-diffusible LBPS was detected in the supernatant after low-speed centrifugation (fraction II), in unfractionated (I) whole culture, and in the cell-gel complex (fraction III). Repeated washing of the cell-gel complex removed diffusible LBPS, but cells washed out of the complex, however, were free of LBPS (fraction VII). Diffusible LBPS was removed from the supernatant (fraction II) by high-speed centrifugation (100,000 × *g*, 120 min) as a pellet (fraction V), demonstrating its macromolecular nature. Cells in all fractions which contained EPS gel (I, III, and VI) were capable of binding fluorescein isothiocyanate-SBA, whereas cells washed free (fraction VII) or centrifuged free of EPS (fraction VIII) failed to bind fluorescein isothiocyanate-SBA.

LBPS associated with the cell surface. Exponential-phase cells (harvested approximately 5 days after inoculation [for the growth curve, see reference 21]), reacted with ferritin-SBA and examined directly by electron microscopy, were observed to be partially embedded by one end in ferritin-rich material of low electron density (Fig. 3A). The low-magnification electron micrograph (Fig. 3B) shows that ferritin particles were distributed in extracellular materials. These materials frequently appeared as an irregular patch associated with one end of the cell, analogous to the polar binding of fluorescein isothiocyanate-SBA described by Bohlool and Schmidt (8). The low-electron-density extracellular material which interacts with ferritin-SBA was previously described as EPS (20); the part of the cell with low electron density (arrowhead

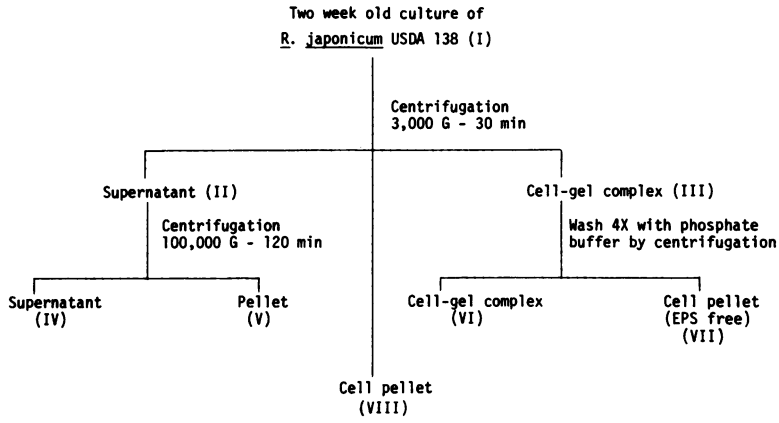

FIG. 2. *Fractionation scheme used to analyze the distribution of LBPS. (Fraction numbers are shown within parentheses.)*

TABLE 1. *Distribution of soybean LBPS in a 2-week-old culture of R. japonicum USDA 138*[a]

Fraction	Test result for LBPS[b]	
	Ouchterlony gel diffusion	Fluorescence microscopy[c]
I	+	+
II	+	NA
III	+	+
IV	−	NA
V	+	NA
VI	−	+
VII	−	−
VIII	−	−

[a] Fractionated as described in the legend to Fig. 2.
[b] +, LBPS present; −, LBPS absent; NA, not applicable.
[c] Cells were stained with fluorescein isothiocyanate-conjugated soybean lectin.

in Fig. 3B) was described as the reserve polymer end (20).

The EPS associated with ferritin is seen in thin-section preparations in Fig. 4A as fibrous materials, distributed both randomly about some cells and concentrated about one end of others. A portion of the EPS which did not react with ferritin-SBA is indicated by the arrowhead in Fig. 4A. This was probably due to the heterogeneity of the EPS since lectin-nonbinding polysaccharide was isolated from the Ouchterlony-diffusible EPS fraction (see below).

The specificity of interaction between *R. japonicum* EPS and ferritin-SBA was demonstrated by the following control experiments: (i) blocking with a specific sugar hapten of SBA (either *N*-acetyl-D-galactosamine or galactose); (ii) saturating binding sites with SBA before the addition of ferritin-SBA; and (iii) using a *Rhizobium* species other than *R. japonicum*. No ferritin was found to be associated with *R. leguminosarum* cells or cell-associated EPS by either direct microscopic examination or by thin sectioning. Figure 4b shows a control with galactose to block the interaction between ferritin-SBA and LBPS. EPS fibrils (arrowheads in Fig. 4B) were visible, but ferritin particles were absent.

Interaction between SBA and EPS was evident in electron microscope preparations of the cell-gel complex agglutinated with SBA. As shown in Fig. 5A, the EPS in such a preparation was stained densely with lead citrate and uranyl acetate. This was in contrast to the control, untreated with SBA, in which the EPS was stained only lightly (Fig. 5B). The intensive staining shown in Fig. 5A is typical of heavy metal interaction with protein. Subsequent addition of ferritin-SBA to preparations already agglutinated with SBA did not alter the staining from that observed in Fig. 5A, nor were ferritin particles found in association with EPS. In no case was there evidence of SBA in association with the outer membrane.

Further evidence that the extracellular SBA-binding material of *R. japonicum* is EPS, an acidic polysaccharide, was presented by a positive ruthenium red staining reaction. Figure 6A shows a ruthenium red-stained cell, with the stained polysaccharide material partially attached to the cell. In an electron micrograph of lower magnification (Fig. 6B), it is also clear that the ruthenium red-stained materials either partially attach to cells or surround them loosely. No staining was observed on the outer membranes.

Fractionation and characterization of diffusible LBPS. Semipurified EPS, obtained from desalted supernatant and concentrated by

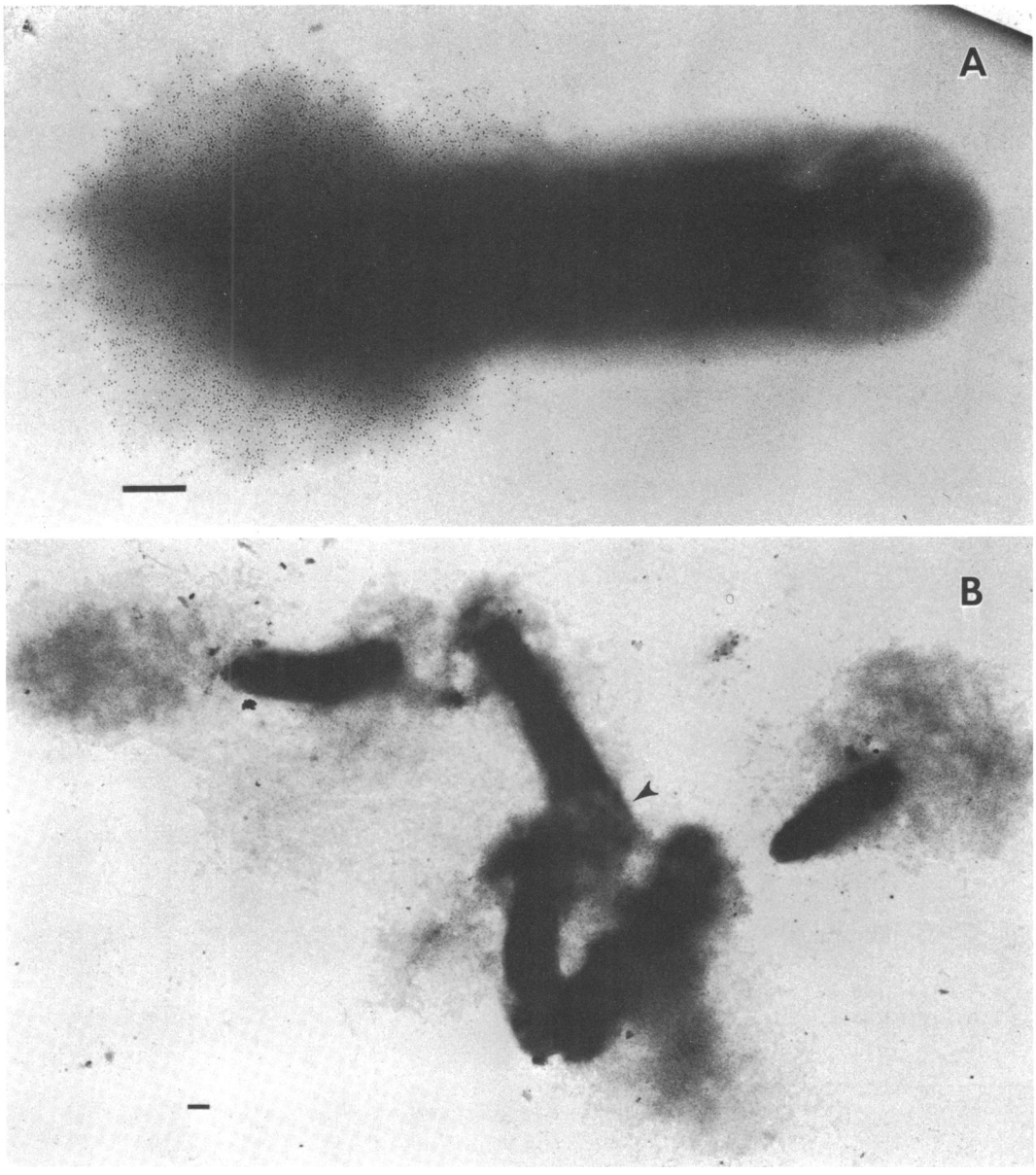

FIG. 3. *Direct examination of unstained cells from an exponential-phase culture of R. japonicum after interacting with ferritin-conjugated SBA. (A) At high magnification, ferritin particles were seen to be associated with amorphous materials of low electron density. No ferritin particle was found in direct association with the cell surface. (B) At low magnification, ferritin particles were bound exclusively to materials of low electron density. The arrowhead indicates the reserve polymer end of a rhizobial cell as described previously (20). Bars, 0.2 μm.*

ultrafiltration was shown by gel diffusion to have lectin-binding activity (Table 2). Ultrafiltrate composed of molecules of less than 300,000 daltons had no reactivity in gel diffusion after dialysis against distilled water, lyophilization, and resolubilization.

Fractionation by Sepharose 4B gel column gave two carbohydrate peaks (Fig. 7). The main peak, eluted immediately after the void volume, was excluded by the Sepharose 6B column and thus contained EPS of about 2×10^6 to 5×10^6 daltons. The second peak represented a low-

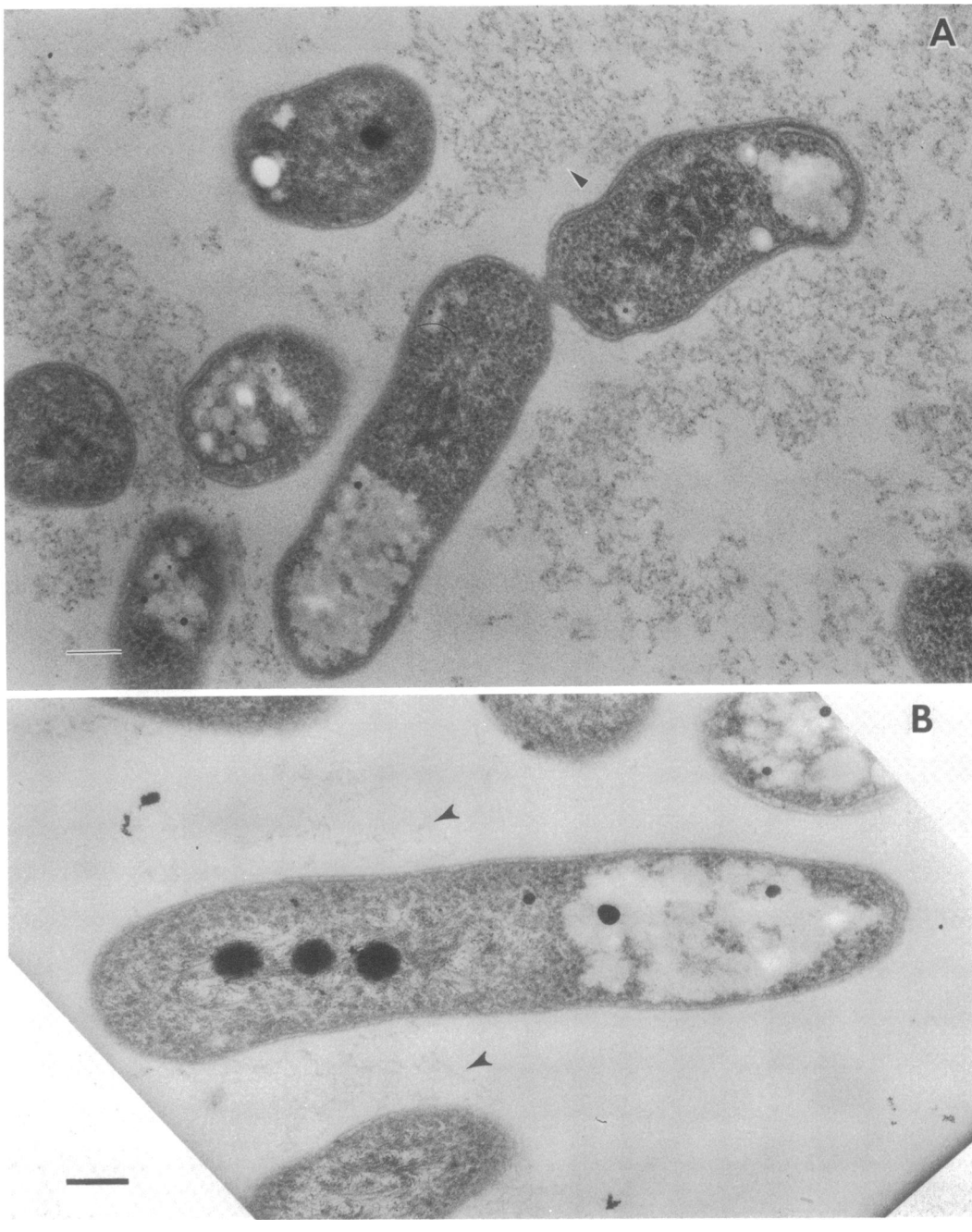

FIG. 4. *Thin-sectioned cells of R. japonicum labeled with ferritin-SBA. (A) Ferritin particles associated with EPS fibrils. A few fibrils (arrowhead) did not bind with ferritin-SBA, indicating the presence of a lectin-nonbinding polysaccharide. No ferritin was found in association with the outer membrane. (B) Lectin-binding inhibited control. Galactose solution (final concentration, 0.2 M) was added to the ferritin-SBA solution before incubating with the cell suspension. No ferritin particles were seen associated with EPS fibrils. EPS fibrils (arrowheads) in this micrograph were sparsely preserved due to loss of fibrils during preparations for embedding. Sections were not stained with heavy metals. Bars, 0.2 μm.*

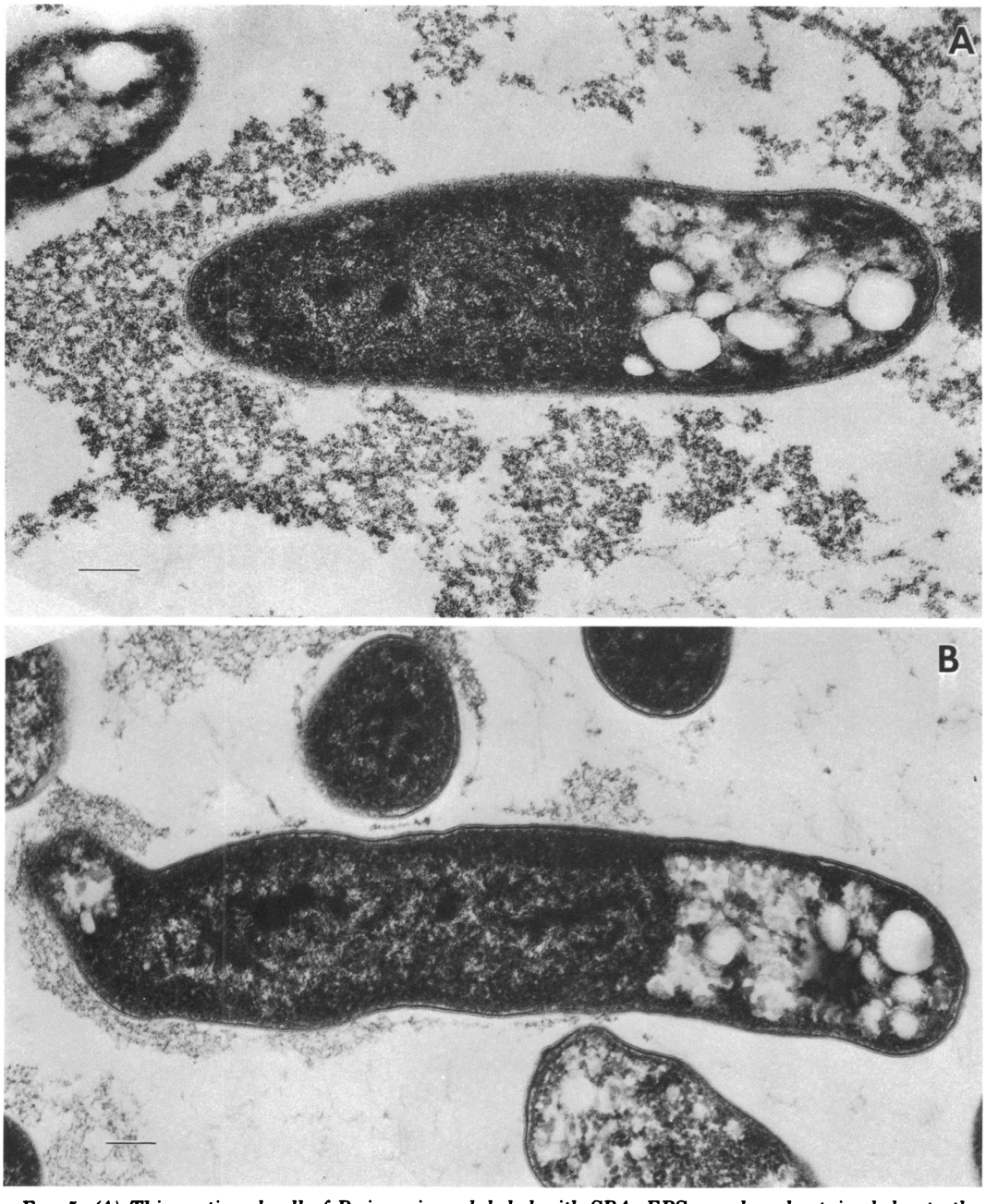

FIG. 5. *(A) Thin-sectioned cell of R. japonicum labeled with SBA. EPS are densely stained due to the association of SBA and EPS. (B) A control cell without SBA labeling. Sections were stained with lead citrate and uranyl acetate. Bars, 0.2 μm.*

molecular-weight polysaccharide of approximately 0.3×10^6 to 1×10^6 daltons. SBA-binding assays by gel diffusion indicated that the higher molecular weight fraction was responsible for interaction with SBA (Fig. 8). Fractions 32 and 58 from elution profile A of Fig. 7 (indicated by arrows) were concentrated to 250 μg/ml in phosphate buffer after dialysis and lyophilization. Only fraction 32 interacted positively with SBA to form a precipitin band.

Some biological and chemical properties of EPS fractions isolated by several chemical pro-

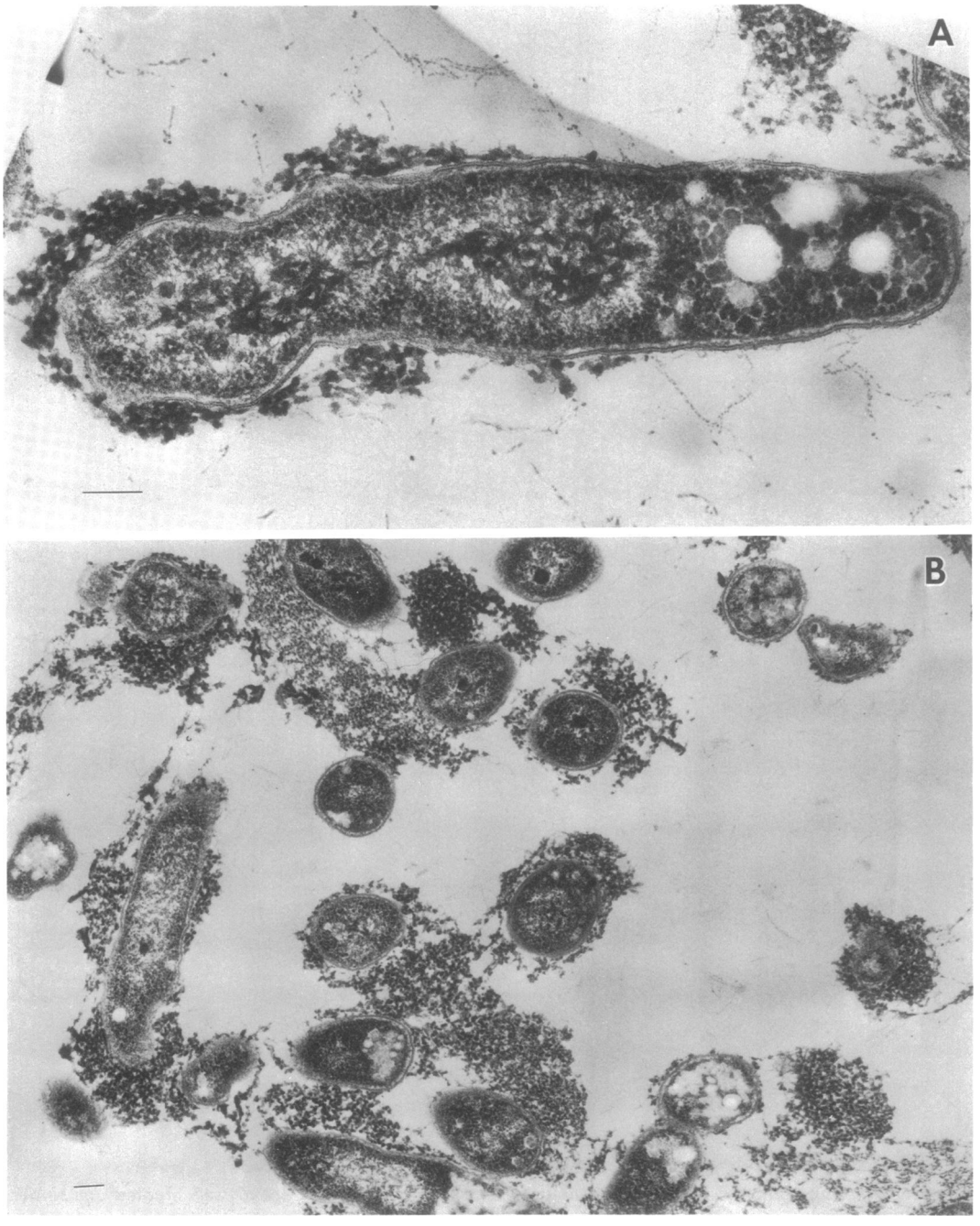

FIG. 6. *Thin-sectioned cells of R. japonicum stained with ruthenium red during fixation. (A) and (B) show cells in different magnifications. Dark ruthenium red-positive materials were deposited extracellularly either partially attached on the outer cell surface or loosely distributed around the cells. These materials have been demonstrated previously to be EPS (20). Sections were stained with lead citrate and uranyl acetate. Bar, 0.2 μm.*

TABLE 2. *Properties of R. japonicum EPS*

Method of isolation[a]	Lectin binding[b]	Antibody interaction[c]	Estimated mol wt	Carbohydrate[d]	KDO[e] (absorbance at 548 nm)
Semipurification[f]	+	−	>0.3 × 10^6	0.47	± (0.03)
Gel filtration[g]					
A	+	−	2 × 10^6–5 × 10^6	NA	−
B	−	−	0.3 × 10^6–1 × 10^6	NA	−
Acetone precipitation[h]	+	−	ND[i]	0.49	−
Cetavlon precipitation[j]	+	−	>0.3 × 10^6	0.44	−
Phenol extraction[k]					
Aqueous	+	+	ND	0.49	+ (0.11)
Phenol	−	+	ND	0.13	++ (0.23)

[a] See the text for details.
[b] Determined by Ouchterlony gel diffusion. +, Positive; −, negative.
[c] Anti-*R. japonicum* USDA 138 antibody was prepared with heat-killed cells, and interaction was determined by Ouchterlony gel diffusion. +, Positive; −, negative.
[d] Milligrams of glucose equivalent per milligram of lyophilized polysaccharide as determined by anthrone reaction. NA, Not applicable.
[e] KDO, 2-Keto-3-deoxyoctonate; determined by the thiobarbituric acid test (+, positive; −, negative).
[f] Purified from low-speed-centrifuged (3,000 × *g*, 30 min) culture supernatant by an Amicon Diaflo membrane ultrafiltration system (XM300 membrane).
[g] Fractionated by passing semipurified EPS through a Sepharose 4B column (2.6 by 32 cm). (A) Fractions 22 to 40; (B) fractions 50 to 70 (see Fig. 7).
[h] Acetone-precipitated EPS from low-speed-centrifuged culture supernatant.
[i] ND, Not determined.
[j] Cetavlon (cetyltrimethylammonium bromide)-precipitated fraction from a semipurified EPS preparation.
[k] Extracted from the cell-gel complex.

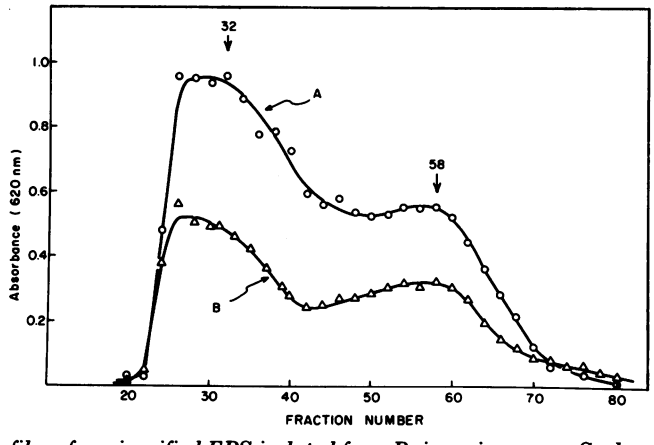

FIG. 7. *Elution profiles of semipurified EPS isolated from R. japonicum on a Sepharose 4B gel bed (2.6 by 32 cm). A 2-ml amount of EPS solution (10 mg/ml of water) (curve A) was layered on the top of gel and eluted with 10 mM phosphate buffer at the rate of 1 drop every 20 to 30 s. Forty drops (2.15 ml) of fraction were collected. Fractions were assayed for carbohydrate by using the anthrone colorimetric method. Absorbance at 620 nm was plotted against the fraction number. In curve B, 1 ml of EPS solution was applied to the column. Slightly better separation was obtained.*

cedures are shown in Table 2. Lectin-binding fractions included EPS precipitated from fraction II by acetone, acidic polysaccharide obtained from semipurified EPS by Cetavlon precipitation, and polysaccharide present in the aqueous phase after hot phenol extraction of the cell-gel complex (fraction VI). All lectin-binding fractions except the phenol extracts were free of lipopolysaccharide as indicated by 2-keto-3-deoxyoctonate analysis and reactivity to homol-

ogous antibody prepared against somatic antigens. Binding of SBA by *R. japonicum* was clearly carried out by a polysaccharide component (LBPS) of the EPS and not by lipopolysaccharide of the outer membrane.

The possibility of SBA binding by DNA, RNA, or protein was tested by treating EPS preparations with DNase, RNase, and pronase, followed by assaying for SBA-binding activity with the hemagglutination inhibition assay and Ouchterlony gel diffusion. All EPS preparations so treated reacted positively with SBA, indicating that DNA, RNA, and protein had no effect on SBA binding.

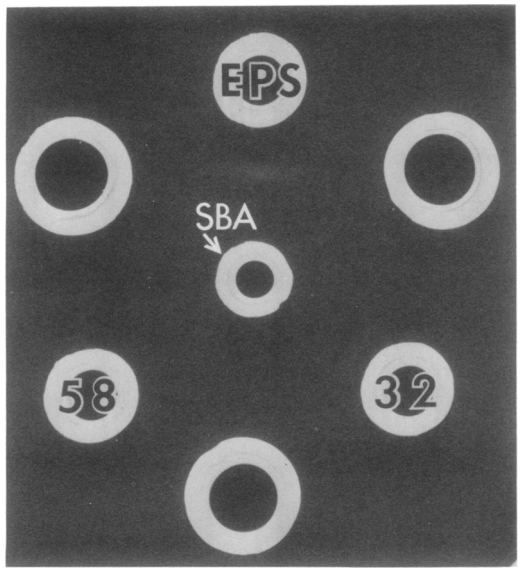

FIG. 8. *Ouchterlony gel diffusion pattern of SBA and the fractions 32 and 58 shown in Fig. 7 (indicated by arrows). Fractions 32 and 58 were concentrated to 250 μg/ml by dialysis, lyophilized, and resolubilized in 50 mM phosphate buffer. In each well, 0.2 ml of the samples was present. The top well contained a semipurified EPS solution as a control. The center well contained 0.1 ml of SBA (2 mg/ml).*

LBPS isolated by gel chromatography was examined by electron microscopy after shadow casting with platinum-carbon (Fig. 9). Homogeneous filamentous structures of various thickness were observed. This is in agreement with observations made by others on the capsular polysaccharides of *Escherichia coli* similarly prepared (3).

DISCUSSION

The several lines of evidence presented here in the isolation and partial characterization of the lectin-binding material synthesized by *R. japonicum* clearly identify that material as extracellular polysaccharide and not lipopolysaccharide. These data are in support of earlier morphological and gel diffusion evidence (5, 7–9, 11, 20) which demonstrated that SBA was associated with extracellular material and not with the outer membrane of the cells. The morphological data of Bal et al. (2) and Shantharam et al. (16) are difficult to interpret in view of the remarkably short generation time (±2 h) reported for their *R. japonicum* cultures. *R. japonicum* strains are included among the slow-growing rhizobia, which have generation times of 6 to 8 h (23). The only work in favor of the original observation of Wolpert and Albersheim (26) that *R. japonicum* lipopolysaccharide was responsible for lectin binding was reported recently by Kato et al. (12). Kato et al. used chemical approaches generally similar to those that we followed, but they found 2-keto-3-deoxyoctonate in lectin-binding fractions. We found all of the lectin-binding activity in purified EPS fractions which were devoid of 2-keto-3-deoxyoctonate. Moreover, we observed that the lectin-binding material under the electron microscope was physically separate from the outer membrane of the cell.

LBPS was found in two forms in the EPS: (i) as a very high-molecular-weight polysaccharide component of a viscous cell-gel complex not

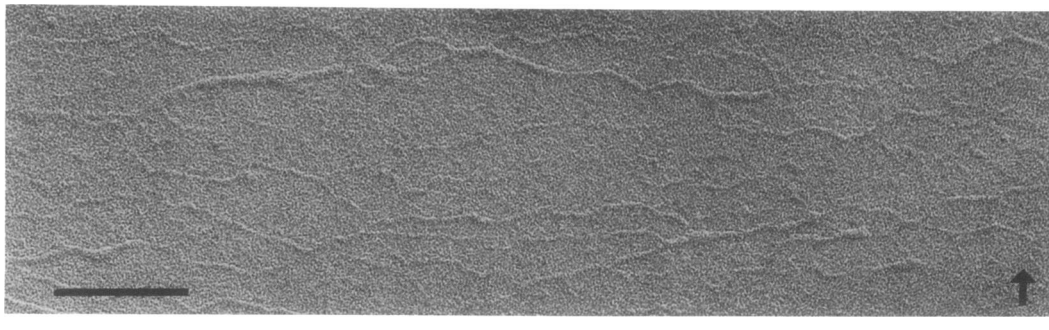

FIG. 9. *A shadow-cast EPS preparation showing filamentous structures of various thicknesses. The arrow indicates the direction of shadowing. Bar, 0.2 μm.*

diffusible in 1% agar gel, and (ii) as lower-molecular-weight polysaccharide (2×10^6 to 5×10^6 daltons) dissociated from the cells and detectable in cell-free supernatant by gel diffusion techniques. The two forms of LBPS are presumed to differ only in their degree of polymerization, since both forms are acidic, and lectin binding by both is blocked by either *N*-acetylgalactosamine or galactose.

Diffusible LBPS in the supernatant was amenable to separation and characterization as summarized in Table 2. It is noteworthy that this is the same lectin-binding material studied recently (21) and found to be produced throughout the growth cycle of *R. japonicum*. LBPS, as observed here, whether in diffusible or highly polymerized form, is not "transient" as reported for *R. trifolii* (10) or restricted to certain stages of growth of *R. japonicum* (5), but synthesized in synchrony with cell division as with synthesis of microbial EPS in general (18, 21). The lectin-binding inhibitor observed by Bhuvaneswari et al. (5) was probably 2×10^6- to 5×10^6-dalton LBPS.

The high-molecular-weight LBPS could not be separated out of the cell-gel complex; therefore, its study was restricted to physical means. Electron micrographs revealed that the LBPS in the cell-gel complex partially surrounded the cells, frequently with a highly polar distribution (Fig. 3). Such localization is consistent with the polar binding of fluorescein isothiocyanate-SBA previously observed (8). Although the very high-molecular-weight LBPS appears to occur as capsular EPS, this partial capsule is not tightly associated with the cell. At least under shaken liquid-culture conditions, it dissociates from some cells in the cell-gel complex (Fig. 3), and cells centrifuged or washed out of the complex no longer bind lectin. The EPS of *R. japonicum* is perhaps better described as a slime than a true capsule.

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