Re-examination of Capsule Development and Lectin-binding Sites on *Rhizobium japonicum* 3I1B110 by the Glutaraldehyde/Ruthenium Red/Uranyl Acetate Staining Method

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Capsular development and soybean lectin binding properties of *Rhizobium japonicum* 3I1B110 were examined by the glutaraldehyde/ruthenium red/uranyl acetate method permitting observation of whole bacteria and their polar capsules by transmission electron microscopy. Ultrastructurally, the polar capsule consisted of (i) an electron-dense aggregated material in close contact with the cell surface which stained with ruthenium red and intensively bound soybean lectin, and (ii) a less electron-dense fibrillar material at the periphery of the capsule which stained with ruthenium red but did not bind soybean lectin. The latter material interconnected with the aggregated capsular material at the cell surface. As the culture advanced into stationary phase, these acidic capsular materials were shed into the culture medium where they kept their respective lectin-binding activity. The cells correspondingly decreased in their lectin-binding activity and encapsulation with culture age. However, the remaining encapsulated cells in late stationary phase were still able to bind the lectin in a hapten-specific way.

INTRODUCTION

According to the lectin-recognition hypothesis (Bohlool & Schmidt, 1974), the specificity in a *Rhizobium*-legume symbiosis involves the interaction between host lectins and bacterial polysaccharides. Soybean lectin (SBL) binds specifically to the majority of *R. japonicum* strains grown in broth (Bal *et al.*, 1978; Bhuvaneswari & Bauer, 1978; Bhuvaneswari *et al.*, 1977; Bohlool & Schmidt, 1974; Kamberger, 1979; Shantharam *et al.*, 1980; Stacey *et al.*, 1980; Tsien & Schmidt, 1981), but also binds to several heterologous strains of the cowpea miscellany group (Bhuvaneswari & Bauer, 1978; Brethauer & Paxton, 1977; Pueppke *et al.*, 1980). Transmission and scanning electron microscopy suggested that *R. japonicum* interacted specifically with root hairs of its soybean host through SBL-binding surface polysaccharides (Bal *et al.*, 1978; Stacey *et al.*, 1980). Polysaccharide receptors for SBL have been described as O-antigen-containing lipopolysaccharides (Wolpert & Albersheim, 1976), capsular polysaccharides (Bal *et al.*, 1978; Mort & Bauer, 1980; Tsien & Schmidt, 1980, 1981), and exocellular diffusible material (Bhuvaneswari *et al.*, 1977; Brethauer & Paxton, 1977; Kamberger, 1979; Mort & Bauer, 1980, 1982; Pueppke *et al.*, 1980; Tsien & Schmidt, 1980, 1981). The degree of binding of SBL to *R. japonicum* also varied among strains (Bhuvaneswari & Bauer, 1978; Bhuvaneswari *et al.*, 1977; Bohlool & Schmidt, 1974; Calvert *et al.*, 1978), and cells of some strains bound SBL only when cultured in

Abbreviations: G/RR/UA, glutaraldehyde/ruthenium red/uranyl acetate; PBS, phosphate buffered saline containing 10 mM-KH₂PO₄/K₂HPO₄, 145 mM-NaCl, 1 mM-MgSO₄, pH 7.0; SBL, soybean lectin; TEM, transmission electron microscopy.

the root environment (Bhuvaneswari & Bauer, 1978) or during certain phases of growth (Bhuvaneswari *et al.*, 1977; Mort & Bauer, 1980). For two reference strains of *R. japonicum* (311B110 and 311B138), the growth phase-dependent variations have been attributed to changes in the composition of capsular and extracellular polysaccharides and to the loss of encapsulation during batch culture (Mort & Bauer, 1980). Other studies of *R. japonicum* 311B138 growing in broth reported that SBL-binding material was not restricted to certain stages of growth, but rather was synthesized in synchrony with cell division throughout the growth cycle (Tsien & Schmidt, 1980, 1981).

In the studies which examined the binding of SBL to broth-grown *R. japonicum* by electron microscopy (Bal *et al.*, 1978; Calvert *et al.*, 1978; Shantharam *et al.*, 1980; Stacey *et al.*, 1980; Tsien & Schmidt, 1981), the cells were subjected to centrifugation, which may have introduced artifacts by shearing the capsular polysaccharides. Therefore, we have re-examined the development and lectin-binding properties of the capsule of *R. japonicum* 311B110 using a new method of specimen preparation for transmission electron microscopy (TEM) (Mutaftschiev *et al.*, 1982). Using this method, cells were not centrifuged or dehydrated. In this way, the development of capsules and lectin-binding sites could be more accurately assessed. A preliminary report of this work was presented at the 82nd Annual Meeting of the American Society for Microbiology (Truchet *et al.*, 1982).

METHODS

Bacterial culture. *Rhizobium japonicum* 311B110, from Dr D. Weber, USDA, Beltsville, Md., USA, was grown aerobically in shaken cultures of YMS broth (Tsien & Schmidt, 1980) at 30 °C. Cells in mid-exponential phase were used as the inoculum (1 ml of a suspension of OD₆₂₀ = 1.0 per 100 ml broth). Culture growth was measured by optical density at 620 nm through a 1 cm cuvette. When necessary, samples were diluted with sterile broth to less than 0.3 OD unit.

Lectins. SBL isolated by affinity chromatography from *Glycine max* (L.) Merr. cv. Portage seeds was a gift of S. G. Pueppke, University of Florida, USA. In addition, SBL and *Lens* lentil lectin were purchased from Miles Laboratories, Elkhart, Ind., USA.

Conjugation of SBL to colloidal gold. Colloidal gold was prepared according to Frens (1973) from an aqueous solution of trichloroauric acid (Polysciences Inc., Warrington, Pa., USA). The average diameter of the gold particles was 16 nm. The lowest concentration of SBL required to stabilize the colloidal gold was determined visually (Horisberger *et al.*, 1975). The stabilized gold colloid was free of aggregates as judged by TEM (figure not shown). SBL was conjugated with colloidal gold (Horisberger *et al.*, 1975) except that the SBL and freshly prepared colloidal gold suspension were mixed for 15 min with constant stirring before centrifugation for 45 min at 30000 g. The pellet from 10 ml of the conjugated colloid was resuspended gently on ice in 2 ml 1% (w/v) polyethylene glycol (av. mol. wt 20000) in PBS (pH 7.0), preserved with sodium azide (0.02%, w/v final concentration), and stored at 4 °C.

TEM of whole bacteria. To localize exocellular acidic polysaccharides, cells in broth culture were examined directly by the glutaraldehyde/ruthenium red/uranyl acetate (G/RR/UA) method (Mutaftschiev *et al.*, 1982). Briefly, this involved deposition of 3 µl of an uncentrifuged bacterial suspension onto a carbon-coated Formvar grid, adsorption of the cells for 3 min, and subsequent removal of the free-standing suspension by blotting with filter paper. Adsorbed cells were stained with a mixture of glutaraldehyde and ruthenium red, and then contrasted with uranyl acetate. In some cases, 3 µl broth culture was deposited on grids and evaporated at room temperature before staining. Grids were then examined by TEM with a Hitachi H600 electron microscope at 75 kV.

To localize anionic sites by cationized ferritin, the labelling procedure described by Schrevel *et al.* (1981) was adapted to study whole bacteria. Equal volumes of bacterial broth culture and 0.5% (v/v) glutaraldehyde in 0.2 M-sodium cacodylate buffer (pH 7.2) were mixed. Two µl of this mixed solution was deposited on a Formvar-coated grid and gently dried under a stream of sterile air. The dried grid was floated on a drop of sterile cationized ferritin (85 µg ml⁻¹) in sodium veronal buffer (0.1 M, pH 7.2). After incubation for 1 min, the remaining solution was blotted with filter paper. Non-specific binding of cationized ferritin was removed by five washes of Veronal buffer for 10 min each without blotting between each wash. After the last rinse, the grid was blotted with filter paper and completely dried under sterile air. Controls were incubated with a sterile solution of native ferritin (Sigma) under the same conditions.

To localize SBL-binding sites, bacteria adsorbed to grids were treated under sterile conditions at room temperature with SBL-colloidal gold for 10 min in a humid chamber. After blotting off the excess SBL-gold, the adsorbed cells were washed with PBS for 10 min and finally stained by the G/RR/UA method. Other grids were incubated with SBL-gold and then washed three times with PBS without staining by G/RR/UA. For controls,

bacteria on grids were incubated with the same stock of unconjugated colloidal gold or with a lentil lectin-gold complex prepared as described above. Cells were then stained by the G/RR/UA method.

To study hapten reversibility of lectin binding, cells on grids which had been treated with lectin-gold complexes were incubated for 3 min in humid air at room temperature with 10, 25 and 50 mM solutions of either *N*-acetyl-D-galactosamine (Lis *et al.*, 1970), *N*-acetyl-D-glucosamine or 2-deoxy-D-glucose. Afterwards, cells were stained by the G/RR/UA method.

To examine bacterial agglutination, 5 µl of a broth culture were incubated with 5 µl SBL-gold in sterile conditions at room temperature. After 15 min, 2 µl of the mixture were deposited on Formvar-coated grids, blotted, and the cells stained by the G/RR/UA method. Controls included bacteria treated with PBS or uncoupled colloidal gold in place of SBL-gold, followed by staining by the G/RR/UA method.

RESULTS

The morphology and size of the capsule on *R. japonicum* 311B110 changed gradually with culture age. In early exponential phase, the capsule began to develop at one cell pole as a very compact structure which became electron-dense following ruthenium red staining (Fig. 1*a, b*). At mid-exponential phase, the capsule was a well-developed structure and extended as far as 3 µm from the cell wall (Fig. 1*c, d*). In late exponential and early stationary phases, the capsule had less-defined borders and dispersed into the culture medium, showing a typical 'radiating' appearance on the Formvar surface (Fig. 1*e, f*). The shedding of polymers in stationary phase was accompanied by a progressive decrease of capsule size. However, capsules were still detected at one pole of some cells after 16 d incubation. At all growth phases, the capsule was exclusively located at one cell pole regardless of how the cells were treated on the grids, i.e. after blotting with filter paper or drying with a gentle stream of air. This result indicated that blotting the specimen on the grid did not affect the distribution of capsular material associated with the cells.

In early exponential phase, the capsule was composed of aggregated, electron-dense and ruthenium red-positive material in close contact with the cell surface (Fig. 1*b*). In older cultures, this aggregated material extended further out from the pole and was interconnected with an ultrastructurally different fibrillar material which also reacted with ruthenium red (Fig. 1*d*). The acidic nature of these two polymers, as suggested by their affinity for ruthenium red (Fig. 1*b-f*), was confirmed at all growth phases by the extensive binding of cationic ferritin to the polar capsule (Fig. 2*a, b*).

During early exponential phase, the uncentrifuged cell-free culture medium was typically devoid of electron-dense material (Fig. 1*a*). Beginning in mid-exponential phase, extracellular amorphous aggregates and loose fibrils were found concurrently with the dispersion of the capsule (Fig. 1*e, f*). These extracellular materials resembled the polymers of the capsule and were particularly abundant in the medium of stationary phase cultures.

The percentage of polarly encapsulated cells changed with culture age (Fig. 3). The highest percentage (68%) of encapsulated cells as exemplified in Fig. 1 (*b, d*) occurred at day 3 in an exponential phase which lasted from day 2 to day 5. Later, this percentage declined to 20% at day 12 in late stationary phase (Fig. 3).

Low-speed centrifugation (3000 *g* for 30 min) separated broth cultures into a hard pellet, an overlying soft pellet and a clear supernatant. The volume of these three fractions changed with culture age. Examination of these fractions by the G/RR/UA method revealed that the hard pellet contained predominantly non-encapsulated cells (Fig. 4*a*), the soft pellet contained predominantly polarly encapsulated cells (Fig. 4*b*), and the supernatant contained both capsular polymers which were more abundant in older cultures (Fig. 4*c*).

The polar aggregated material on uncentrifuged cells in early exponential phase intensively bound SBL-colloidal gold (Fig. 5*a*). Later, in mid- and late exponential phases, SBL receptors exclusively matched the location of the enlarged polar capsule (Fig. 5*b*). However, the SBL-colloidal gold did not bind to the naked cell surface, the flagellum, or the fibrillar material of the outer capsule, regardless of the growth phase (Fig. 5*a-c*).

As the percentage of encapsulation decreased with culture age (Fig. 3), there was a progressive increase of SBL-binding material in the surrounding medium, indicating that shedding had

Fig. 1. *Rhizobium japonicum* 311B110 stained by G/RR/UA showing the progressive steps of encapsulation. (a, b) Early exponential phase: (a) the beginning of the capsule; (b) the nascent capsule (arrows) is seen as an aggregated material in close contact with one pole of the cell. (c, d) Mid-exponential phase: (c) well-developed capsule; (d) detail of a polar capsule showing the aggregated (single arrows) and the fibrillar (double arrows) components. (e, f) Late stationary phase: (e) accumulation of material in the medium concurrent with the shedding of the capsule; (f) both the aggregated (single arrows) and the fibrillar (double arrows) components of the capsule were shed into the medium. In all these and subsequent micrographs, bar markers represent 1 μ m.

occurred (Fig. 5c, d). Consistent with the above results, the cell-free SBL receptors were associated with the aggregated rather than the fibrillar type polymer (Fig. 5e).

Fig. 2. Localization of anionic sites by cationic ferritin. (a) Early exponential phase: cationic ferritin is densely accumulated at one pole of the cell. (b) Mid-exponential phase: the distribution of ferritin particles matches the spatial distribution of the capsule. The dense deposition of ferritin probably corresponds to the aggregated capsular component (arrows).

Fig. 3. Lectin-binding activity and encapsulation of *R. japonicum* 3I1B110 during the first 12 d of growth in YMS broth. ■, Growth curve (OD₆₂₀); ○, percentage of lectin-binding cells. At each day of growth, the total percentage of encapsulated cells included both the percentage of cells with a well-developed (▲) and nascent (△) capsule.

Fig. 4. Different centrifuged fractions of a 6-day-old culture stained with G/RR/UA. (a) Hard pellet containing the majority of unencapsulated cells; (b) soft pellet enriched with polarly encapsulated cells; (c) supernatant containing both aggregated (single arrows) and fibrillar (double arrows) components.

Several controls established the biochemical specificity of the interaction of SBL-colloidal gold with the cell-bound and cell-free aggregated polymer. *N*-Acetyl-D-galactosamine, a specific hapten of SBL, reversed the interaction at a concentration of 10 mM (Fig. 6*a, b*). No hapten reversal was obtained with the non-inhibitory analogues *N*-acetyl-D-glucosamine or 2-deoxy-D-glucose at even 50 mM (Fig. 6*c*). Uncoupled colloidal gold or lentil lectin-gold complex did not bind to the bacteria or extracellular materials (figures not shown). Cells incubated with SBL-colloidal gold without further treatment had lectin bound predominantly to one pole, presumably where the aggregated polymers of the capsule were located (Fig. 7*a, b*). The few SBL-gold particles on the opposite pole of the cell (Fig. 7*a*) were easily removed by subsequent washing with PBS or treatment by the G/RR/UA method, indicating that they were not bound to the cell surface in a biochemically specific manner. This control also indicated that treatment of cells by the G/RR/UA method did not redistribute the SBL-binding receptors on the capsule. The same treatment showed that the cell-free aggregated polymer, but not the cell-free fibrillar polymer, was able to bind the SBL-gold complex (Fig. 7*c*).

The percentage of cells which bound SBL was maximal at day 3 and then progressively decreased during growth (Fig. 3). At each growth phase, the percentage of SBL-binding cells changed in parallel with the percentage of encapsulated cells (Fig. 3). Encapsulated cells in late stationary phase (up to day 16) were still able to bind SBL in a hapten-reversible way (Fig. 8).

Consistent with previous studies (Truchet *et al.*, 1983), the G/RR/UA method revealed that *R. japonicum* could be agglutinated by SBL-gold (Fig. 9). Bacteria incubated with PBS or uncoupled colloidal gold did not agglutinate (figures not shown).

DISCUSSION

The G/RR/UA method has been applied here to study the capsule development and the lectin-binding properties of *R. japonicum* 3I1B110. The capsule of this slow-growing strain developed at one pole and remained exclusively at this cellular location regardless of the phase of growth. Ultrastructurally, it consisted of two materials which stained with ruthenium red and bound cationic ferritin, but differed in electron density, morphology, location within the capsule, and reactivity with soybean lectin. The electron-dense aggregated material bound SBL

Fig. 5. Treatment of cells with SBL-colloidal gold followed by staining with G/RR/UA. SBL-colloidal gold bound to the capsule of bacteria collected in early (a), mid- (b) and late (c, d) exponential phase. SBL-colloidal gold bound to the aggregated materials (arrows) of well-developed (b) or diffusing (c, d) capsules, but not to the naked cell surface (a-d) or to the flagellum (a, fl.). (e) Extracellular material of a 6-day-old culture (early stationary phase). SBL-colloidal gold was exclusively bound to the aggregated component (single arrows). The double arrows indicate the fibrillar component which did not bind SBL-gold.

in a biochemically specific manner and represented the capsular material retained at the 'cytoplasmic and nuclear pole' of thin-sectioned and freeze-etched bacteria (Calvert *et al.*, 1978; Tsien & Schmidt, 1977), and the 'very high molecular weight form' of the capsule which was isolated by ultracentrifugation at 100000 *g* (Tsien & Schmidt, 1981). In contrast, the fibrillar material which did not bind SBL-gold was probably lost during specimen preparation in other studies as suspected (Tsien & Schmidt, 1981). Both of these capsular polymers were shed into the culture medium as the culture aged and retained their differential hapten-specific reactivity with SBL. A relationship between the capsular polysaccharide and exocellular acidic polysaccharides was also suggested by their chemical similarity (Mort & Bauer, 1980, 1982), although exopolysaccharides tended to be more polydisperse in molecular weight (Tsien & Schmidt, 1981; I. Sutherland, personal communication).

The polar capsule as described here seemed to be a general feature of most slow-growing rhizobia which develop capsules (Vasse, 1982; J. M. Vasse & G. L. Truchet, unpublished observations). The appearance of a capsule completely surrounding the *R. japonicum* cell in thin

Fig. 6. Reversion of the SBL-colloidal gold binding to the capsular (a) or the extracellular (b) materials with 10 mM-*N*-acetyl-D-galactosamine. No reversion was observed with 50 mM-*N*-acetyl-D-glucosamine (c).

Fig. 7. Treatment by SBL-colloidal gold without subsequent washings (a) or followed by washings with uranyl acetate (b) or PBS (c). (a) Cell in mid-exponential phase. The distribution of lectin receptors, particularly concentrated in certain places of the capsule (arrows), probably corresponds to the spatial distribution of the aggregated component. The few SBL-gold particles at the opposite pole could easily be removed by subsequent washings. (b) Cell in early exponential phase. SBL-colloidal gold intensively bound to the nascent polar capsule but not to the flagellum (arrow) or to the cell itself. (c) SBL-colloidal gold bound to the extracellular aggregated material (arrows) of cells in early stationary phase.

Fig. 8

Fig. 9

Fig. 8. Treatment of cells in late stationary phase (16-day-old culture) with SBL-colloidal gold followed by staining with G/RR/UA. SBL-gold bound to the capsule (single arrows) and to the extracellular material (double arrows).

Fig. 9. SBL-colloidal gold mediated agglutination of a 4-day-old culture.

sections (Bal *et al.*, 1978; Calvert *et al.*, 1978; Shantharam *et al.*, 1980) or in negative stains using India ink (Mort & Bauer, 1980) may be due to the orientation of the thin-section which did not encompass the entire length of the cell or to a redistribution of capsular materials during centrifugation or other steps of specimen preparation. This hypothesis was supported by the observation that cells from 9-day-old uncentrifuged cultures had polar capsules when examined by the G/RR/UA method but were unencapsulated when processed for thin-sectioning (figure not shown).

Our results are in general agreement with other studies showing that SBL receptors were located on the capsule and not on unencapsulated cell surfaces of *R. japonicum* growing in broth culture (Bal *et al.*, 1978; Calvert *et al.*, 1978; Mort & Bauer, 1980; Shantharam *et al.*, 1980; Stacey *et al.*, 1980; Tsien & Schmidt, 1981). In addition, the results confirmed that the percentage of cell encapsulation and SBL-binding ability of broth cultures reached their maxima in mid-exponential phase and both declined thereafter. Thus, the overall agreement of our results with previous studies attested to the validity of this new method. Its main advantage in comparison with others is that it optimizes the relationship of encapsulation and binding of lectin with a minimum of loss due to specimen handling. This was illustrated by the failure of India ink exclusion techniques (Mort & Bauer, 1980) and fluorescence microscopy (Bhuvaneswari *et al.*, 1977; Mort & Bauer, 1980; Truchet *et al.*, 1983) to detect encapsulated cells of *R. japonicum* 311B110 in late stationary phase which were still able to bind SBL specifically. This positive result obtained by the G/RR/UA method was not an artifact created by deposition of extracellular material on the grid since its location was exclusively polar rather than random on cells. The complication of accumulated extracellular material obscuring the

observations in older cultures was minimized by preventing the grids from drying until the entire sequence of steps of the G/RR/UA method was completed.

The progressive increase of lectin-binding material in the culture medium concurrent with the capsule dispersion explains why the culture as a whole retained its SBL-binding activity (Tsien & Schmidt, 1980). The methylation of galactose residues has been shown to result in a decrease in affinity of SBL for the bacterial polymer (Mort & Bauer, 1980). However, we were unable to detect a change in affinity of SBL-gold for the aggregated capsular material on cells or in the culture medium at all growth phases based on a differential inhibition by *N*-acetyl-D-galactosamine within the concentration range of 10–50 mM.

Schmidt & Bohlool (1981) suggested that different methods used in different laboratories could account for contradictory results and proposed the use of common procedures among workers. Because the G/RR/UA method is simple and allows the observation of entire bacteria by TEM, it could be of value to study mutant strains of rhizobia (Law *et al.*, 1982; Rolfe *et al.*, 1981) and clarify the morphological basis of recognition events mediated by lectin-poly-saccharide interactions in this nitrogen-fixing symbiosis.

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