Development and Trifoliin A-Binding Ability of the Capsule of *Rhizobium trifolii*†

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The age-dependent lectin-binding ability of *Rhizobium trifolii* 0403 capsular polysaccharide (CPS) was examined by following the development of the capsule and its ability to interact with the white clover lectin trifoliin A. Bacteria grown on agar plates for 3, 5, 7, 14, and 21 days were examined by electron microscopy and immunofluorescence microscopy with antibodies prepared against either *R. trifolii* 0403 CPS or trifoliin A after pretreatment with the lectin. The capsule began to develop at one pole by day 3 and completely surrounded the cells in cultures incubated for 5 days or longer. The capsular polysaccharide on cells cultured for 3 and 5 days was completely reactive with trifoliin A, became noticeably less reactive by day 7, and was only reactive with the lectin at one pole of a few cells after that time. The quantity and location of lectin receptors on bacteria of different ages directly correlated with their attachment in short-term clover root hair-binding studies. Cells from 3- or 21-day-old cultures attached almost exclusively in a polar fashion, whereas cells grown for 5 days attached to root hairs randomly and in the highest numbers. CPS isolated from a 5-day-old culture had higher lectin-binding ability than CPS from 3- and 7-day-old cultures, whereas the CPS from a 14-day-old culture had the lowest. Chemical analyses of the isolated CPS showed changes in the levels of uronic acids (as glucuronic acid), pyruvate, and O-acetyl substitutions with culture age, but the neutral sugar composition remained relatively constant. These results provide evidence that the age-dependent distribution of lectin receptors dictates the level and orientation of attachment of *R. trifolii* 0403 to clover root hairs.

The capsular polysaccharide (CPS) surrounding bacteria is important for various cellular functions in nature, including adhesion to solid surfaces, pathogenicity, and protection from predation, desiccation, and other environmental stresses (9, 19). The development of bacterial capsules has been examined microscopically with temperature-sensitive mutants of *Escherichia coli* (2) and *Haemophilus influenzae* transformants of mutants lacking capsules (7). *E. coli* appears to have 10 to 40 random sites of capsular excretion around the cell. The majority of newly encapsulated *H. influenzae* cells show thin capsules completely surrounding the cells, although some capsules appear to develop polarly. Bacteria of the genus *Rhizobium*, which form a nitrogen-fixing symbiosis with leguminous plants, differ in terms of encapsulation (18). Slow-growing *R. japonicum* strains typically exhibit a polar capsule (29; J. Vasse et al., submitted for publication), whereas *R. trifolii*, *R. leguminosarum*, and *R. phaseoli* form a complete capsule (15, 18). *R. meliloti* cells typically lack a capsule (18, 24), although polar fibrils can generally be seen (24). The percentage of encapsulated cells of *R. trifolii* and the amount of polysaccharide synthesized vary with the strain and cultural conditions (18). The development of the rhizobial capsule is relevant to the symbiosis due to the specific interaction between the CPS and lectins synthesized by the legume host (4, 6, 13). It has been hypothesized that the specific attachment of rhizobia to host root hairs, an early event in the infection process, results from the interaction between the CPS and lectin on the host root (13, 22, 25, 27).

A phenomenon which has been noted for several *Rhizobium* species is the age-dependent nature of lectin-binding. *R.*

japonicum 311b138 loses its ability to interact with the soybean lectin after mid-exponential growth, apparently due to the methylation of galactosyl residues in the polysaccharide and a reduction in encapsulation (23). Transient lectin-binding ability also occurs with certain strains of *R. leguminosarum*, which interact with the pea lectin optimally when the bacteria are in early-exponential phase (30). *R. trifolii* 0403, grown either in broth or on agar plates, shows a transient ability to bind the clover lectin, trifoliin A (15, 21). The lipopolysaccharide (LPS) of *R. trifolii* 0403 grown in broth culture has growth phase-dependent increases in the level of quinovosamine, which occur at the same time (early stationary phase) that the LPS becomes optimally reactive with trifoliin A (21). Although it has been shown that an acidic polysaccharide from the capsule of *R. trifolii* 0403 binds to trifoliin A (11), the transient ability of the isolated CPS to bind trifoliin A was not examined. Cadmus et al. (5) found age-dependent variations in the levels of pyruvate and O-acetyl substitutions in several fast-growing *Rhizobium* species, including *R. trifolii*, although lectin binding was not investigated.

To characterize the age-dependent nature of the binding of the capsule of *R. trifolii* to trifoliin A, we examined cells of *R. trifolii* 0403 for (i) the development of the capsule, (ii) the ability of the capsule at various stages of development to interact with the lectin, and (iii) the effect of culture age on the binding of the cells to clover root hairs.

MATERIALS AND METHODS

Bacterial cultures. *R. trifolii* 0403 was obtained from the Rothamsted Experimental Station (Harpenden, England). Cultures were maintained on BIII agar medium (10) and grown at 30°C. Broth cultures were grown in BIII medium as previously described (21). All transfers were made with a 5-day-old plate culture as inoculum.

Immunofluorescence microscopy. *R. trifolii* 0403 of various ages was suspended from BIII plates in phosphate-buffered saline (PBS) (16) and centrifuged at 3,000 × *g* for 20 min at 4°C. The encapsulated cells in the upper, soft layer of the pellet were suspended and washed twice in PBS under the same conditions. The soft pellet was suspended in PBS, heat fixed to microscope slides, rinsed with distilled water, and air dried. Antiserum prepared in rabbits against whole encapsulated cells of *R. trifolii* 0403 was adsorbed exhaustively with steamed, washed, broth-grown bacteria in mid-exponential and early-stationary growth phases. This adsorbed antiserum no longer reacted with unencapsulated cells. Trifoliin A and rabbit antibody against this clover lectin were prepared as previously described (16). The cells were labeled for indirect immunofluorescence with fluorescein isothiocyanate goat anti-rabbit immunoglobulin G (Miles-Yeda, Ltd., Rehovot, Israel).

Electron microscopy. Bacteria for electron microscopy were prepared as described above. The cells were contrasted by the glutaraldehyde-ruthenium red-uranyl acetate (G/RR/UA) procedure (24). In some cases, bacteria were pretreated for 30 min at room temperature with immunoaffinity-purified trifoliin A (12) coupled with colloidal gold (20) before treatment by the G/RR/UA method. Specimens were observed on a Phillips 300 transmission electron microscope. Cells were also treated by freeze-drying and shadowing (24). The bacteria were treated by G/RR/UA, frozen in liquid nitrogen, and sublimed in a vacuum (1×10^{-7} torr [ca. 1.33×10^{-5} Pa]) for 10 min each at -120, -100, and -80°C. The specimens were shadowed by platinum-carbon evaporation at -150°C at a 45° angle and examined with a Hitachi 600 electron microscope at 75 kV.

Root hair-binding assay. A suspension of washed, encapsulated *R. trifolii* 0403 was adjusted to a concentration of 10^6 or 10^8 cells per ml in Fahraeus (N-free) medium (10). Surface-sterilized seeds of *Trifolium repens* var. Louisiana Nolin were germinated into humid air for 48 h. The seedlings were incubated with 5 ml of the bacterial suspension in polypropylene beakers for 1 h at room temperature with gentle circular shaking. The seedlings were gently washed in sterile Fahraeus medium, mounted on a microscope slide, and observed by phase-contrast microscopy. Bacteria attached to root hairs 200 μm long were counted (10), noting both the total number of attached cells per root hair and the orientation of attachment.

Isolation of CPS. Lawns of *R. trifolii* 0403 were prepared with an inoculum from a 5-day-old culture of cells which had their capsules removed by vigorous agitation in sterile PBS, centrifugation at 12,000 × *g*, and two washes in PBS. The bacteria were grown at 30°C for 3, 5, 7, 14, or 21 days and prepared as described above. The encapsulated cells were rapidly stirred at room temperature with 0.5 M NaCl in PBS for 1 h to extract the capsular polysaccharide. The cells were removed by centrifugation at 10,000 × *g* at room temperature until the supernatant was clear. The extracted polysaccharide was precipitated in 3 volumes of cold ethanol, redissolved, dialyzed against deionized distilled water at 4 to 10°C, and lyophilized.

Agglutination inhibition assay. Bacterial agglutination inhibition assays were performed as described previously (15) to measure the level of binding of CPS to trifoliin A. *R. trifolii* 0403 cells used for the assay were grown on BIII agar at 30°C for 5 days. Purified trifoliin A was adjusted to a concentration of 260 μg/ml as measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.). Solutions of the isolated polysaccharides (200 μg of glucose equivalents per

ml) in M buffer (16) were preincubated with equal volumes of the lectin solution for 1 h at 30°C. Serial dilutions were then made in M buffer, and bacteria were added (200 μl of a suspension containing 1.2×10^9 cells per ml) and then incubated at 30°C for an additional 4 h. The degree of inhibition is expressed as agglutination inhibition units (decrease in titer per 100 μg of glucose equivalent) (21).

Carbohydrate analysis. The isolated polysaccharides were examined for neutral sugars by the phenol-sulfuric acid assay (17), for uronic acids by the method of Blumenkrantz and Asboe-Hansen (3), and for pyruvate and O-acetyl substitutions as described by Sutherland (28). The level of pyruvylation was also measured by high-performance liquid chromatography after hydrolysis of the pyruvate groups in 0.005 N H_2SO_4 at 100°C for 3 h. The acid-treated polysaccharide was precipitated in 3 volumes of ethanol containing 0.1 mM $MgSO_4$. After centrifugation at 1,000 × *g* for 5 min,
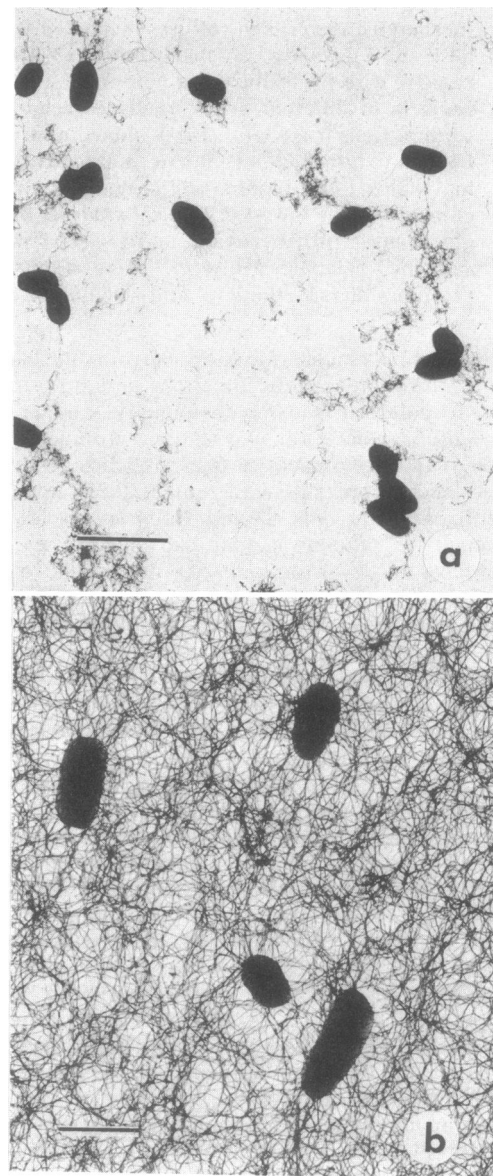

FIG. 1. *R. trifolii* 0403 grown in shaken broth culture. Cells from (a) early exponential and (b) stationary phases were examined by electron microscopy. Bars, 2 μm.

TABLE 1. Effect of culture age on the encapsulation and lectin-binding ability of R. trifolii 0403 grown on BIII agar[a]

Culture age (days)	% Encapsulation		Distribution of capsular trifoliin A receptors	
	Full	Partial	% Uniform	% Nonuniform
3	0	23	1	16
5	72	16	76	12
7	76	12	70	21
14	76	11	4	14
21	42	12	5	20

[a] Expressed as a percentage of the total population examined by immunofluorescence with anti-capsule or trifoliin A and anti-trifoliin A antibodies.

the ethanol in the supernatant was evaporated under nitrogen. Pyruvate in the remaining solution was quantitated on a Waters high-performance liquid chromatograph with a Bio-Rad HPX-87H Organic Acid Analysis column at 50°C, 0.005 N H_2SO_4 as the solvent, and detected at 210 nm with a Gilson Holochrome UV monitor. The sugar composition of the CPS was determined by analyzing trimethylsilyl derivatives prepared with Tri-Sil Z (Pierce Chemical Co., Rockford, Il.) after overnight methanolysis at 80°C in 1 N HCl in methanol (8). The derivatized sugars were separated on 3% SE-30 on Chromosorb W(HP) (Pierce Chemical Co.) at 150°C with a Varian 3740 gas chromatograph. Peak areas were quantitated on a Hewlett-Packard 3390A integrator and corrected for detector response by comparison with derivatized standard sugars.

RESULTS

The development of the capsule of R. trifolii 0403 was found to be directly related to the conditions of growth. In shaken broth cultures, no encapsulation was detected by the G/RR/UA method, regardless of the culture age. Fibrils were produced at one cell pole in early-exponential phase (Fig. 1a) and emitted into the growth medium, forming a fibrillar net (Fig. 1b). Encapsulated cells were occasionally observed (<1%) in broth culture at early-stationary phase when examined by immunofluorescence (figure not shown).

In contrast, R. trifolii 0403 frequently formed capsules when cultivated on agar plates (Table 1). Immunofluorescence microscopy with anti-capsule antibody showed that the capsule began to develop at one pole by 3 days (Fig. 2a) and completely surrounded the majority of cells by day 5 (Fig. 2b). Bacteria remained completely encapsulated throughout the remaining 16 days of incubation (Fig. 2c, d,

and e), although the size of the capsule and the intensity of immunofluorescence staining of the capsular antigen varied (Fig. 2c). The percentage of encapsulated cells remained constant in 5- to 14-day-old cultures, but decreased in the 21-day-old culture (Table 1).

Examination of cells by electron microscopy confirmed that the capsule was polar in early development (Fig. 3a and b) and completely surrounded most cells in 5-day-old cultures (Fig. 3c). The capsule remained intact through the remainder of the incubation period as shown by freeze-drying (Fig. 4) and the G/RR/UA method (data not shown).

The ability of encapsulated cells to bind to the clover lectin, trifoliin A, changed with culture age (Table 1). Indirect immunofluorescence showed that lectin bound to few cells grown for 3 days, and, in those cases, only to one pole (Fig. 5a). By 5 days, lectin receptors were distributed evenly in the fully developed capsule (Fig. 5b). The intensity and evenness of fluorescence decreased by day 7 (Fig. 5c), although the capsule did retain the ability to bind trifoliin A at that time. The ability of encapsulated cells to bind trifoliin A decreased in intensity and percentage of reactive cells by days 14 and 21, when lectin receptors were predominately detected at one pole (Fig. 5d, Table 1).

These immunofluorescence studies were confirmed by electron microscopy with trifoliin A coupled to electron-dense colloidal gold to locate lectin receptors. The newly developed polar capsule on cells in 3-day-old cultures uniformly bound trifoliin A (Fig. 6a), as did the complete capsule on cells from 5-day-old cultures (Fig. 6b). In contrast, fully encapsulated cells cultured for 14 and 21 days generally bound the lectin more at one pole (Fig. 6c). The capsules of cells cultured for 21 days and treated by the G/RR/UA method were much less electron dense than cells from younger cultures (compare Fig. 6a, b, and c), and other extracellular fibrillar structures which stained negatively were also evident (Fig. 6c).

The relationship between these developmental changes of the capsule and the attachment of R. trifolii 0403 to clover root hairs was examined after 1 h of incubation of the bacteria at two different inoculum sizes with clover seedlings. When a 3-day-old culture was used at the higher inoculum (10^8 bacteria per ml), the bacteria attached almost exclusively in a polar orientation (Fig. 7a), matching the polar location of the lectin-binding capsule (Fig. 2a, 3a, 5a, and 6a). Similarly, R. trifolii 0403 from 5-day-old cultures, which have lectin receptors uniformly surrounding the fully encapsulated bacteria, attached to root hairs in a random orientation and clumped extensively at the root hair tips
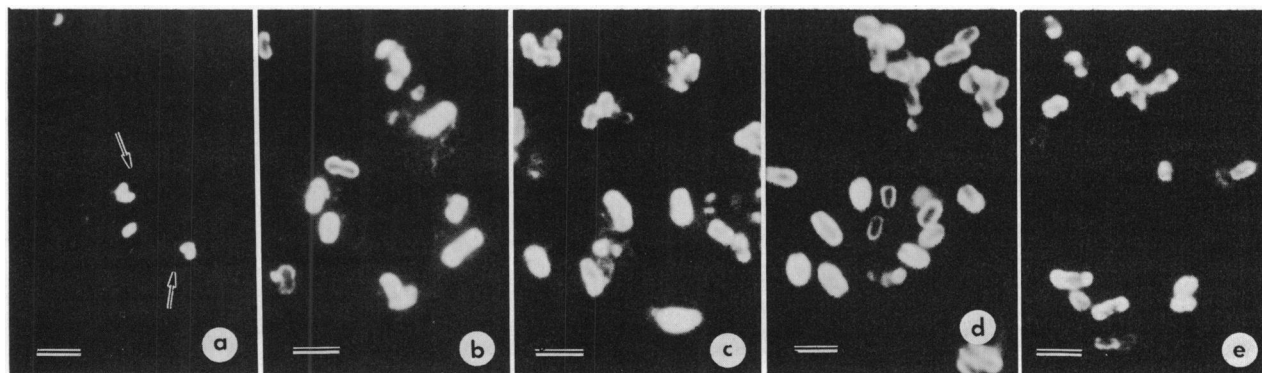

FIG. 2. Encapsulation of R. trifolii 0403 detected by indirect immunofluorescence with homologous anti-CPS antiserum. Bacteria were grown for (a) 3, (b) 5, (c) 7, (d) 14, or (e) 21 days. Arrows indicate location of cells when viewed by phase-contrast microscopy. Bars, 2 μm.
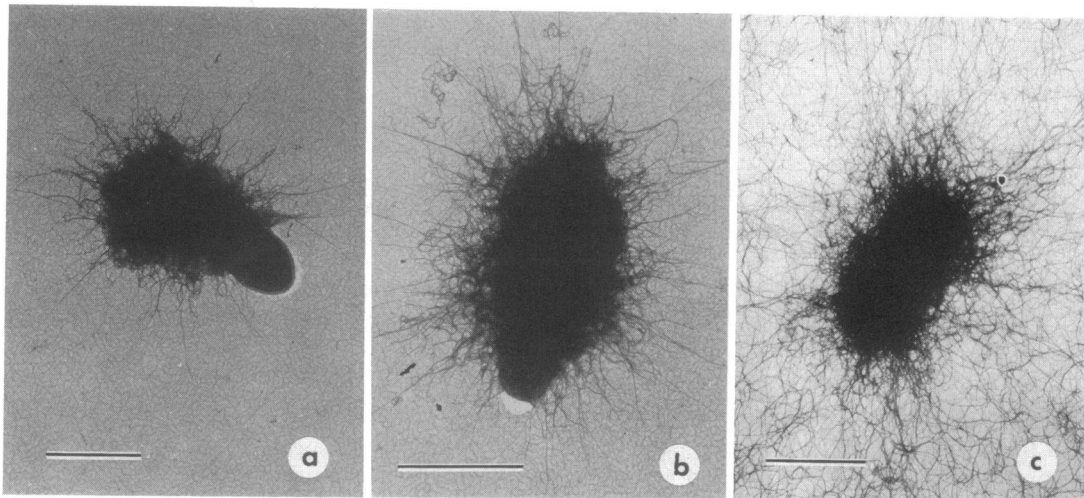
FIG. 3. Development of the capsule of *R. trifolii* 0403 examined by transmission electron microscopy. Bacteria are from 4- (a and b) or 5-day (c) cultures. Bars, 2 μm.

(Fig. 7b). The few cells from cultures grown for 14 or 21 days which bound to root hairs did so polarly (Fig. 7c). These results were confirmed by direct microscopic counts of attached bacteria and their orientation of attachment with a lower inoculum of 10^6 cells per ml (Fig. 8). Bacterial attachment to root hairs was greatest in number with the least amount of polar attachment with cells from 5-day-old cultures. When receptors for trifoliin A were more abundant at one cell pole (i.e., in 3- and 21-day-old cultures), attachment was almost exclusively polar. Although this also coincided with the location of the developing capsule of young cells, such was not the case with bacteria from 14- and 21-day-old cultures, which were most often fully encapsulated (Table 1).

The CPS was extracted from plate cultures of *R. trifolii* 0403 at different culture ages and examined for the ability to

bind purified trifoliin A by bacterial agglutination inhibition (Table 2). Lectin-binding activity of the isolated CPS was highest from 5-day-old cultures, intermediate from 3- and 7-day-old cultures, and least from cells grown for 14 days.

Changes were found in both the glycosyl and nonglycosyl composition of the CPS isolated from cultures at different ages (Fig. 9). The relative level of neutral hexoses remained constant (60% of the total weight) throughout the incubation period, as did the ratio of glucose to galactose (5:1), measured as their trimethylsilyl derivatives. Both the colorimetric assay and gas chromatographic analysis detected a slight increase in the relative level of glucuronic acid in the CPS of cells grown for 5 and 7 days. More striking, however, were the transient increases in the nonglycosyl substitutions. The level of pyruvate substitutions increased from 9.7% (wt/wt) in CPS from 3-day-old cultures to a maximum of 10.8% (wt/wt) in 7-day-old cultures. This increase was detected by the colorimetric assay (Fig. 9) and confirmed by high-performance liquid chromatography analysis. The level of O-acetylation rose from 7.5% (wt/wt) in the CPS from 3-day-old cultures to 8.3% (wt/wt) in the CPS from 5- and 7-day-old cultures. The levels of both pyruvate and O-acetyl substitutions decreased slightly in the CPS from a 14-day-old culture, although not to the level found with very young (3-day) cultures.

DISCUSSION

The development of the capsule and localization of the lectin receptors in the capsule of *R. trifolii* 0403 were examined by immunofluorescence and electron microscopy. There is a fundamental difference between the relationship of encapsulation and ability to bind trifoliin A, based on whether the cells are grown in shaken broth culture or on agar surfaces. In shaken broth culture, these bacteria rarely form complete capsules, although >90% of the cells uniformly bind trifoliin A at a specific time in early-stationary phase (21). The interaction between the lectin and cells in shaken broth culture can therefore be attributed primarily to the lectin-binding LPS (21). In contrast, cells grown on agar surfaces form capsules which also bind trifoliin A at a specific culture age (15). Therefore, to study the development of the lectin-binding capsule of *R. trifolii* 0403, we used cultures grown on solid medium. In addition, growth on an
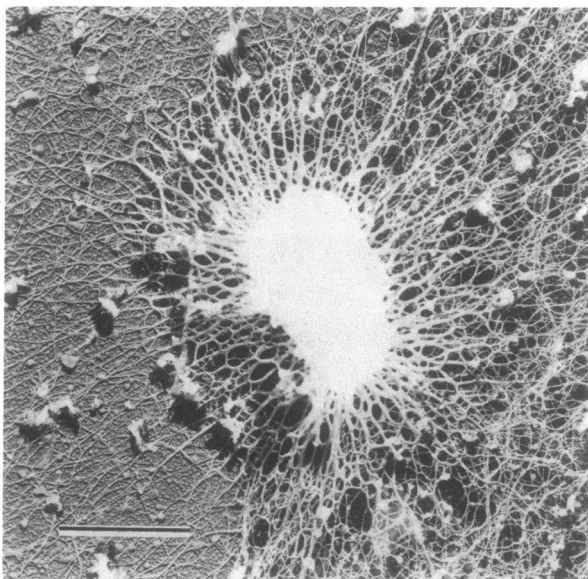

FIG. 4. *R. trifolii* 0403 from 21-day-old cultures examined by transmission electron microscopy after freeze-drying and shadowing. Bar, 1 μm.
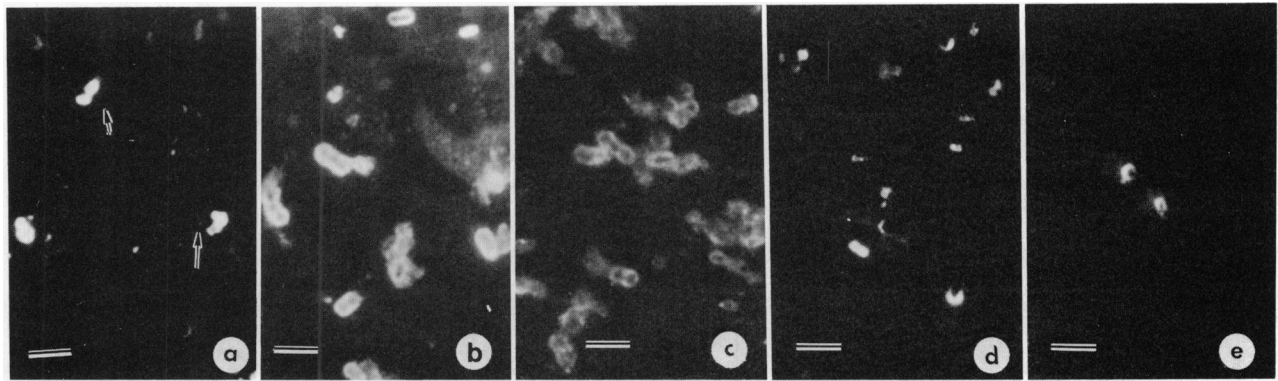
FIG. 5. Binding of trifoliin A to the capsule of *R. trifolii* 0403 as shown by indirect immunofluorescence. Bacteria were grown for (a) 3, (b) 5, (c) 7, (d) 14, or (e) 21 days. Arrows indicate position of cells when viewed by phase-contrast microscopy. Bars, 2 μm.
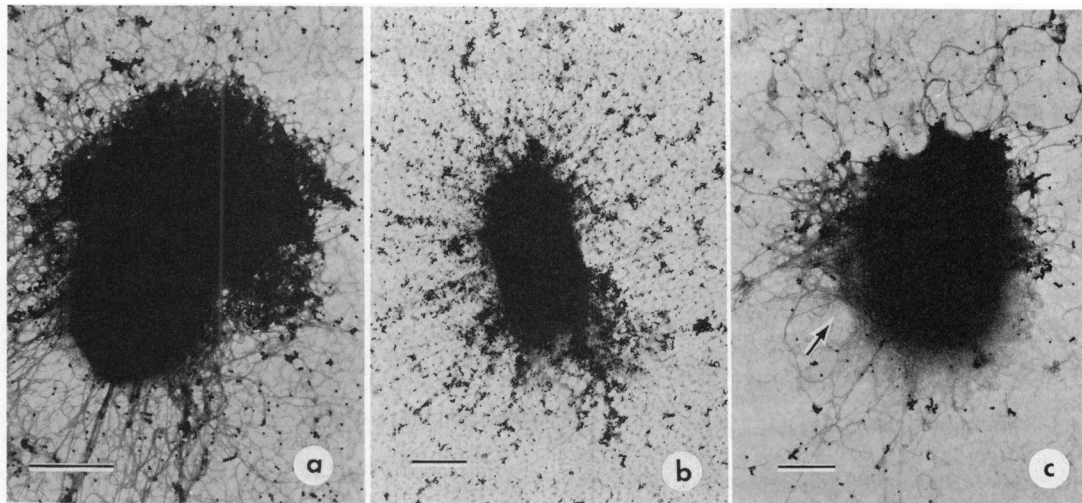

FIG. 6. Localization of trifoliin A binding sites on the capsule of *R. trifolii* 0403 by transmission electron microscopy. Bacteria were grown for (a) 3, (b) 5, or (c) 21 days and reacted with trifoliin A coupled with colloidal gold. Note the negatively stained fibrillar material in (c) (arrow). Bars, 0.5 μm.
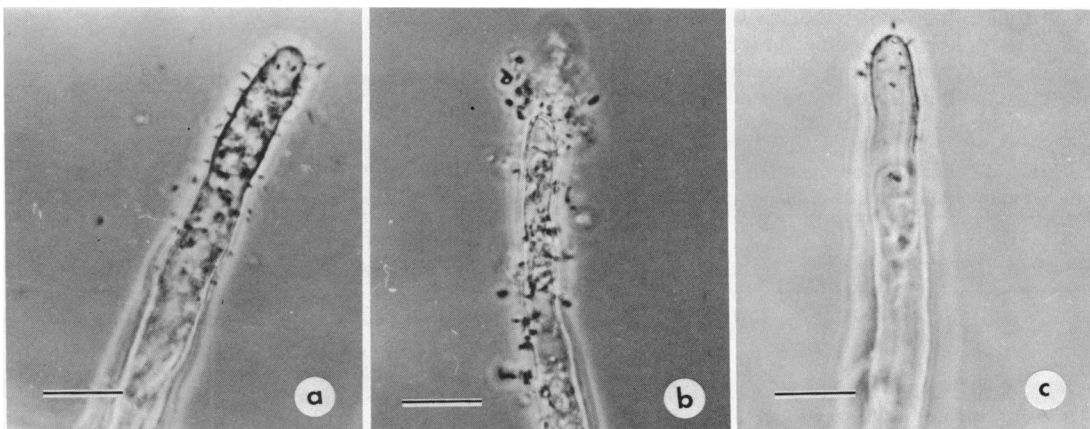

FIG. 7. Attachment of *R. trifolii* 0403 to clover root hairs. Bacteria were grown for (a) 3, (b) 5, and (c) 21 days and incubated with clover seedlings for 1 h. Bars, 20 μm.
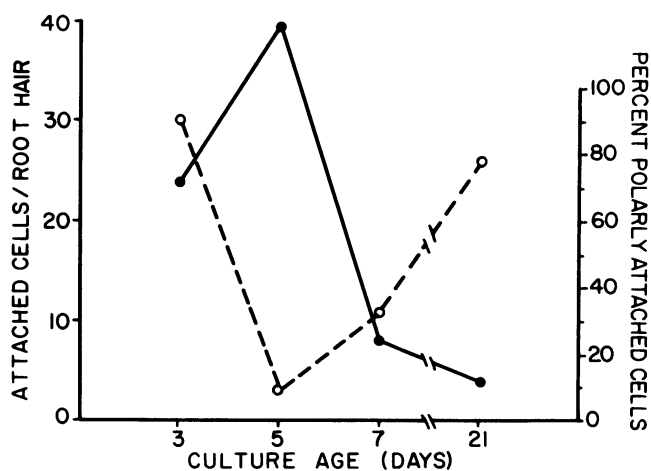
FIG. 8. Effect of culture age on the quantitative attachment of *R. trifolii* 0403 to clover root hairs. Numbers of bacteria attached (●) and orientation of attachment (○) are shown.
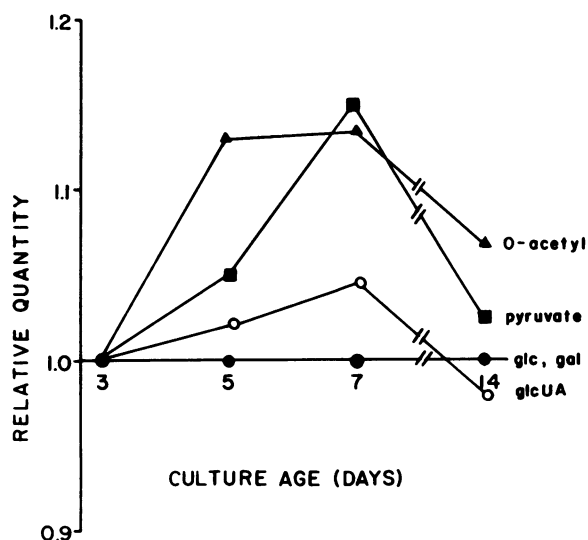

FIG. 9. Relative quantities of the components of the capsular polysaccharide of *R. trifolii* 0403 at different culture ages. All values are standardized to the levels found in the CPS from 3-day-old cultures.

agar surface may more closely approximate growth in the natural environment than does growth in shaken broth culture (9). Our studies confirm the age-dependent ability of encapsulated cells of *R. trifolii* 0403 to bind trifoliin A and that encapsulated cells cultivated for 5 days on BIII agar react most strongly with trifoliin A (15). In addition, a direct correlation between the distribution of the lectin receptors within the capsule and the orientation of attachment of *R. trifolii* 0403 to clover root hairs was demonstrated. The direct interaction between isolated CPS and purified lectin was quantitated and also found to change with culture age.

The capsule of *R. trifolii* 0403 grown on BIII agar would be described, according to the scheme of Costerton et al. (9), as rigid, since it can exclude India ink particles (unpublished observation), and peripheral, since it remains intact under certain conditions but may be shed directly into the medium under other conditions. The CPS forms a discrete layer bound to the bacterial surface and is distinct from extracellular polysaccharide (EPS), which is not bound to the cell. Thus, the CPS of plate-growth cells can be physically distinguished and separated from EPS.

Immunofluorescence and electron microscopy indicated that the capsule of *R. trifolii* 0403 is first excreted from the cell at one pole. This pattern of capsule development differs from that in *E. coli*, in which the polysaccharide is excreted from many places around the cell (2). Synthesis of the capsule by *R. trifolii* could remain polar, with the capsule expanding from one pole to "engulf" the cell, or, alternatively, the points of synthesis and excretion of capsular polysaccharide from the fluid outer membrane may redistribute more evenly around the cell as the culture ages. Evi-

TABLE 2. Effect of culture age on the interaction between isolated CPS and trifoliin A

Culture age (days)[a]	Bacterial agglutination inhibition[b]
3	502
5	852
7	686
14	0

[a] CPS isolated from plate cultures at different ages and adjusted to 100 µg of glucose equivalents per ml.

[b] Inhibitory units per milligram of glucose equivalents; trifoliin A-specific activity was 985 agglutination units per mg protein.

dence suggesting that the first possibility is more likely includes: (i) the acidic fibrils of cells grown in shaken broth culture remain polar during growth; (ii) the capsules of cells in young, plate-grown cultures are polar; and (iii) root exudate enzymes partially degrade the capsule of living *R. trifolii* 0403 cells, leaving a polar capsule, but completely degrade the capsule of killed cells (14). The lack of capsules on 7-day-old plate cultures of *R. trifolii* 0403 reported previously (15) was probably due to the centrifugation steps used to prepare the cells for electron microscopy. The more gentle methods used in this study clearly show that the cells remain fully encapsulated after 5 days of growth.

The ability of the capsule of *R. trifolii* 0403 to bind to trifoliin A is maximal on cells grown for ca. 5 days. The binding of trifoliin A to encapsulated cells grown for 7 days is weaker and uneven, whereas lectin receptors are generally polar after that time. The orientation of bacterial attachment to root hairs reflects the location of lectin receptors rather than the presence of the capsule itself. Cells with polar lectin receptors, therefore, bind to root hairs polarly, regardless of whether they have a polar or uniform capsule, and cells with a uniform distribution of lectin receptors in the capsule bind to root hairs randomly. The quantity of cells which attach reflects the relative proportion of cells in the inoculum which can bind trifoliin A. Thus, 5-day-old cultures, which contain the highest percentage of cells which uniformly bind trifoliin A, attach to clover root hairs in the highest numbers and in a random orientation. The bacterial clumping at the root hair tip may be mediated by trifoliin A, since it is *Rhizobium*-specific and inhibited by 2-deoxy-D-glucose (which blocks the interaction between trifoliin A and its saccharide receptors), at inoculum sizes below 10^9 bacteria per seedling (F. B. Dazzo et al., submitted for publication). Although cells from 14- and 21-day-old cultures have uniform capsules, they bind to root hairs polarly, which rules out a nonspecific adherence of the capsule itself. The polar attachment of cells from 3- and 21-day-old cultures to clover root hairs occurs within 1 h, and therefore does not involve rhizosphere enzymes, which require at least 4 h to convert a full capsule into a polar capsule (14).

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A relatively crude preparation of isolated CPS was used for lectin-binding assays and chemical analysis to minimize the possibility of removing any capsular fractions which might interact with the lectin, although ethanol precipitation and dialysis may have resulted in the loss of low-molecular-weight compounds which affect root hair infection (1). The CPS isolated from cells at all culture ages contained less than 3% (wt/wt) neutral polysaccharides, which are soluble in 2% cetylpyridinium chloride (unpublished data) and lack LPS-specific sugars of R. trifolii 0403 (21) which would have been detected by gas chromatography. Agglutination inhibition studies showed that the degree of lectin binding by the isolated CPS parallels the age-dependent ability of R. trifolii 0403 cells to react with trifoliin A (15) and is optimal in both cases with 5-day-old cultures. Changes in the CPS, therefore, can explain the age-dependent interaction of plate-grown cells with the lectin. Identification of these changes should provide clues to explain the chemical basis for binding of CPS to trifoliin A. The glycosyl composition is consistent with that reported for the EPS of broth-grown R. trifolii 0403 isolated during stationary phase (26), in which the repeating oligosaccharide subunit contains five glucose, one galactose, and two glucuronic acid moieties. Although the neutral sugar composition of the CPS remained unchanged during growth, slight changes were found in uronic acid levels by procedures reported to optimize the recovery of uronic acids from polysaccharides (8). These changes may be due to nonglycosyl substitutions which affect their detection and quantitation or to synthesis of CPS with less uronic acid per repeating unit. Pyruvate and O-acetyl substitutions increase during growth to a greater degree than do the uronic acids. Although similar increases have been shown for the EPS of broth-grown R. trifolii L-158 (5), the degree of substitution begins to decrease in the CPS after 7 days of growth on agar plates. The excretion of less-substituted polysaccharides could explain the reduction in the percentage of these substitutions in the EPS of R. meliloti L-89 reported by Cadmus et al. (5). The age-dependent changes in levels of pyruvate and O-acetyl substitutions occurred in parallel with the ability of the isolated CPS to bind trifoliin A. Separate studies with one- and two-dimensional ^{1}H-NMR analysis of oligosaccharide fragments of the CPS of R. trifolii 0403 confirm that the noncarbohydrate substitutions change with culture age and that these changes affect the affinity of the oligosaccharides for trifoliin A (M. Abe et al., manuscript in preparation).

The bacterial capsule appears to be a dynamic structure surrounding the cell rather than a simple homogeneous entity. In pure culture, the ability of encapsulated Rhizobium cells to interact with the lectins of their host plants changes with culture age (15, 22, 23, 30). In addition, localized alterations in the capsule of R. trifolii which affect its affinity for trifoliin A occur when the bacterium is inoculated into the clover root environment (14). These modifications have a profound impact on the ability of R. trifolii to attach to clover root hairs, an early step in the formation of the nitrogen-fixing symbiosis.

ACKNOWLEDGMENTS

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