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Alteration of the Trifoliin A-Binding Capsule of *Rhizobium trifolii* 0403 by Enzymes Released from Clover Roots†

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The effect of white clover root exudate on capsules of *Rhizobium trifolii* 0403 was examined. The clover lectin trifoliin A was detected in root exudate of two clover varieties by indirect immunofluorescence with antibody against this lectin purified from clover seed. Trifoliin A bound uniformly to encapsulated, heat-fixed cells during 1 h of incubation with root exudate. After 4 to 8 h of incubation, trifoliin A was only bound to one pole of the cells. Transmission electron microscopy showed that the capsule itself was altered. The disorganization of the acidic polymers of the capsule began in the equatorial center of the rod-shaped cell and then progressed toward the poles at unequal rates. Trifoliin A could no longer be detected on heat-fixed cells after 12 h of incubation with root exudate. However, trifoliin A was detected in situ on one pole of cells grown for 4 days in the clover root environment of Fahraeus slide cultures. Inhibition studies with the hapten 2-deoxy-D-glucose showed that trifoliin A in root exudate had a higher affinity for one of the cell poles. Immunoelectrophoresis was used to monitor the alteration of the extracellular polysaccharides from *R. trifolii* 0403 by concentrated root exudate. These polysaccharides were converted into products which eventually lost their ability to immunoprecipitate with homologous antibody. This progressive loss of antigenic reactivity proceeded more rapidly with root exudate from seedlings grown under nitrogen-free conditions than with root exudate from plants grown with 15 mM KNO_3. The root exudate, depleted of trifoliin A by immunoaffinity chromatography, was still able to alter the capsule of *R. trifolii* 0403. Reconstitution experiments showed that the substance(s) in root exudate which induced this alteration of the capsule was of a high molecular weight, heat labile, trypsin sensitive, and antigenically unrelated to trifoliin A. A variety of glycosidase activities were also detected in the fraction depleted of trifoliin A. These results suggest that enzymes in clover root exudate alter the trifoliin A-binding capsule in a way which would favor polar attachment of *R. trifolii* to clover root hairs.

The rhizosphere environment provides unique ecological niches for soil microorganisms through exudation of organic compounds from the living root. *Rhizobium* spp., the nitrogen-fixing symbionts of leguminous plants, would presumably encounter these compounds before the bacteria infect the root hairs of their legume hosts. Clover root exudates have been shown to stimulate infection and nodulation of clover by its symbiont, *Rhizobium trifolii* (27, 32; C. A. Napoli and D. H. Hubbell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, N3, p. 176). Similarly, nodulation of cowpeas is stimulated by their root exudate (3). In recent studies (10), we found that root exudates of axenically grown white clover seedlings (*Trifolium repens* var. Louisiana Nolin) contain trifoliin A, a lectin which specifically binds to *R. trifolii* (13). Thirtyfold less trifoliin A was detected in root exudates of white clover seedlings (*T. repens* var. Louisiana Nolin) when they were grown in a rooting medium supplemented with 15 mM KNO_3 (10), a concentration of combined nitrogen which completely suppresses root hair infection by *R. trifolii* 0403 (8). The presence in root exudate of lectins which specifically bind to rhizobia raises the possibility that cellular recognition events may begin in the rhizosphere before the microorganism attaches to the root hair that it later infects.

In this paper, we describe the alteration of the trifoliin A-binding capsule of *R. trifolii* 0403 by

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enzymes which are released from clover roots and accumulate in root exudate. These studies provide evidence for a unique organization of lectin receptors at one pole of the encapsulated cell.

(Preliminary reports of this work have been presented [F. B. Dazzo, E. Hrabak, J. Sherwood, and G. L. Truchet, Abstr. 4th Int. Symp. Nitrogen Fixation 1980, abstr. no. 212, p. 424; F. B. Dazzo, E. M. Hrabak, J. E. Sherwood, and G. L. Truchet, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K96, p. 153].)

MATERIALS AND METHODS

Bacterial and plant cultures. *R. trifolii* 0403, from the Rothamsted Experimental Station, United Kingdom, was grown at 30°C in BIII medium (19). Encapsulated cells were obtained from a culture grown for 5 days on plates (9). Cells for adsorption of antiserum were grown in BIII broth (19). White clover (*T. repens* var. Louisiana Nolin and Ladino) and alfalfa (*Medicago sativa* var. Vernal) were obtained commercially. Plants were grown from surface-sterilized seeds (10) in axenic, hydroponic culture with a 14-h photoperiod at 22°C (26,900 lx) and 10-h darkness at 20°C.

Reagents and chemicals. Rabbit antisera against sonicated cells and lipopolysaccharide of *R. trifolii* 0403 and against trifoliin A from clover seeds were prepared as previously described (9, 11, 13, 19). Antiserum against capsular polysaccharide was prepared by use of fully encapsulated cells as the immunogen followed by exhaustive adsorption of the antiserum with washed, mid-exponentially growing cells (optical density at 660 nm of 0.32 in shaken BIII broth culture) and steamed-washed cells from the early stationary phase (optical density at 660 nm of 0.61 in shaken BIII broth culture). The adsorption procedure was previously described (19). Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., and solutions were prepared with distilled, deionized water (resistance, 18 MΩ cm).

Preparation of root exudate. Root exudates were obtained as previously described (10). Briefly, seedling roots were grown through purified agar blocks supported above stainless steel wire mesh into nitrogen-free Fahraeus medium (14) or Fahraeus medium supplemented with 15 mM KNO_3. This design protects the rooting medium from contaminating seed exudates. After 5 days of plant growth, the root exudates were collected, treated with 2% (wt/vol) acid-washed insoluble polyvinylpyrrolidone, clarified by centrifugation for 20 min at 12,000 × *g*, and concentrated approximately 50-fold to 40 μg of protein per ml by ultrafiltration on an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.) at 4°C. This concentrated root exudate was fractionated by immunoaffinity chromatography (30) with rabbit anti-trifoliin A immunoglobulin G (IgG) coupled to CNBr-activated Sepharose 4B (10).

Assays. Protein was measured by the Folin phenol method (23). Trifoliin A was detected in root exudate by indirect immunofluorescence (10) of encapsulated cells of *R. trifolii* 0403 heat fixed to slides. For hapten inhibition, root exudate was incubated with sugars (30 mM final concentration) for 1 h at 22°C and then assayed for trifoliin A by immunofluorescence (10). Glycosidase activity was measured with *p*-nitrophenyl-substituted glycosides (1). These substrates were examined for purity by thin-layer chromatography through Silica Gel 60 (E. Merck AG, Darmstadt, Germany) in chloroform-methanol-acetic acid (85:10:5, vol/vol/vol) at room temperature and detected after spraying with 20% H_2SO_4 and heating at 100°C.

Plant-bacterium cultures. For root environment studies, 10^7 to 10^8 encapsulated bacteria were inoculated into sterile Fahraeus slide assemblies (14) without agar supports or into 10 ml of sterile nitrogen-free Fahraeus medium in tubes containing approximately 20 seedlings grown for 2 days through agarose blocks supported by wire mesh (8). After incubation, the bacteria were removed from the rooting medium and examined for in situ accumulation of trifoliin A on the entire cell by immunofluorescence (10) and by immunoelectron microscopy with anti-trifoliin A IgG directly coupled to colloidal gold as described by Horisberger et al. (18). For immunoelectron microscopy, the bacteria were removed directly from the rooting medium and deposited on Formvar-coated copper grids as previously described (25). The bacteria were then treated for 30 min with gold-coupled anti-trifoliin A IgG and finally stained by the glutaraldehyde-ruthenium red-uranyl acetate method (25). In addition, cells were fixed in the presence of ruthenium red, embedded, thin sectioned, post stained, and examined by transmission electron microscopy as described elsewhere (29). Exocellular structures of whole, uncentrifuged bacteria were also examined with ruthenium red staining (25). Examinations were performed on a Philips 300 transmission electron microscope.

Interaction of root exudate with cell-bound extracellular polysaccharides. *R. trifolii* 0403 grown in shaken BIII broth culture to the early stationary phase (optical density at 660 nm of 0.61) was harvested by centrifugation at 9,150 × *g*. The cell pellet was suspended in 0.5 M NaCl, stirred at room temperature for 1 h, and centrifuged at 9,150 × *g* for 30 min. The extracted polysaccharides were precipitated in 70% ethanol for 30 min at 4°C. The precipitated polysaccharide was dissolved in water (pH 7), reprecipitated twice in 70% ethanol at 4°C, redissolved in water, dialyzed exhaustively against water, and lyophilized. Previous analyses had shown that this crude extracellular polysaccharide material bound to trifoliin A (contains 10,160 agglutination-inhibitory units per mg of glucose equivalents [E. M. Hrabak and F. B. Dazzo, unpublished data]). This material contained no detectable 2-keto-3-deoxyoctanoic acid (28) or heptose (34) as markers of lipopolysaccharide contamination (19) and displayed no absorption maxima at 260 or 280 nm as evidence of nucleic acid or protein contamination. The lyophilized polysaccharide was dissolved in water (1 mg/ml), filter sterilized, analyzed for carbohydrate content by the phenol-sulfuric acid method (26), and finally adjusted to 600 μg of glucose equivalents per ml. The polysaccharide solution (100 μl) was mixed with 300 μl of filter-sterilized concentrated root exudate (40 μg of protein per ml of Fahraeus medium), 100 μl of filter-sterilized 1 mM potassium phosphate buffer (pH 7.2), and 6 μl of 0.6% sodium azide as a preservative. The solution of extracellular polysaccharide or root exudate was replaced with sterile phosphate buffer for

control experiments. The tubes were sealed and incubated at 30°C. Samples of the reaction mixture (15 μl) were removed periodically and analyzed by immunoelectrophoresis on glass slides (8.5 by 10 cm). The polysaccharide antigens were electrophoresed through 0.7% (wt/vol) agarose in 50 mM barbital buffer (pH 8.6) for 2 h at 40 mA and developed for 2 days at room temperature with antiserum (10 mg/ml) against encapsulated cells of *R. trifolii* 0403. After 4 days of incubation at 30°C, the reaction mixtures of polysaccharide and root exudate were also analyzed for quantitative changes in reducing sugars (26).

RESULTS

Interaction of root exudate with encapsulated cells. Trifoliin A was detected by immunofluorescence in the root exudates from the two varieties of white clover. Trifoliin A in root exudate bound uniformly to encapsulated cells after 0.5 to 1 h of incubation (Fig. 1A and B). Controls with preimmune serum were negative. Incubation of root exudate with cells for 4 to 8 h showed a progressive reduction in bound trifoliin A (Table 1), beginning with a loss of immunofluorescence around the equatorial center of the rod-shaped cell which still fluoresced at its poles (Fig. 2). Photomicrographic negatives showed that the fluorescent intensity was brighter at one cell pole. Later, cells had a flare of immunofluorescence at only one cell pole (Fig. 3). By 8 h of incubation, immunofluorescent detection of trifoliin A was restricted to approximately 5% of the population of cells (compared with 95% at 1 h) and appeared as a tight, fluorescent cap at only one cell pole (Fig. 4). Finally, after 12 h of incubation of heat-fixed cells with root exudate, trifoliin A could no longer be detected.

The detection of trifoliin A in concentrated root exudate prompted us to examine whether the bacteria had trifoliin A bound to them in situ when inoculated into the root environment of growing plants. Fully encapsulated cells were introduced into the rooting medium and incubated for different times before being collected and examined for bound trifoliin A. Immunofluorescence showed that 95% of the control, encapsulated cells bound trifoliin A uniformly (Table 1). Among the cells which bound trifoliin A after 1 h of incubation in the simulated rhizosphere, 70% appeared to be uniformly fluorescent whereas the remainder had more trifoliin A bound at the pole(s) (Fig. 5A). After 2, 4, and 6 days of incubation, trifoliin A was bound to the cells at only one pole. Results are shown after 4 days of incubation (Fig. 5B). Controls in which normal preimmune serum was substituted for immune anti-trifoliin A IgG were negative. Streak plating of the inoculated rooting medium on BIII plates

TABLE 1. Progressive reduction in binding of trifoliin A from clover root exudate to heat-fixed, encapsulated cells of *R. trifolii* 0403

Time of incubation with root exudate (h)	% of immunofluorescent cells[a]		
	Uniform	Polar[b]	Unreactive
0[c]	95	0	5
1	90	0	10
4	13	26	61
8	0	5	95
12	0	0	100

[a] Calculated from the average of three to five microscope fields, ca. 130 cells per field.

[b] Includes cells with fluorescence at either one or both poles, but no fluorescence in the equatorial center.

[c] At time = 0 h, only purified trifoliin A was assayed.

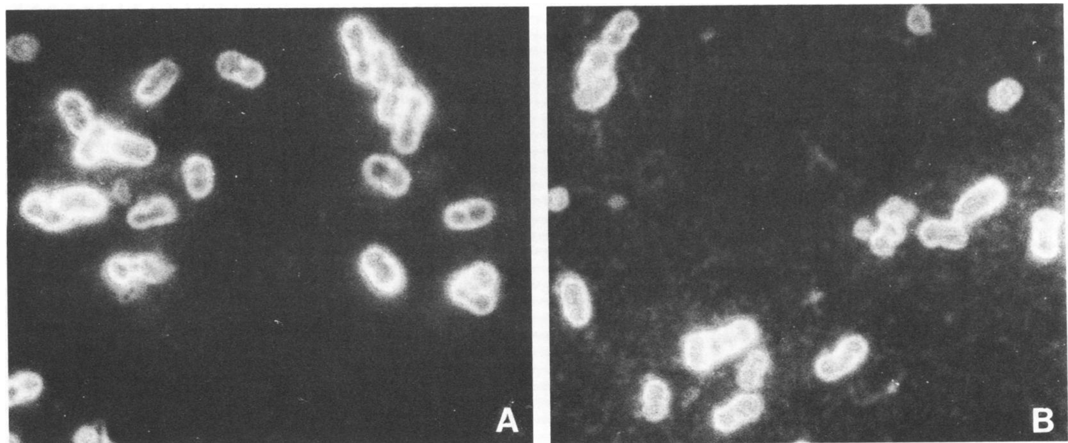

FIG. 1. Immunofluorescent detection of trifoliin A on encapsulated cells of *R. trifolii* 0403 after 1 h of incubation with clover root exudate from Louisiana Nolin (A) and Ladino (B). ×4,270.

FIG. 2. Immunofluorescent detection of trifoliin A on *R. trifolii* 0403 after 4 h of incubation with clover root exudate. Note reduced fluorescence in the equatorial center of the cell. ×9,650.

produced only colonies resembling *R. trifolii* 0403.

The results above were confirmed and extended at the electron microscopy level with ruthenium red as a stain for the acidic polymers of the capsule. Bacteria were incubated in concentrated root exudate for 1, 2, or 4 h and then centrifuged, fixed, embedded, and thin sectioned. The successive steps of capsular disorganization started in an equatorial band (Fig. 6A) and then proceeded toward the poles, producing two polar caps (Fig. 6B) and finally only one cap (Fig. 6C). This remaining polar material was very often dislodged from the cells during this procedure (not shown). These results were strikingly different from control cells in which the electron-dense fibrillar acidic polymers remained stable on the capsule for 4 h in Fahraeus medium (Fig. 6D).

Encapsulated bacteria incubated in the rooting medium of growing seedlings for 1, 2, and 4 days were treated by the glutaraldehyde-ruthenium red-uranyl acetate method without centrifugation (25) and observed by electron microscopy. Cells progressively lost the uniform capsules but had fibrils at one cell pole (Fig. 7A, at 4 days of incubation). These bacteria represent a sample of the same population which showed a polar reaction when treated by the immunofluorescent assay for bound trifoliin A (see above; Fig. 5B). In contrast, a full capsule was always detectable on bacteria incubated in sterile Fahraeus medium (Fig. 7B, at 4 days of incubation).

FIG. 3. Immunofluorescent detection of trifoliin A on *R. trifolii* 0403 after 6 h of incubation with clover root exudate. Note flare of fluorescence at one pole of the cell. ×9,650.

Finally, bacteria incubated in the clover root environment were collected directly and examined for bound trifoliin A by immunoelectron microscopy. After 6 h of incubation, bipolar or unipolar material on the bacteria strongly reacted with the anti-trifoliin A IgG–gold complex (Fig. 8A and B, respectively). Control cells suspended in Fahraeus medium only (no root exudate) did not bind the anti-trifoliin A IgG–gold complex (not shown). Furthermore, control cells from the rhizosphere did not bind uncoupled gold colloid substituted for immune anti-trifoliin A IgG–gold complex.

Hapten inhibition studies showed that the binding of trifoliin A in the root exudate to encapsulated *R. trifolii* 0403 was hapten specific and was inhibited by 2-deoxy-D-glucose but not by 2-deoxy-D-galactose or α-D-glucose. In the presence of 5 mM 2-deoxy-D-glucose, 30% of the population of encapsulated cells bound trifoliin A at only one pole (Fig. 9), whereas the binding of trifoliin A to cells was completely inhibited in the presence of 2-deoxy-D-glucose at a concentration of 10 mM or higher (Fig. 9). These results suggest that trifoliin A in root exudate has a higher affinity for one of the poles of the encapsulated cell.

Interaction of clover root exudate with isolated extracellular polysaccharides. The modification of extracellular polysaccharide from *R. trifolii* 0403 after incubation with clover root exudate was examined by immunoelectrophoresis. After 1 day of treatment, a product appeared which showed a slower electrophoretic mobility and an immunological reaction of identity with the native polysaccharide (Fig. 10A). The relative quantity of this product increased with further incubation of the antigenic polysaccharide and the root exudate (Fig. 10B and C). Another product, detected after 4 days of incubation of polysaccharide with the root exudate, formed an immunoprecipitin band closer to the antibody trough (Fig. 10C). The extracellular polysaccharide no longer immunoprecipitated after incubation for 7 days with the root exudate. The rate at which these intermediate products formed was slower when the capsular polysaccharide was incubated with concentrated root exudate from

FIG. 4. Immunofluorescent detection of trifoliin A on *R. trifolii* 0403 after 8 h of incubation with clover root exudate. Note polar fluorescence. ×9,650.

seedlings grown in the presence of 15 mM KNO_3 than when it was incubated with root exudate from seedlings grown under nitrogen-free conditions (Fig. 10A and B). Despite the antigenic changes in the extracellular polysaccharide, there was no change detected in reducing sugar equivalents of the complete reaction mixture after 4 days of incubation with the root exudate.

Enzymatic activity of fractionated root exudate. Fractionation of concentrated root exudate on an immunoaffinity column of immobilized anti-trifoliin A IgG (Fig. 11) resulted in a fraction of trifoliin A (peak 2) and a fraction of antigenically unrelated material (peak 1). Reconstitution experiments indicated that the peak 1 fraction contained the active substances responsible for the alteration of the trifoliin A-binding sites on encapsulated *R. trifolii* 0403 (Table 2). This activity of peak 1 was heat labile, destroyed by incubation with trypsin immobilized on agarose beads (Table 2), and retained by ultrafiltration on a PM-10 ultramembrane (approximate cutoff, 10,000 daltons). In the absence of a previous incubation with the peak 1 fraction, the capsules of *R. trifolii* 0403 bound trifoliin A (peak 2) uniformly, even after 12 h of incubation (Table 2).

The extent to which the peak 1 fraction altered the surface antigens of encapsulated *R. trifolii* 0403 was measured. Heat-fixed cells were treated with the peak 1 fraction on slides for 1, 4, and 20 h and then rinsed, treated with homologous antibodies to surface antigens, and finally examined by indirect immunofluorescence (Table 3). The cell surfaces remained reactive with antibodies raised against the sonicated cells throughout the 20-h treatment with peak 1. However, there was a marked reduction in the percentage of heat-fixed cells reactive with anti-capsular polysaccharide IgG after incubation with peak 1 of clover root exudate. By contrast, there was no clear evidence during the 20 h of incubation that peak 1 material affected the reactivity of cells to antibodies against the immunodominant determinants of their lipopolysaccharides.

These results were compared with treatment of encapsulated cells with alfalfa root exudate collected in nitrogen-free Fahraeus medium and concentrated to 40 μg of protein per ml. After incubation of heat-fixed cells with the root exudate for 26 and 48 h, 94 and 81% of the remaining cells immunofluoresced with the anti-capsular polysaccharide IgG, respectively. All control cells treated for 48 h with Fahraeus medium alone were immunofluorescent with this antibody. Thus, the capsular antigens were destroyed much quicker with root exudate from clover than with that from alfalfa, suggesting some host specificity in these enzymatic reactions.

Fractions of Ladino root exudate obtained by immunoaffinity chromatography were assayed for various glycosidase activities with *p*-nitrophenyl-substituted glycosides as substrates. Each of the substrates was homogeneous by thin-layer chromatography (data not shown). A wide variety of glycosidase activities were detected in the peak 1 fraction, and none were detected in peak 2 (Table 4). α-Galactosidase was examined further, since previous reports have indicated that this enzyme from certain legumes behaves as a phytohemagglutinin (15, 16). All of the α-galactosidase activity in Ladino root exudate was destroyed by boiling for 1 min and eluted from the immunoaffinity column without interacting with anti-trifoliin A IgG (Table 4).

DISCUSSION

This study indicates that the capsule of *R. trifolii* 0403 is altered by an enzyme, or enzymes, released from clover roots. This alteration affects the distribution of trifoliin A-binding sites on cells heat fixed to slides or on living cells in situ in the root environments of hydro-

ponic cultures. Synthesis of new, polar exocellular material which binds trifoliin A in root exudate presumably keeps pace with the modification of the capsule by the host enzyme(s). As a result, many of the cells growing in the root environment have trifoliin A bound in situ at

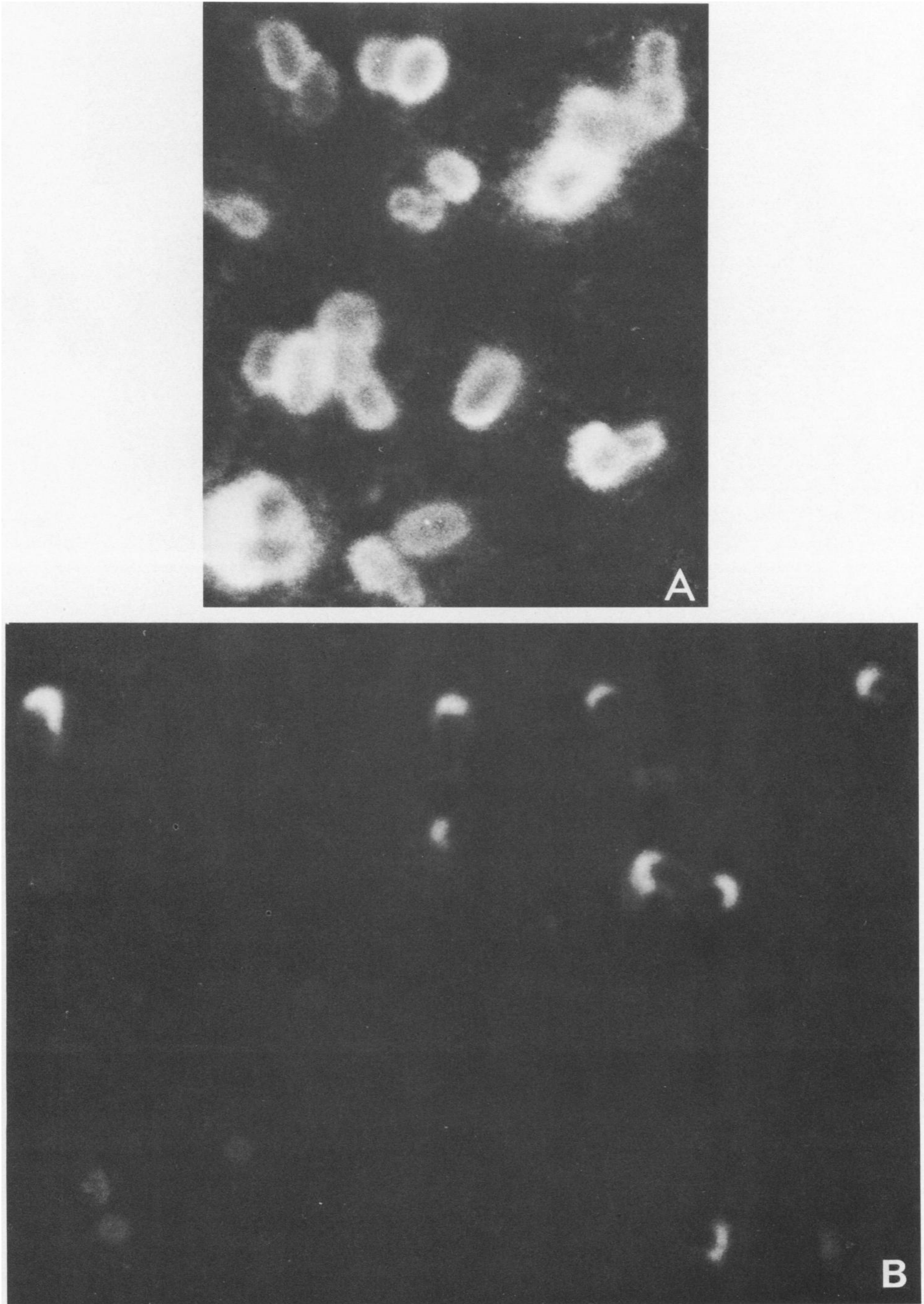

FIG. 5. Immunofluorescent detection of trifoliin A on *R. trifolii* 0403 in the simulated clover rhizosphere after 1 h of incubation (A) and after 4 days of incubation (B). Note that fluorescence in (B) is only at one cell pole. ×9,650.

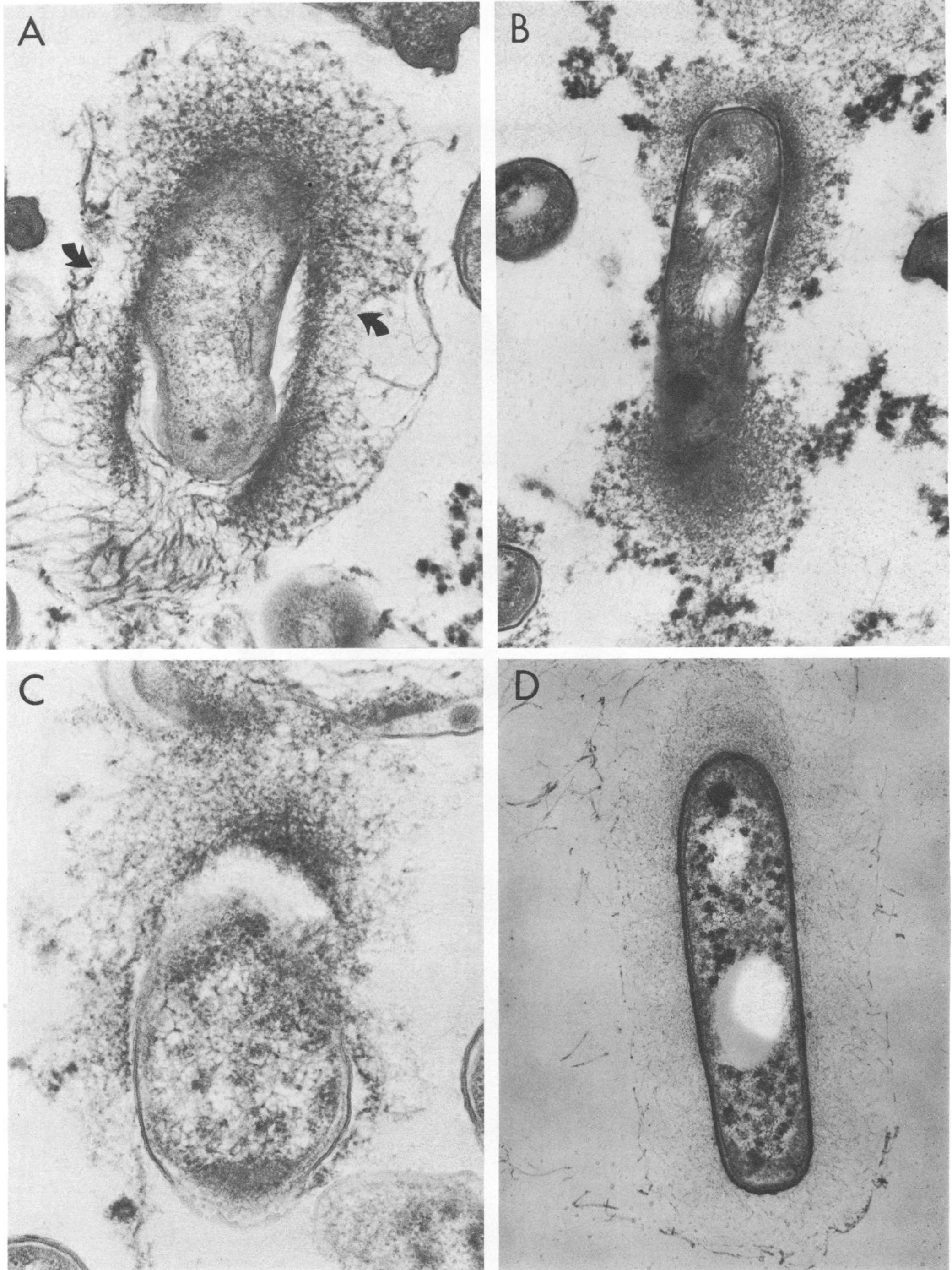

FIG. 6. Transmission electron microscopy of ultrathin sections of *R. trifolii* 0403 suspended in concentrated root exudate. Cells were stained with ruthenium red and poststained with uranyl acetate and lead citrate. (A) Cells incubated for 1 h, with an equatorial region of the capsule (arrows) disorganized (×20,000); (B) acidic polymers of the capsule located at both cell poles but no longer detected in the equatorial region (×14,000); (C) cells incubated for 4 h, with the capsule localized at only one pole (×30,000); (D) control cell with uniform capsule after 4 h of incubation with Fahraeus medium (×17,000).

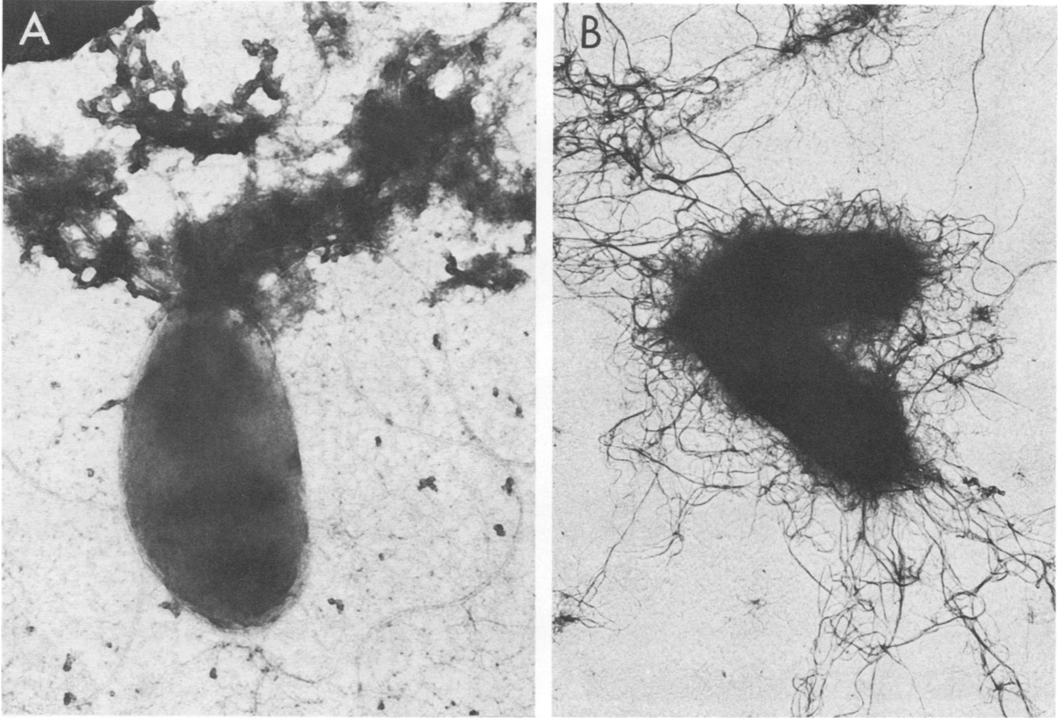

FIG. 7. Transmission electron micrographs of *R. trifolii* 0403 incubated for 4 days in the simulated clover rhizosphere and stained by the glutaraldehyde-ruthenium red-uranyl acetate method. (A) Cells devoid of uniform capsules and having only exocellular fibrils at one pole (×23,500); (B) control cells incubated in sterile Fahraeus medium only and retaining a dense, fibrillar capsule (×12,000).

only one cell pole. We propose that the activity of these host proteins (enzymes and trifoliin A) contributes to the polarity of the lectin-binding capsule in a way which favors the polar attachment of *R. trifolii* to clover root hairs (12, 17, 21, 24, 31, 35).

The modification of the capsular and extracellular polysaccharides by these host enzymes was demonstrated by immunofluorescence, electron microscopy, and immunoelectrophoresis. The most straightforward interpretation of the immunoelectrophoresis result (Fig. 10) is that intermediate products of the enzymatic reaction carry less negative charge and antigenic reactivity than does the polysaccharide substrate. Any interpretation would have to account for the lack of detectable change in reducing sugar equivalents upon conversion of the extracellular polysaccharides to the respective products under the conditions described. We would have anticipated an increase in reducing sugar equivalents of the reaction mixture if glycosidases were solely responsible for the results observed. However, since neither the chemical nature of the enzymatic activity nor the products of the reaction are known at this time, it is premature to completely rule out this possibility.

Clover root exudate from axenically grown seedlings was fractionated to determine whether trifoliin A itself was responsible for the activity which altered the capsule of *R. trifolii*. Such was not the case. Trifoliin A isolated from root exudate (Fig. 11, peak 2) remained bound to the capsule after many hours of incubation and gave no indication of an alteration of the capsule surrounding the cells (Table 2). By contrast, other proteins in root exudate (Fig. 11, peak 1) which did not bind to anti-trifoliin A IgG did alter the capsule of cells (Table 2), producing an intermediate stage in which trifoliin A receptors were localized at one cell pole. Since the activity in root exudate responsible for the alteration of the capsule was only found in peak 1, we conclude that these enzymes are antigenically unrelated to trifoliin A and that trifoliin A is not an enzyme which degrades the capsule of *R. trifolii* 0403. Furthermore, separate studies (J. E. Sherwood, G. L. Truchet, and F. B. Dazzo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K79, p. 149; G. L. Truchet, J. E. Sherwood, and F. B. Dazzo, manuscript in preparation) have shown that particles containing trifoliin A isolated from root exudate bind to encapsulated cells without subsequent alteration of the capsule.

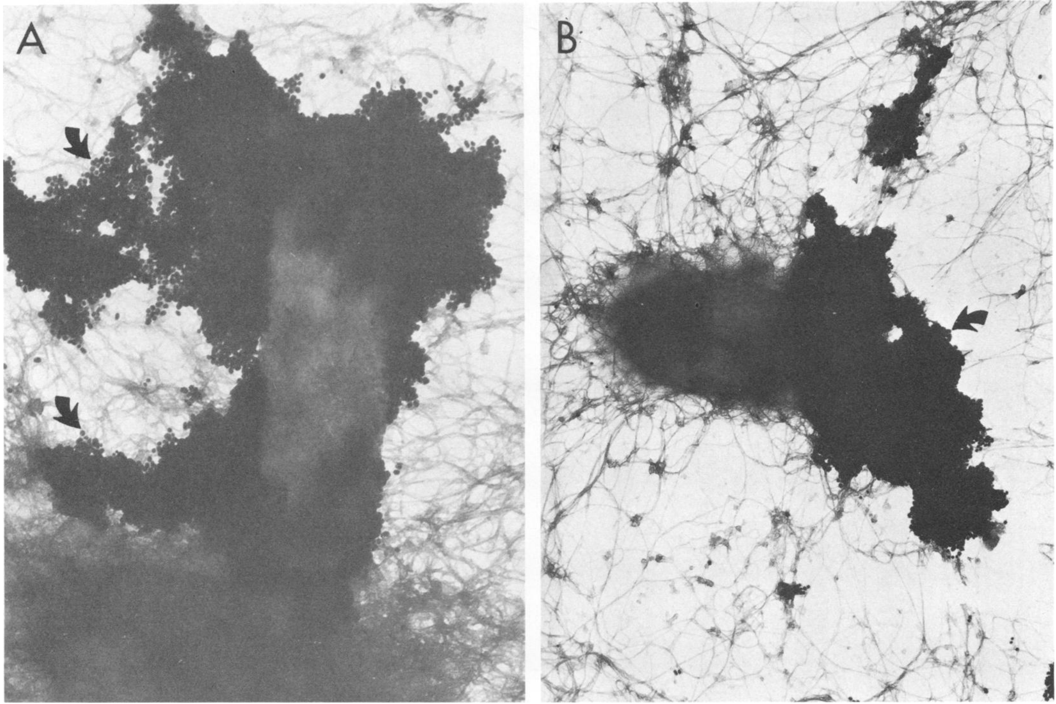

FIG. 8. Immunoelectron microscopic localization of trifoliin A on *R. trifolii* 0403 after 4 days of incubation in the clover root environment. Cells were treated with the anti-trifoliin A IgG–colloidal gold complex and then contrasted by the glutaraldehyde-ruthenium red-uranyl acetate method. (A) Trifoliin A detected on fibrils at both cell poles (arrows) (×15,900); (B) trifoliin A detected on fibrils at one cell pole (arrow) (×20,000). Uncoupled gold colloid did not bind to the cells from the simulated rhizosphere, and cells suspended in Fahraeus medium for 4 days did not bind the anti-trifoliin A IgG–colloidal gold complex.

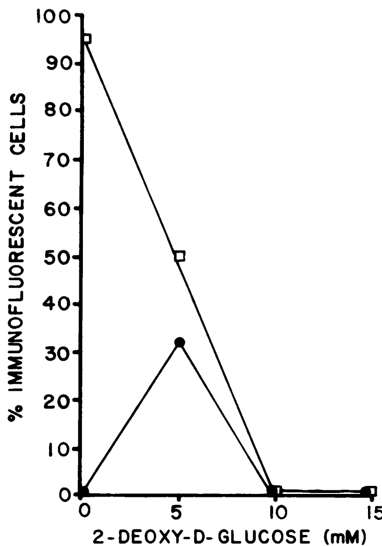

FIG. 9. Hapten inhibition of trifoliin A binding to encapsulated cells of *R. trifolii* 0403 by 2-deoxy-D-glucose showing percentage of cells with uniform (□) or polar (●) fluorescence.

Various glycosidase activities have been detected previously in clover root exudates (6, 7, 22). In addition, root extracts of white clover (*T. repens*) and peas (*Pisum sativum*) contain glycosidases which act specifically on capsular polysaccharides from homologous, compatible rhizobia (K. E. Fjellheim and B. Solheim, personal communication). We found a wide variety of glycosidase activities in the peak 1 fraction of clover root exudate (Table 4), the same fraction which alters the capsule of *R. trifolii* 0403. Conversely, trifoliin A isolated from clover root exudate did not contain any of the glycosidase activities found in the peak 1 fraction (Table 4). Thus, the trifoliin A in root exudate does not seem to belong to the same class of enzymatic phytohemagglutinins previously reported to occur in other legumes (2, 15, 16) or to fit the qualifications of the recently proposed lectin enzyme model (20).

Root exudates from clover seedlings grown with 15 mM KNO_3 instead of in nitrogen-free medium contain less trifoliin A (10) and seem to alter the extracellular polysaccharides of *R. trifolii* 0403 at a slower rate (Fig. 10A and B). A

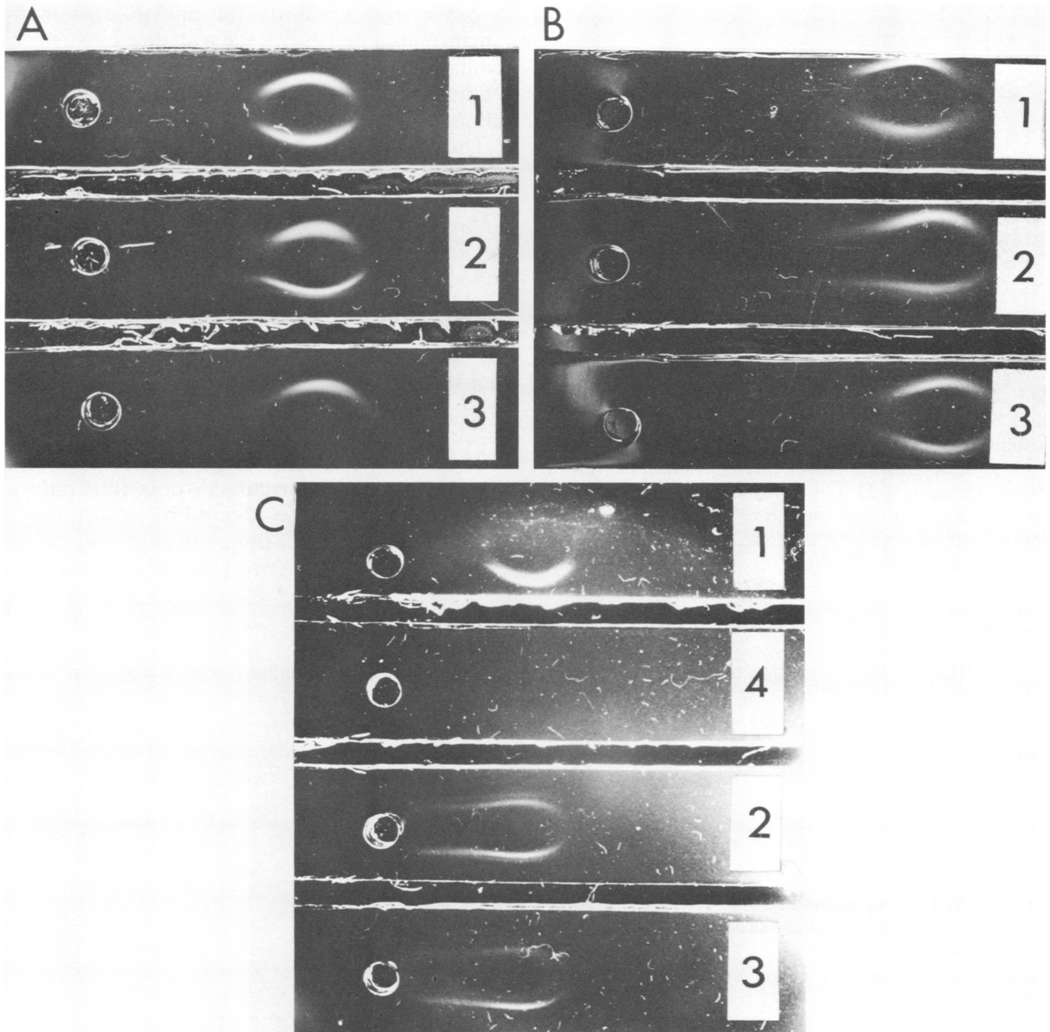

FIG. 10. Modification of extracellular polysaccharide antigen from *R. trifolii* 0403 after incubation with Ladino clover root exudate. Polysaccharides were incubated with root exudate for 1 (A), 2 (B), and 4 (C) days, electrophoresed through agarose, and immunoprecipitated with homologous antibody for 2 days. Concentrated root exudate was obtained after growth of seedlings in nitrogen-free rooting medium or this medium supplemented with 15 mM KNO_3. Tracks correspond to: (1) polysaccharide control (no root exudate treatment); (2) polysaccharide treated with nitrogen-free root exudate; (3) polysaccharide treated with nitrogen-supplemented root exudate; (4) root exudate control (no polysaccharide antigen added).

similar effect of combined nitrogen was found by Ljunggren (22), in which case less polygalacturonase activity was detected in both root exudates and root extracts of clover plants inoculated with *R. trifolii* if nitrate was added to the plant growth medium. These observations are of possible relevance to the regulation of host-symbiont recognition by combined nitrogen in the *R. trifolii*-clover symbiosis (8).

The alteration of the *R. trifolii* 0403 capsule in clover root exudate begins with a disorganization around the equatorial center of the cell and then proceeds toward the opposite cell poles at unequal rates (Fig. 2 to 7). This temporal sequence in the disorganization of the capsule was an unexpected result. A clue to explain the phenomenon was obtained by examining the binding of trifoliin A to encapsulated cells in the presence of 5 mM 2-deoxy-D-glucose. Under this condition, trifoliin A could only bind to one pole of many of the encapsulated cells (Fig. 9). Higher concentrations of this hapten completely inhibited the binding of trifoliin A to both encapsulated poles. These results indicate that trifoliin

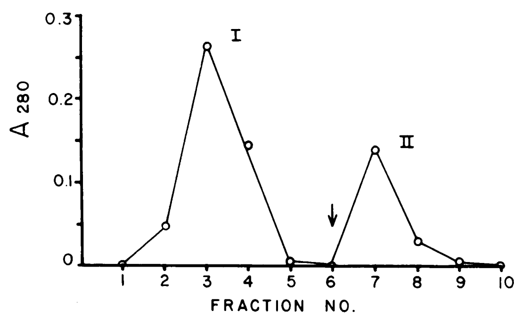

FIG. 11. Fractionation of concentrated root exudate (nitrogen-free, Ladino) by immunoaffinity chromatography on a column of anti-trifoliin A IgG coupled to CNBr-activated agarose. Unbound material (peak 1) was eluted with phosphate-buffered saline. Trifoliin A (peak 2) was eluted by a pH shift with glycine hydrochloride buffer (arrow) and collected in tubes containing 1 M Tris-hydrochloride (pH 7.4) for immediate neutralization. Similar chromatograms were produced with root exudate of Louisiana Nolin. A_{280}, Absorbance at 280 nm.

A has a higher affinity for one pole of many encapsulated cells. We suggest that clover root exudate disorganizes the capsule at the lower-affinity pole more rapidly than at the higher-affinity pole.

The difference in affinity of the lectin for opposite poles of fully encapsulated cells could explain the biphasic affinity binding curves of soybean seed lectin for some strains of *Rhizobium japonicum* (4). Some strains of *R. japonicum* have exopolysaccharide organized in the form of an "extracellular polar body" which has a higher affinity for the soybean seed lectin than does the opposite cell pole (5, 33). It has been shown that this polar organization of the capsule functions in the tip-to-tip attachment of cells in rosettes (33).

The activity of the capsule-modifying enzymes released from clover roots into natural rhizospheres could be affected by adsorption to soil particles, denaturation, and degradative processes. Nevertheless, we propose that the idea

TABLE 2. Reconstitution of activity in clover root exudate after immunoaffinity chromatography[a]

Peak fraction added first	Incubation time (h)	Subsequent treatment with peak 2 (1 h)[b]	Distribution of trifoliin A on *R. trifolii* 0403
1	1	−	None
2	1	−	Uniform
2	12	−	Uniform
1	4	+	Polar
1	8	+	None
2 + 30 mM 2-deoxy-D-glucose	1	−	None
2 + 30 mM 2-deoxy-D-galactose	1	−	Uniform
1 ("trypsinized")	4	+	Uniform
1 (boiled)	4	+	Uniform
None		−	None

[a] Root exudate was fractionated on anti-trifoliin A IgG–CNBr-activated agarose into peak 1 and peak 2 fractions (Fig. 11), treated as indicated in the table, incubated with fully encapsulated cells of *R. trifolii* 0403 heat fixed to microscope slides, and then assayed for trifoliin A by indirect immunofluorescence.

[b] −, No treatment; +, treatment.

TABLE 3. Effect of peak 1 fraction of clover root exudate on surface antigens of encapsulated *R. trifolii* 0403 as measured by immunofluorescence

Source of root exudate	Time of incubation of cells with fraction 1[a] (h)	% of fluorescent cells[b] after staining with homologous antisera against:		
		Sonicated cells	Capsular polysaccharide	Lipopolysaccharide isolated from early-stationary-phase cells
None	0	100	98	100
Louisiana Nolin	1	98	100	98
Louisiana Nolin	4	98	79	97
Louisiana Nolin	20	99	34	94
Ladino	1	100	94	100
Ladino	4	100	93	98
Ladino	20	99	54	97

[a] Ca, 20 μg of protein per ml.

[b] Calculated from the average of three microscope fields, ca. 130 cells per field.

TABLE 4. Glycosidase activity in fractions of Ladino clover root exudate

Enzyme activity assayed[a]	Sp act of root exudate fractions (enzyme units per mg of protein)[b]	
	Peak 1[c]	Peak 2[d]
β-D-Fucosidase	56.0	ND[e]
β-D-Glucosidase	50.0	ND
β-D-Glucuronidase	44.0	ND
α-D-Mannosidase	37.4	ND
β-*N*-Acetyl-D-galactosaminidase	37.0	ND
β-*N*-Acetyl-D-glucosaminidase	33.6	ND
α-L-Arabinosidase	33.6	ND
α-L-Fucosidase	29.0	ND
α-D-Galactosidase	27.4	ND
α-D-Glucosidase	26.6	ND
β-D-Galactosidase	14.6	ND
β-D-Xylosidase	3.1	ND
β-D-Galacturonidase	0.8	ND
6-Phospho-β-D-galactosidase	0	ND
Endo-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucosidase[f]	0	ND

[a] With *p*-nitrophenyl-substituted glycosides (1).
[b] One unit produces 1 μmol of product per h at 30°C.
[c] Represents protein which did not bind to agarose–anti-trifoliin A IgG column.
[d] Represents trifoliin A isolated by immunoaffinity chromatography.
[e] ND, No activity was detected.
[f] Substrate was *p*-nitrophenyl-α-D-maltoside.

of the rhizobial cell surface as an inert structure should be replaced by the concept of a dynamic entity in which certain polymers are altered and resynthesized during bacterial growth in the rhizosphere. Enzymes in the root exudate of the host legume may play a major role in this turnover, but probably because of the low amounts present, they have remained previously undetected. More studies are necessary to determine whether these capsule-modifying enzymes are the components in clover root exudate which regulate the rate of the infection process in the nitrogen-fixing *R. trifolii*-clover symbiosis (27, 32, C. A. Napoli and D. H. Hubbell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, N3, p. 176).

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ADDENDUM IN PROOF

The soybean lectin which specifically binds to *Rhizobium japonicum* is, like trifoliin A, distinct from the class of enzymatic lectins possessing α-galactosidase activity (E. D. Campello and L. M. Shannon, Plant Physiol. 69:628–631, 1982).

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