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Topical Review

Interactions of Lectins and their Saccharide Receptors in the *Rhizobium*-Legume Symbiosis*

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Introduction

The process of cellular recognition between microorganisms and higher plants is receiving considerable attention in light of its effect on plant morphogenesis, nutrition, and protection against infectious disease. These positive cellular recognitions are believed to arise from a specific union, reversible or irreversible, between chemical receptors on the surface of interacting cells (Burnet, 1971). This hypothesis implies that communication occurs when cells that recognize one another come into contact, and therefore the complementary components of the cell surfaces have naturally been the focus for most biochemical studies. Such is the case for studies on the infection of legume roots by the nitrogen-fixing symbiont, *Rhizobium*. According to the lectin-recognition hypothesis (Hamblin & Kent, 1973; Bohlool & Schmidt, 1974; Dazzo & Hubbell, 1975), specific, complementary lectin-polysaccharide interactions serve as a basis of host specificity in this nitrogen-fixing symbiosis.

There are many cellular recognition phenomena which occur during the infection of legume root hairs by *Rhizobium*, e.g., bacterial attachment, root-hair deformation, infection thread formation, infection thread growth directed by the root hair nucleus, etc. Lectin-mediated specific attachment of rhizobia to legume host root hairs has been the step of cellular recognition studied in greatest detail. During early stages of the infection process, the bacteria attach via hapten-reversible interactions, and then later become irreversibly anchored to the host cell. The ability of the bacteria to attach to root hairs is controlled by Roa (root hair attachment) genes (Vincent, 1980), which occur on large, transmissable plasmids (Zurkowski, 1980). Roa^- phenotype is illustrated by noninfective mutant strains which are defective in hapten-specific attachment steps (Dazzo, Napoli & Hubbell, 1976; Zurkowski, 1980; Kato, Maruyama & Nakamura, 1981; Paau, Leps & Brill, 1981). However, recent studies have clearly shown that successful infection of root hairs by rhizobia requires additional events of cellular recognition. An understanding of the recognition code to host specificity in the *Rhizobium*-legume symbiosis could provide ways to broaden the range of agricultural crops which can enter efficient nitrogen-fixing associations. Studies on lectin-*Rhizobium* interactions have been exhaustively reviewed (Broughton, 1978; Sequeira, 1978; Schmidt, 1979; Dazzo, 1980*a–d*; Kauss, 1980; Dazzo, 1981*a, b*; Bauer, 1981; Graham, 1981; Dazzo, 1982; Dazzo & Hubbell, 1982; Kijne & van der Schaal, 1982). In this article, we highlight some of the earlier work (1970's), and then expand on more recent studies which focus on lectin-saccharide interactions in specific rhizobial attachment, with particular emphasis on how this process of cellular recognition is regulated.

Infection of Legume Root Hairs by *Rhizobium*

Rhizobium is a genus of Gram negative bacteria that selectively infects legume roots and then forms root nodules (Fig. 1*a*) that "fix" atmospheric nitrogen into ammonia, which becomes immediately available to the plant for growth. The infection process is very selective for certain combinations of rhizobia and legume, and this high degree of host-range specificity is used to define the various

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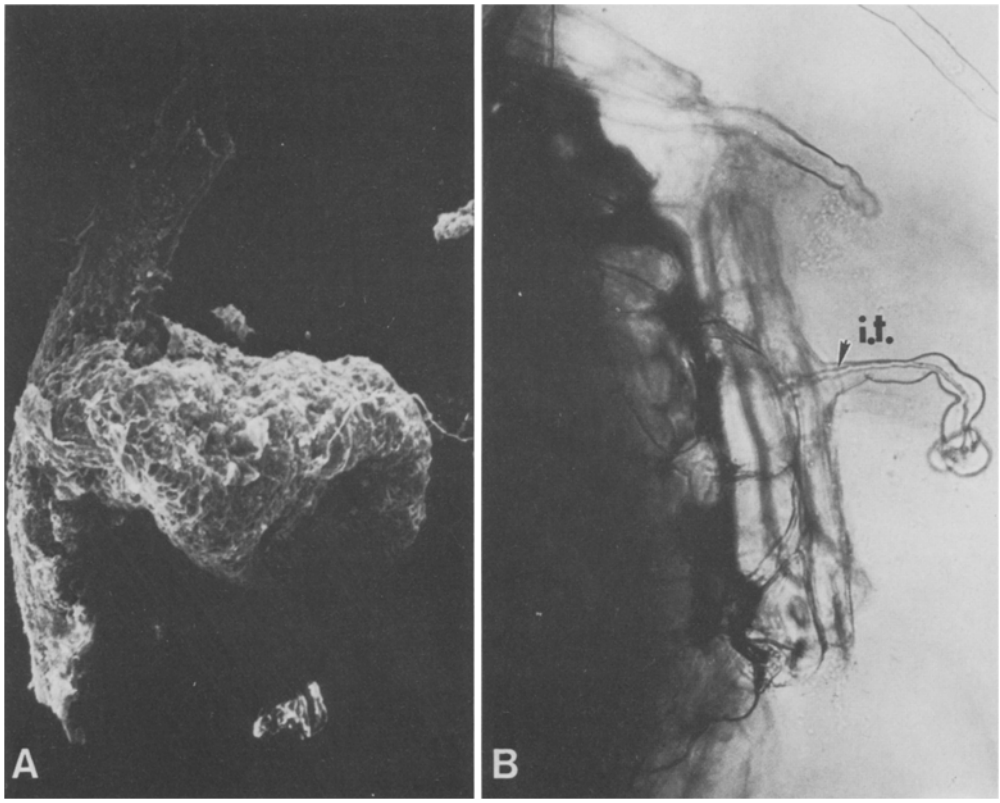


Fig. 1. Infection of legume roots by *Rhizobium*. (*A* Scanning electron micrograph of a root nodule on an alfalfa root. 43×. (*B*). The infection process in the *Rhizobium trifolii*-clover symbiosis begins with bacterial attachment, curling, and penetration of the host root hair. The bacterial, confined to the tubular infection thread (*I. T.*) are transported to the base of the root hair, where they penetrate into the root and form the nodules which fix nitrogen into ammonia (phase contrast micrograph). 630×

species of *Rhizobium*. For example, *R. trifolii* infects clover root hairs (Fig. 1*b*) and *R. meliloti* infects alfalfa root hairs. Neither species infects root hairs of the heterologous plant host. Successful infection of legume roots by these nitrogen-fixing bacteria in soil is of immense importance in the nitrogen cycle on earth.

Bacterial Attachment is an Early Recognition Step of Root-Hair Infection

Rhizobium attaches to the root hairs that are later infected. Quantitative light microscopic assays (Dazzo et al., 1976; Dazzo, 1980*a*) and transmission electron microscopic studies (Dazzo & Hubbell, 1975; Napoli & Hubbell, 1975; Kumarasinghe & Nutman, 1977; Dazzo, 1980*c*) of the *Rhizobium*-clover symbiosis have revealed multiple mechanisms of bacterial attachment to the root hairs. A nonspecific mechanism allows all species of rhizobia to attach in low numbers (2–4 cells per 200 μm root hair length per 12 hr using low inoculum per seedling). In addition, a specific mechanism allows selective attachment in significantly ($P=0.005$) larger numbers (22–27 cells per 200 μm root hair length per 12 hr) under identical conditions (Dazzo et al., 1976). Host-specific attachment has also been demonstrated in *R. japonicum*-soybean (Stacey, Paau & Brill, 1980) and *R. leguminosarum*-pea root systems (Kato et al., 1980). *R. japonicum* also selectively attaches to soybean root cells in suspension culture (Reporter, Raveed & Norris, 1975). However, specificity was not found in quantitative root attachment studies employing very high densities of radiolabelled rhizobia [10^9–10^{10} cells per seedling (Chen & Phillips, 1976)], but many unattached bacteria could have contributed to this result. In a recent study employing marble chips to dislodge bacteria attached to the root system and quantitative plating assays, "firm" attachment was found to be host-specific in *R. trifolii*-clover and *R. meliloti*-alfalfa systems, and "loose" attachment was nonspecific (Van Rensburg & Strijdom, 1982). It is therefore apparent that bacterial attachment to host roots is an

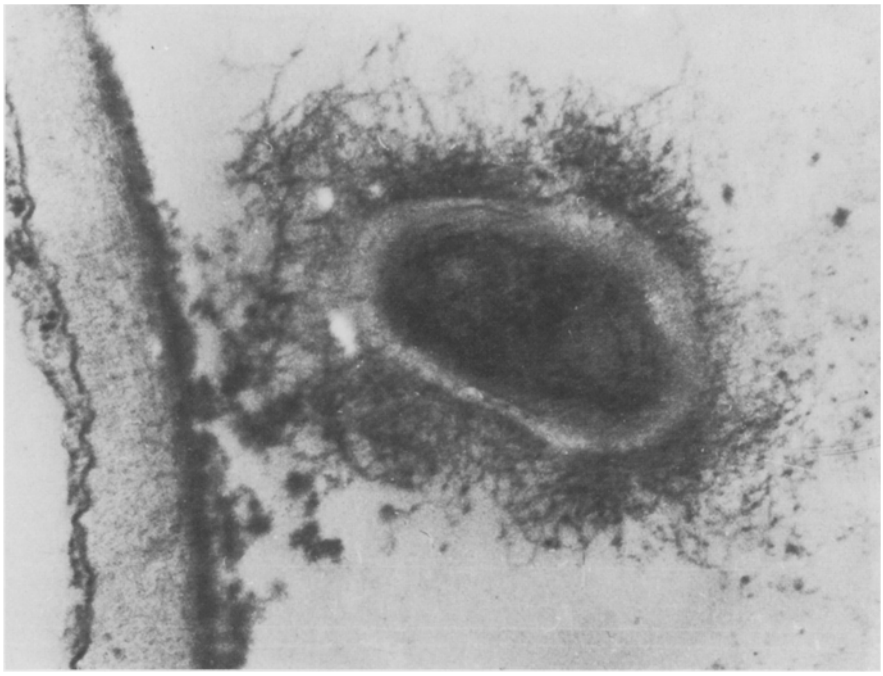


Fig. 2. Docking stage of Phase 1*a* attachment of *Rhizobium trifolii* NA-30 to the clover root hair cell wall (transmission electron micrograph). Note the fibrillar capsule which contacts electron-dense aggregates on the outer periphery of the root hair cell wall. (From Dazzo and Hubbell (1975), and courtesy of the American Society for Microbiology. 4,000×

early expression of cellular recognition in the *Rhizobium*-legume symbiosis.

Phase I Attachment

Transmission electron microscopy (Dazzo & Hubbell, 1975) disclosed that the initial bacterial attachment step consisted of contact between the fibrillar capsule of *R. trifolii* and electron-dense globular aggregates lying on the outer periphery of the clover root hair cell wall (Fig. 2). This "docking" stage is the first point of physical contact between the microbe and the host (Phase I Attachment) and occurs within minutes after inoculation of encapsulated cells of *R. trifolii* on the host clover.

Our strategy to identify the cell surface molecules involved in Phase I attachment has been to examine the surface components of the bacterium and the host that interact with the same order of specificity as is observed with the adhesion of the bacterial cells. Immunochemical and genetic studies have demonstrated that the surfaces of *R. trifolii* and clover epidermal cells contain a unique carbohydrate antigen that is immunochemically cross-reactive (Dazzo & Hubbell, 1975; Dazzo & Brill, 1979), suggesting its structural relatedness on both symbionts. This antigen contains receptors that bind hapten-reversibly to a multivalent clover lectin called trifoliin A (originally trifoliin) which has been isolated from seeds and seedling roots (Dazzo, Yanke & Brill, 1978; Dazzo & Brill, 1979). A specific hapten inhibitor of trifoliin A binding to these receptors is 2-deoxy-D-glucose (Dazzo & Hubbell, 1975; Dazzo & Brill, 1977). The first clue that trifoliin A on the root may be involved in rhizobial attachment came from the observation that 2-deoxy-D-glucose specifically inhibited the attachment of *R. trifolii* to clover root hairs (Dazzo et al., 1976), reducing the high level of bacterial adhesion to that characteristic of background. Subsequent studies showed that 2-deoxy-D-glucose specifically facilitated the elution of trifoliin A from the intact clover root (Dazzo & Brill, 1977; Dazzo et al.; 1978) and inhibited the binding of *R. trifolii* capsular polysaccharide to clover root hairs (Dazzo & Brill, 1977). In contrast, 2-deoxy-D-glucose did not inhibit adsorption of *R. meliloti* or its capsular polysaccharide to alfalfa root hairs (Dazzo et al., 1976; Dazzo & Brill, 1977). Consistent with the above results in the *R. trifolii*-clover system, lectins on pea, alfalfa, and soybean roots accessible for binding to the appropriate rhizobia have been demonstrated (Gatehouse & Boulter, 1980; Kato et al., 1980, 1981; Kijne, van der Schaal & deVries, 1980; Gade et al., 1981; Stacey et al., 1980; Paau et al., 1981; van der Schaal & Kijne, 1981; Kijne, 1982). In addition, specific hapten-facilitated elution of lectin from pea, alfalfa, and soybean roots has also been found (Kijne et al., 1980; van der Schaal & Kijne, 1981; Gade et al., 1981; W. Kamberger, *personal commu-*

nication). In such hapten-facilitated elution techniques, it is believed that the sugar acts by combining specifically with the site on the lectin which is normally occupied by the natural saccharide receptor. This implies a close but not necessarily identical structure of the hapten and the native determinant. However, some lectins undergo conformational changes when associated with saccharide binding (Reeke et al., 1975), and so this possibility must also be considered in the interpretation of hapten inhibition studies.

Lectin Cross-Bridging Model

Dazzo and Hubbell (1975) proposed a model to explain this early recognition event of Phase I attachment on the clover root hair surface prior to infection. According to this hypothesis, the multivalent trifoliin A (it agglutinates cells) recognizes similar saccharide residues on *R. trifolii* and clover and cross-bridges them. This complementary interaction forms the correct molecular interfacial structure that inititates the preferential and specific adsorption of the bacteria to the root hair surface. Therefore, the lectin may also function as a "cell recognition molecule" since it could feasibly influence which cells associate in sufficient proximity to the root hairs to allow subsequent specific recognition steps to occur. Figure 3 is a revision of the original cross-bridging model, which takes into account the recent finding that *R. trifolii* has multiple receptors for trifoliin A on its surface (described in more detail below).

The model predicts that there exists host-specific receptor sites on the legume root which interact specifically with surface molecules of the rhizobial symbiont. A key experiment that demonstrated these receptor sites and localized them on the root surface was performed by first labeling the trifoliin A-binding capsular polysaccharide of *R. trifolii* with the fluoresescent dye fluorescein isothiocyanate (FITC), incubating the conjugate with sterile seedling roots for a brief period, and then examining the roots by epifluorescence microscopy (Dazzo & Brill, 1977). The receptor sites on clover roots that immediately bound the FITC-capsular polysaccharide from *R. trifolii* were located at discrete root hair sites that had differentiated on the epidermal root surface (Fig. 4). They accumulate at root hair tips and diminish toward the base of the root hair. This unique location exactly matched both the distribution of trifoliin A on the surface

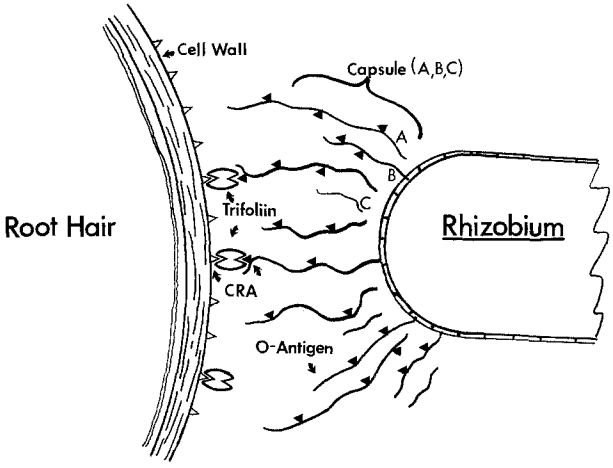
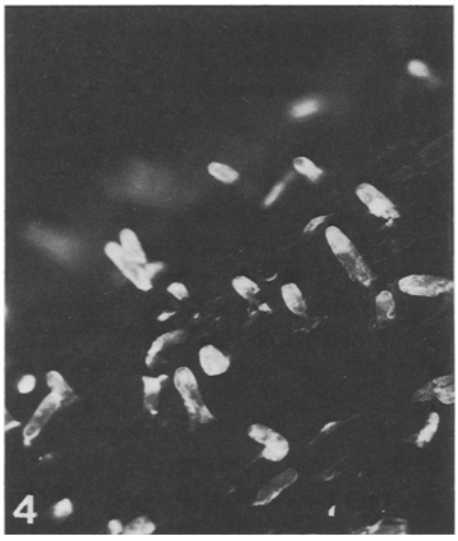


Fig. 3. Proposed cross-bridging of *Rhizobium trifolii* receptors to clover root hairs by the host lectin, trifoliin A. Modified from Dazzo and Hubbell (1975)



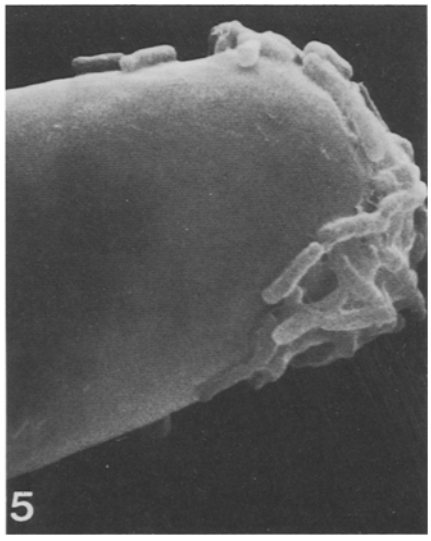


Fig. 4. Specific binding of FITC-labeled capsular polysaccharide from *Rhizobium trifolii* 0403 to clover root hairs (epifluorescence micrograph). (From Dazzo and Brill (1977), and courtesy of the American Society for Microbiology.) 321 ×

Fig. 5. Scanning electron micrograph of encapsulated *Rhizobium trifolii* 0403 attached to the tip of a clover root hair after short-term incubation. (From Dazzo and Brill (1979), and courtesy of the American Society for Microbiology.) 7,500 ×

of the clover seedling root (Dazzo et al., 1978) and the sites that immediately bound encapsulated *R. trifolii* in Phase I attachment (Fig. 5, Dazzo & Brill, 1979). The result also highlighted the importance of epidermal cell differentiation in the development of receptor sites that recognize rhizobia. Close inspection of the photomicrographs revealed that undifferentiated epidermal cells in the root hair region did not bind the bacterial polysaccharide, whereas epidermal root hair primordia had this surface property (Fig. 4).

Specificity of these receptor sites was demonstrated by the ability of unlabeled capsular polysaccharide from *R. trifolii* but not from *R. meliloti*, to block the binding of the labeled polysaccharide to clover root hairs. Similar specific binding of bacterial polysaccharides to legume host root hairs has been demonstrated in the *R. meliloti*-alfalfa (Dazzo & Brill, 1977), *R. leguminosarum*-pea (Kato et al., 1980), and *R. japonicum*-soybean systems (Hughes, Leece & Elkan, 1979; Hughes & Elkan, 1981).

The results of immunochemical and genetic studies suggest that trifoliin A and cross-reactive anti-clover root antibody bind to the same or similar overlapping saccharide determinants on *R. trifolii*. First, the antibody and the lectin bind specifically to the same isolated polysaccharides from *R. trifolii* (Dazzo & Brill, 1979; Hrabak, Urbano & Dazzo, 1981). Second, this interaction is specifically inhibited by the hapten, 2-deoxy-D-glucose. Third, the genetic markers of *R. trifolii* that bind trifoliin A and the antibody cotransform into *Azotobacter vinelandii* with 100% frequency (Bishop et al., 1977). And fourth, monovalent Fab fragments of IgG from anti-clover root antiserum strongly block the binding of trifoliin A to *R. trifolii* (Fig. 6, Dazzo & Brill, 1979). Considered collectively, these studies suggest that *R. trifolii* and clover roots have similar saccharide receptors for trifoliin A. However, the definitive test of their identity as antigenically related structures will require knowledge of the minimal saccharide sequence that binds the clover lectin.

The results of three experiments indicate that trifoliin A and antibody to the cross-reactive antigen bind to the same *R. trifolii* saccharide determinants that bind these bacteria to clover root hairs (all in Dazzo & Brill, 1979). First, Fab fragments of anti-clover root IgG blocked Phase I attachment of *R. trifolii* to clover root hairs. Second, only the *A. vinelandii* hybrid transformants that carried the trifoliin A receptor bound to clover root hairs in Phase I attachment assays (Fig. 7). Third, competition assays using fluorescence microscopy indicated that the *R. trifolii* polysaccharides that bound trifoliin A had the highest affinity for clover root hairs.

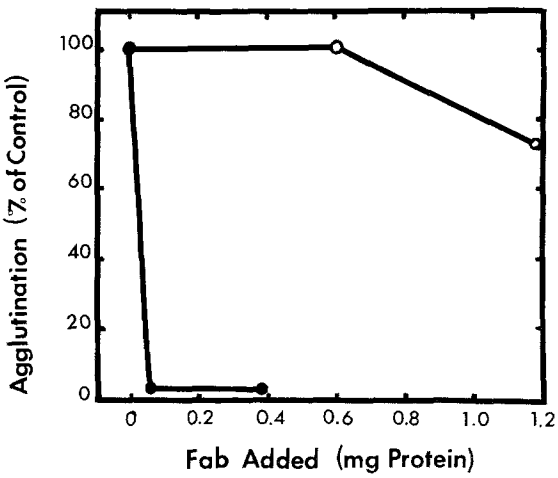


Fig. 6. Effect of Fab of immune anti-clover root antigen (•) and of preimmune serum (o) on trifoliin A-mediated agglutination of *Rhizobium trifolii* 0403. Washed cells were pretreated with Fab, washed, and assayed for agglutination with purified trifoliin A. The specific agglutination activity of the uninhibited control was 9,142 units per mg protein. (From Dazzo and Brill (1979), and courtesy of the American Society for Microbiology)

As with the *R. trifolii*-clover system (Dazzo et al., 1976; Dazzo, 1980*a*; Zurkowski, 1980), heterologous rhizobia (Kato et al., 1980) or non-nodulating mutant strains of *R. leguminosarum* which produce less extracellular/capsular polysaccharide (Saunders et al. 1978; Napoli & Albersheim, 1980) adhere in smaller numbers to pea root hairs as compared with the wild-type nodulating strains (Kato et al., 1980, 1981; C. Napoli, *personal communication*).

Phase II Adherence

Phase II adherence is characterized by the firm anchoring of the bacterial cell to the root hair surface (Dazzo, 1980*c*; Dazzo et al., 1981). Phase II adherence may be important in maintaining the firm contact between the bacterium and the host root hair necessary for triggering the tight root hair curling (shepherd's crook formation) and successful penetration of the root hair cell wall during infection (Napoli, Dazzo & Hubbell, 1975).

During Phase II adherence, fibrillar materials, recognized by scanning electron microscopy, are characteristically found associated with the adherent bacteria (Fig. 8). The nature of these microfibrils is unknown. One possibility is that they are bundles of cellulose microfibrils, known to be produced by many rhizobia (Deinema & Zevenhuizen,

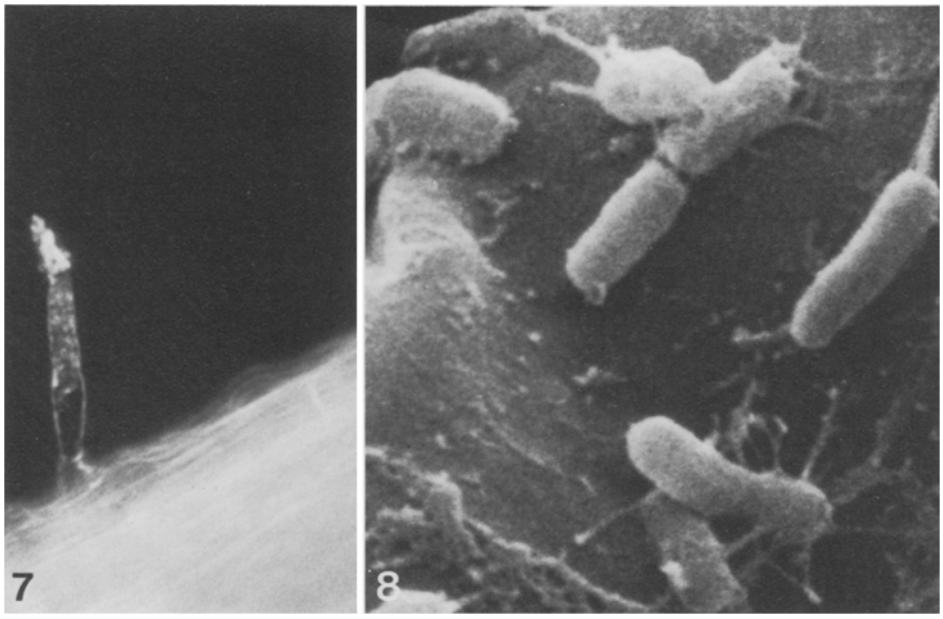


Fig. 7. Binding of *Azotobacter vinelandii* hybrid cells to clover root hair tips. Strain RtAv 10–54 is transformed with DNA from *Rhizobium trifolii* 0403 and carries the *R. trifolii*-specific clover root trifoliin A receptor. Hybrid transformants which did not bind trifoliin A failed to attach to clover root hairs. (From Dazzo and Brill (1979), and courtesy of the American Society for Microbiology.) 700 ×

Fig. 8. Scanning electron micrograph of aggregated microfibrils associated with *Rhizobium trifolii* 0403 firmly attached to the clover root hair surface after prolonged incubation (Phase 2 adhesion). 20,000 ×

1971; Napoli et al., 1975). Another possibility is that they are collections of pili, which have been recently demonstrated in *Rhizobium* (Stemmer & Sequeira, 1981; *see also* Kijne et al., 1982). Future studies should be directed to isolate and characterize these fibrils associated with the adherent bacteria in order to better understand the Phase II adhesion process. This is particularly important in light of the recent demonstration that the degree of host-specific firm attachment of rhizobial strains to the root shows a significant positive correlation with the degree of their success in interstrain competition for nodule sites on the root (Van Rensburg & Strijdom, 1982).

Rhizobial Attachment is Only a Piece of the Puzzle

Although attachment of infective rhizobia to target root hairs is a prerequisite for infection, several observations indicate that other undefined events must occur to initiate root hair infection. First of all, very few root hairs to which infective rhizobia attach eventually become infected. This may be due to a transient susceptibility of the root hairs to infection by the rhizobial symbiont (Bhunvaneswari, Turgeon & Bauer, 1980; Bhuwaneswari, Bhagwat & Bauer, 1981). Secondly, genetic hybrids of *A. vinelandii*, which carry the trifoliin A-binding saccharide receptor on their surface as a result of intergeneric transformation with DNA from *R. trifolii* (Bishop et al., 1977), have acquired the ability to adhere specifically to clover root hairs (Fig. 7, Dazzo & Brill, 1979) but do not infect them. Finally, although mutant strains which fail to bind the host lectin neither attach well to the host root hairs nor infect them (Dazzo et al., 1976; Paau et al., 1981), another class of noninfective mutant strains has been shown to bind the host lectin and attach to the host root hairs (Kamberger, 1979; Paau et al., 1981). Each of these cases serves to illustrate the importance of lectin-mediated root hair attachment to the infection process, but makes it clear that other post-attachment events of cell recognition must occur to advance the infection process to the stage of root hair penetration. Possible genes or gene products which may not have been expressed in the above situations include those controlling cell-wall hydrolytic enzymes (Hubbell, Morales & Umali-Garcia, 1978; Martinez-Molina, Morales Hubbell, 1979), inducers of host polygalacturonase (Ljunggren & Fahraeus, 1961; Palomares, Montega & Olivares, 1978), root hair curling factors (Yao & Vincent, 1976), and peri-

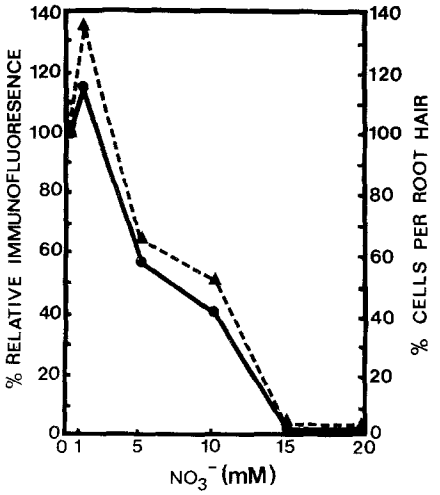


Fig. 9. The effect of NO_3^- on adsorption of *Rhizobium trifolii* 0403 to root hairs (solid line) and on immunologically detectable trifoliin A (dotted line) in the root hair region of clover seedlings. Bacterial adsorption was measured by direct microscopic counting and trifoliin A was measured by cytofluorimetry using indirect immunofluorescence. Values from roots grown in nitrogen-free nutrient solution are taken as 100% and represent 980 photovolts/mm^2 and 21 cells/root hair 200 μm in length. Points along the curve are means from 10–15 root hairs or seedling roots, standard deviations vary within 10% of the means. Values are corrected for nonspecific adsorption of conjugated goat antirabbit gamma globulin. (From Dazzo and Brill (1978), and courtesy of the American Society of Plant Physiologists)

plasmic extrinsic substance ES-6000, which promotes root hair infection (Higashi & Abe, 1980*a*).

Lectins and their Saccharide Receptors are Regulated by Combined Nitrogen

Combined nitrogen limits the development of the *Rhizobium*-legume root nodule symbiosis. For instance, white clover becomes resistant to infection by *R. trifolii* when the roots are grown with 15 mM nitrate. In fact, nitrate supplied at critical concentrations inhibits all of the morphogenetic steps of the nodulation process known to require the bacterial symbiont (Truchet & Dazzo, 1982). Microscopic assays indicated that the specific binding of *R. trifolii* 0403 to clover root hairs and the levels of trifoliin A on these epidermal cells declined in parallel as the nitrate concentration was increased from 1 to 15 mM in the rooting medium (Fig. 9, Dazzo & Brill, 1978). The inhibition was due specifically to nitrate ion and 15 mM nitrate did not stunt seedling growth.

How does nitrate modulate levels of trifoliin A on clover roots? The first possibility tested (Dazzo & Hrabak, 1982) was that nitrate binds to trifoliin A and prevents its interaction with root walls or its detection by homologous antibody. Possible binding of nitrate to trifoliin A was examined by incubating trifoliin A with radioactive ^{13}N-nitrate (produced in a cyclotron), and then testing for the presence of a radioactive ^{13}N-nitrate/trifoliin A complex by selective molecular ultrafiltration. The results showed that nitrate does not bind to trifoliin A. Also, there was no deviation in the quantitative immunoprecipitin curve using trifoliin A as antigen and homologous anti-trifoliin A IgG as antibody in the presence of 15 mM nitrate. In addition, this concentration of nitrate caused no reduction in rhizobial attachment to clover root hairs in a 1-hr assay, in contrast with the significant reduction in rhizobial attachment to clover root hairs if the period of exposure to nitrate was extended to 12 hr (Dazzo & Brill, 1978). Furthermore, the specific agglutinating activity of trifoliin A was unaffected by 15 mM nitrate.

In summary, these results provide evidence that nitrate does not bind directly to trifoliin A or its glycosylated receptors in a way that would reduce the levels of this lectin on clover roots or block attachment of *R. trifolii* 0403 to root hairs. Rather, it is more likely that some intervening process, modulated by nitrate supply over periods greater than 1 hr, regulates these early recognition events of the infection process (Dazzo & Hrabak, 1982).

Other studies have shown that nitrate supply affects root cell wall composition (Dazzo et al., 1981; Diaz, Kijne & Quispel, 1981). For instance, nitrate supply increases the levels of extensin, the hydroxyproline-rich glycoprotein in root cell walls (Dazzo et al., 1981). Since rhizobia must penetrate the host cell wall, changes in the chemistry of the wall could have an important impact on the infection process. In addition, the accessibility of trifoliin A receptors on clover root cell walls is reduced when the plant is grown with nitrate. Isolated root cell walls were assayed for the ability to bind trifoliin A and reduce its agglutination titer with *R. trifolii* 0403. Walls from nitrogen-free grown plants adsorbed three-to-fourfold more trifoliin A agglutinating activity per mg dry wt of walls than of walls from roots grown with 15 mM nitrate (Dazzo et al., 1981). Nitrate supply also seem to affect the accumulation of pea lectin and its receptors on pea roots (Kijne et al., 1982). More studies are needed to determine how the accumulation of *Rhizobium*-binding lectins on legume root surfaces is regulated by combined nitrogen.

Since trifoliin A did not accumulate on or bind

well to root cell walls grown in 15 mM nitrate, we wondered if trifoliin A would be released from the roots and accumulate in root exudate. Thirtyfold higher levels of trifoliin A (per constant total protein concentration) were detected from root exudate of clovers grown under nitrogen-free conditions than when grown with 15 mM nitrate (Dazzo & Hrabak 1981). The presence of trifoliin A in root exudate of two white clover varieties was detected by an immunofluorescence assay and was confirmed by its purification using immunoaffinity chromatography (Dazzo & Hrabak, 1981; Dazzo et al., 1982b).

The presence of trifoliin A in clover root exudate which can bind to receptors on *R. trifolii* provides supporting evidence for a lectin recognition model proposed by Solheim (1975). According to this model, a glycoprotein lectin excreted from the legume root binds to the rhizobia. This active complex then combines with a receptor site on the root. Thus, both partners in the symbiosis could benefit from the discriminatory reaction of a cross-bridging lectin which could be either bound to a glycosylated receptor on the root hair cell wall (Dazzo & Hubbell, 1975) or released from the root to bind to the rhizobial cell (Solheim, 1975). This event would help to ensure that only the symbiotic bacterium could establish the proper intimate contact with the host cell required to trigger other recognition events that lead to successful infection. Combined nitrogen (e.g., nitrate) would play a role in regulating the recognition process as proposed by Solheim (1975).

Lectin Receptors are Transient on Rhizobium

The selective ability of *R. trifolii* to adhere to clover root hairs is also influenced by the accumulation of the saccharide receptor on the bacterium. Evidence supporting this hypothesis came from data that showed that the transient appearance of trifoliin A receptors on *R. trifolii* may influence the ability of these bacteria to attach to clover root hairs (Dazzo, Urbano & Brill, 1979). Cells grown on agar plates of a defined medium were most susceptible to agglutination by trifoliin A when they were harvested at 5 days of growth. In broth cultures, the antigenic determinants on the bacteria that are cross-reactive with clover roots were "exposed" for only short periods as cultures left their lag phase of growth and again as they entered stationary phase. Clover roots adsorbed the bacteria in greatest quantity when the cells were harvested from plate culture incubated for 5 days and

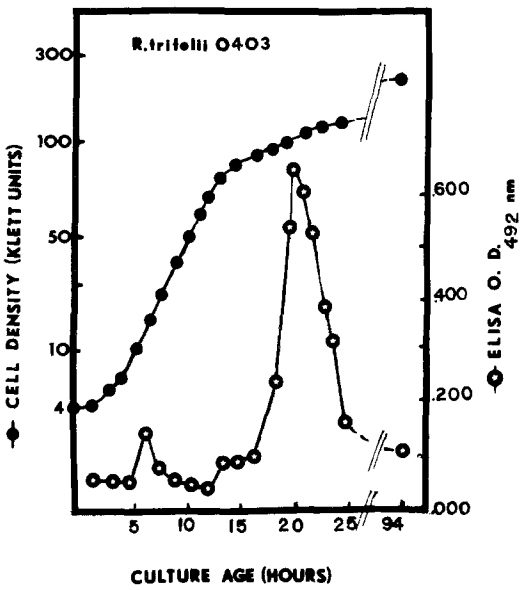


Fig. 10. Effect of culture age on the binding of antibody specific for unique determinants in lipopolysaccharide of *Rhizobium trifolii* 0403 in early stationary phase. Cells were grown in a chemically defined medium and monitored for cell density with a Klett-Summerson colorimeter (red filter). Samples were adjusted to 10^7 cells, and assayed by ELISA. From Hrabak et al. (1981), and courtesy of the American Society for Microbiology

from broth cultures in early stationary phase (Dazzo et al., 1979; G.L. Truchet, J.E. Sherwood and F.B. Dazzo, *in preparation*).

We recently found that growth-phase dependence for trifoliin A binding to *R. trifolii* in broth culture is related to the appearance of a unique determinant in the lipopolysaccharide (LPS) of the bacteria (Hrabak et al., 1981). As the culture advanced from exponential to early stationary phase, changes in the immunochemistry of the LPS were detected with antisera made specific for lipopolysaccharides of cells in early stationary phase by exhaustive adsorption with exponentially growing cells (Fig. 10). Gas chromatography and combined gas chromatography-mass spectrometry showed culture-phase dependent differences in the quantities of several glycosyl components (e.g., quinovosamine, which is 2-amino-2,6-dideoxyglucose) in the LPS that bound trifoliin A. D-quinovosamine, N-acetyl-β-D-quinovosamine, and its *n*-propyl-β-glycoside were found to be effective hapten inhibitors of trifoliin A. In addition, LPS increased in apparent size as the culture aged as shown by gel filtration chromatography. The new immunochemical determinants that occur in LPS as cells enter stationary phase were apparently recognition sites for trifoliin A binding, since immune monovalent

Fab fragments of IgG specific for these unique determinants block the agglutination of cells with trifoliin A. The potential importance of this finding to the infection process was suggested by root hair infection studies using standardized inocula. White clover plants had more infected root hairs after incubation with an inoculum of cells in the early stationary phase than with cells in the mid-exponential phase. As previously predicted (Dazzo & Brill, 1979), trifoliin A and the cross-reactive anti-clover root antibody bind to unique determinants in the LPS of *R. trifolii* 0403, which are not immunodominant and which appear for only a transient period on cells in batch culture (Hrabak et al., 1981). In plate culture, the development of the capsule on *R. trifolii* 0403 coincides with the appearance of trifoliin A receptors; and these receptors are transient on the cell since the encapsulated cells lose their ability to bind the lectin uniformly as the culture ages (Dazzo et al., 1979; G.L. Truchet, J.E. Sherwood, and F.B. Dazzo, *in preparation*).

There is a transient appearance and disappearance on *R. japonicum* of the receptor that specifically binds soybean lectin (Bhuvaneswari, Pueppke & Bauer, 1977; G.L. Truchet, J. Vasse, F.B. Dazzo, S.G. Pueppke, *submitted*). Most strains of *R. japonicum* have the highest percentage of soybean lectin-binding cells and the greatest number of soybean lectin-binding sites per cell in the early and mid-log phases of growth. The proportion of galactose residues in the capsular polysaccharide is high at a culture age when the cells bind the galactose-reversible soybean lectin (Mort & Bauer, 1980). A decline in lectin-binding activity of cells accompanying culture aging is concurrent with a decline in galactose content and a rise in 4-0-methyl galactose residues in the capsular polysaccharides. The latter methylated sugar has low affinity for the galactose-binding soybean lectin. These results suggest that the galactose residues in the capsular polysaccharide become methylated and, as a consequence, the cell loses its ability to combine specifically with the soybean lectin. Shedding of soybean lectin-binding capsular polysaccharides from the cells (Truchet et al., *submitted*) explains why the broth culture as a whole continues to bind soybean lectin (Tsien & Schmidt, 1980).

The profound influence of the growth phase on the composition of lectin-binding polysaccharides of *Rhizobium* may be a major underlying cause of conflicting data among laboratories testing the lectin-recognition hypothesis. Furthermore, the growth-phase dependent modifications of the lectin-binding polysaccharides of rhizobia (Mort & Bauer, 1980; Hrabak et al., 1981) may reflect mechanisms which regulate cellular recognition in the *Rhizobium*-legume symbiosis.

There are Multiple Lectin Receptors on Rhizobium

Rhizobium produces several different polysaccharides in pure culture, including acidic heteropolysaccharides, lipopolysaccharides, and neutral glucans. Their presence in crude extracts has necessitated the development of complex techniques for purification. Because of this, and the need to know when most cells in culture bind the lectin, most of the earlier work on polysaccharides from *Rhizobium* did not reveal information on the chemical nature of the saccharide receptor which binds the host lectin. A major controversy was whether the lectin receptor on *Rhizobium* was the capsular polysaccharide (Dazzo & Hubbell, 1975), LPS (Wolpert & Albersheim, 1976), or glycans (Planque & Kijne, 1977). The picture now emerging is that *R. japonicum* binds soybean lectin through its capsular and extracellular polysaccharides (Bal, Shantharam & Ratnam, 1978; Calvert, Lalonde, Bhuvaneswari & Bauer, 1978; Mort & Bauer 1980, 1982; Tsien & Schmidt, 1981; Truchet et al., *submitted*); *R. meliloti* binds alfalfa lectin through its LPS (Kamberger, 1979*a*; J. Handelsman, *personal communication*); and the related species *R. trifolii* and *R. leguminosarum* specifically bind clover and pea lectin, respectively, through both their extracellular/capsular polysaccharide and their LPS at certain culture ages (Kamberger, 1979*a*; Dazzo & Brill, 1979; Kijne et al., 1980; Kato et al., 1979, 1980, 1981; Hrabak et al., 1981). Similarily, peanut lectin binds LPS and capsular polysaccharide of peanut rhizobia (Bhagwat & Thomas, 1980). Since the compositions and immunodominant structures of LPS vary widely among strains of a single *Rhizobium* species (Carlson, Saunders, Napoli & Albersheim, 1978), the lectins from clover, pea, alfalfa, and peanut may be interacting specifically with a portion of the symbiont's LPS which is poorly immunogenic and common to different strains of the same *Rhizobium* species. The discovery that the lectin-binding sites on the polysaccharides of *R. trifolii* are not immunodominant is thus of paramount importance (Dazzo & Brill, 1979; Hrabak et al., 1981).

A powerful, new technique of specimen preparation for transmission electron microscopy has recently been developed for *Rhizobium* (Mutaftschiev, Vasse & Truchet, 1982). This technique

reveals the details of acidic polymer exostructures on the cell without introducing the artifacts and loss of capsular material associated with centrifugation (Fig. 11). The technique has been expanded to include lectin-markers in the form of colloidal gold-lectin conjugates (Fig. 12*a* and *b*, Truchet et al., *submitted*) and may be of value in further studies of mutant strains of rhizobia which fail to reveal capsules using the traditional method of negative staining with India ink followed by light microscopy (Rolfe et al., 1981; Law, Yamamoto, Mort & Bauer, 1982).

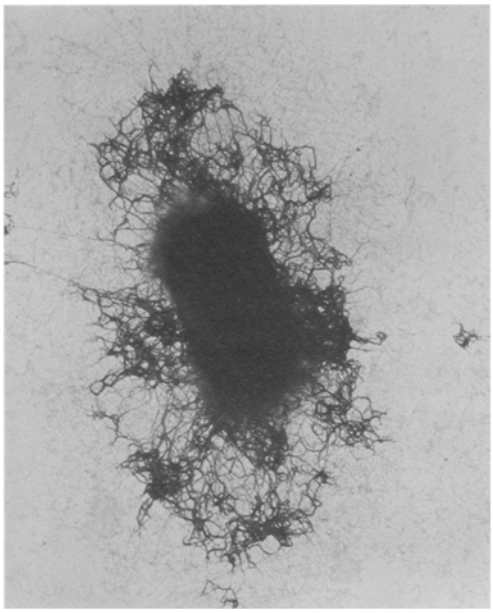


Fig. 11. Transmission electron micrograph of encapsulated cell of *Rhizobium trifolii* 0403 from 5-day-old cultures grown on BIII plates (defined medium), and then contrasted by the glutaraldehyde/ruthenium red/uranyl acetate method of Mutaftschiev et al. (1982). 28,000 ×

The presence of multiple lectin receptors on rhizobia raises the question of whether each one has a different role in root hair infection. Infection studies by Kamberger (1979*b*) suggest that the lectin cross-bridging hypothesis (Dazzo & Hubbell, 1975) needs to be modified. For example, the capsular polysaccharides could be responsible for attachment of high numbers of rhizobial cells to the target root hairs via cross-bridging lectins as an early recognition event. This would be followed by secondary recognition events requiring the host-range specific binding of lectin on localized sites of the root hair to LPS, which triggers subsequent invasive steps (Kamberger, 1979*b*). One challenge of the next few years is to test the validity of this hypothesis.

Host-Specificity Genes are Plasmid Encoded

Exciting evidence is beginning to emerge that genetic elements important to surface polysaccharides and symbiotic recognition are encoded on very large, transmissible plasmids of *Rhizobium* (Johnston et al., 1978; Zurkowski & Lorkiewicz,

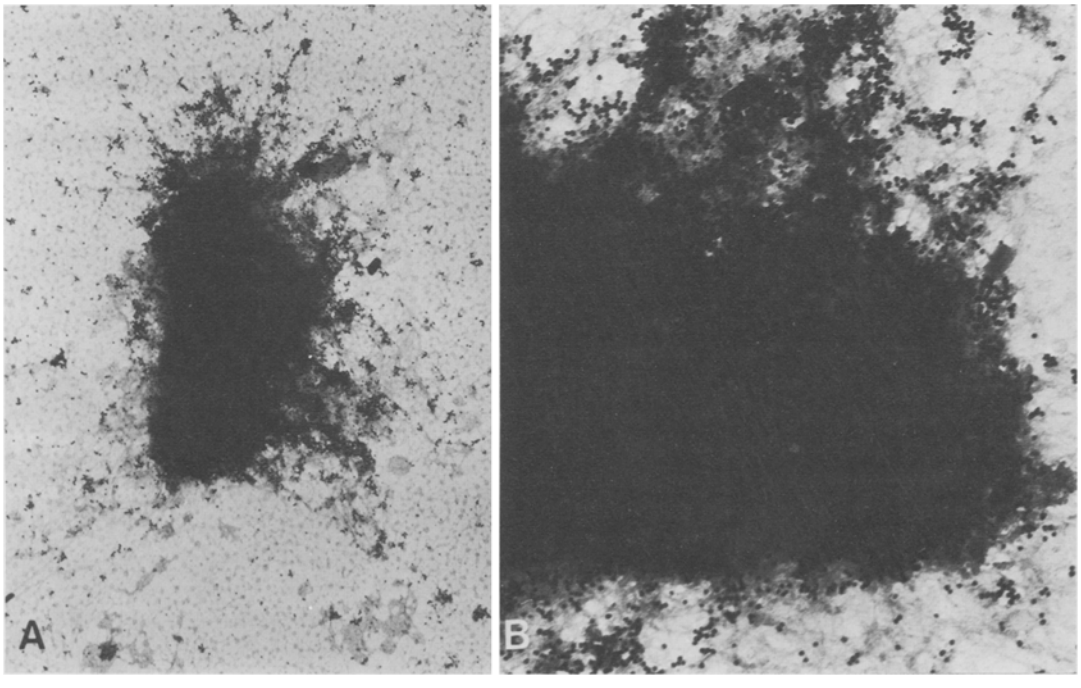


Fig. 12. Binding of trifoliin A-colloidal gold particles to an encapsulated cell of *R. trifolii* 0403 processed as in Fig. 11. Note binding of trifoliin A-gold colloid to the entire cell in *A* (32,000 ×) and its higher magnification in *B*. 88,000 ×

1979; Prakash et al., 1980; Zurkowski, 1980, 1981, 1982; Hooykaas et al., 1981; Rosenberg, Boistard, Denarie & Casse-Delbart, 1981; Bafalvi et al., 1981). For instance, the genes responsible for the 2-deoxy-D-glucose inhibitable attachment of *R. trifolii* to clover root hairs are encoded on the large nodulation plasmid designated pWZ2 (Zurkoski, 1980), and incorporation of the trifoliin A binding sugar, quinovosamine into the LPS of *R. trifolii* is controlled by the clover nodulation plasmid (Russa, Urbanik, Kowalczuk, & Lorkiwicz, 1982). The ability of *R. meliloti* to induce polygalacturonase production in alfalfa roots also seems to be controlled by a plasmid (Palomares et al., 1978). Genes required for interstrain competition of rhizobia for root nodulation and the synthesis of a unique 24,000 dalton protein expressed in the host rhizosphere are located on the pea nodulation plasmid of *R. leguminosarum* (Brewin, 1982). Conjugal transfer of the nodulation plasmid from *R. trifolii* to *Agrobacterium tumefaciens* results in a hybrid which nodulates clover roots (Hooykaas et al., 1981) and which binds trifoliin A (F.B. Dazzo, G.L. Truchet, P.J. Hooykaas, *in preparation*).

Analysis of symbiotically defective mutant strains of rhizobia is complicated by multiple pleiotropic effects when the mutated genes affect production of polysaccharides. For instance, the *R. leguminosarum* mutant strain, EXO-1, does not nodulate peas, and Saunders, Carlson, and Albersheim (1978) reported that 27% of its total LPS mass is anthrone-reactive carbohydrate, as compared to 63% of the total LPS mass from the wild-type *R. leguminosarum* strain from which it was derived. The glycosyl and antigenic compositions of the O-antigen of the mutant and wild-type strain do not seem to be different, and both strains are lysed by the same bacteriophages. The hypothesis advanced by the authors was that the mutant strain EXO-1 has reduced its production of extracellular polysaccharide (it excretes 5% of the amount of the wild type into the culture medium), but not of LPS (Saunders et al., 1978). However, and alternative hypothesis is that the EXO-1 phenotype could be due to a defective O-antigen polymerase, which would fail to polymerize in a block fashion the repeating O-antigen on the polyisoprenoid acyl carrier lipid (Osborne et al., 1972). When the O-antigen oligosaccharide is transferred to the R-core lipid A via the translocase reaction, an LPS with reduced O-antigen polymerization would result. Bacteriophage with receptors for the O-antigens could still recognize those defective LPS structures, and their glycosyl composition would be the same or similar, exactly matching the phenotype of these non-nodulating *R. leguminosarum* mutant strains described by Saunders et al. (1978).Polymerase mutations, as described above, which reduce the chain length of the O-antigen, cause pleiotropic negative effects on the biogenesis and assembly of the outer membrane of the Gram negative cell. The reduction in chain length of this carbohydrate moiety of the LPS causes a concomitant decrease and sometimes virtual loss of outer membrane proteins (Gmeiner & Schlecht, 1979). This pleiotropic negative effect complicates any direct interpretation of the significance of EXO-1 mutant strain to the infection process. Non-nodulating mutant strains of *R. japonicum* have been found that have discrete saccharide changes in the somatic antigens (Maier & Brill, 1978), and further analysis of these strains should determine what role LPS plays in root nodulation.

The Trifoliin A-Binding Capsule of *R. trifolii* is Altered by Enzymes Released from Clover Roots

The ultimate level of regulation of lectin receptors in the root environment is one in which both the bacterium and the host plant play key roles. For instance, Bhuvaneswari and Bauer (1978) showed that some strains of *R. japonicum* bind soybean lectin in the root environment but not in pure culture. This suggests that the host plays some role in expression of lectin-binding receptors on the rhizobial cells. Other strains of *R. japonicum* could bind soybean lectin better when grown in soil extract than in standard bacteriological media (Shantharam & Bal, 1981). These and our finding summarized below illustrate that an understanding of the biochemical basis of *Rhizobium*-legume interactions will require detailed studies of the microorganism in the rhizosphere of the host root as the normal case.

The first clue that the clover root environment altered the lectin receptors on *R. trifolii* 0403 came from detailed studies on the orientation of attachment of these bacteria to clover root hairs (Dazzo et al., 1976, 1981, 1982*b*; Dazzo & Brill, 1977; Dazzo et al., *submitted*).

If an inoculum of 10^9 fully encapsulated cells of *R. trifolii* (which bind trifoliin A uniformly around the cell) were incubated for 15 min with clover seedlings, cells attached with no preferred orientation to root hair tips (*see* Fig. 5). However, after 4 hr of incubation, additional cells began to attach along the sides of the root hair in a distinct polar orientation (Fig. 13). If lower inoculum densities were used (10^5–10^6 per seedling), most cells attached polarly to root hairs within 12 hr without preference to root hair tips (Dazzo et al., 1976).

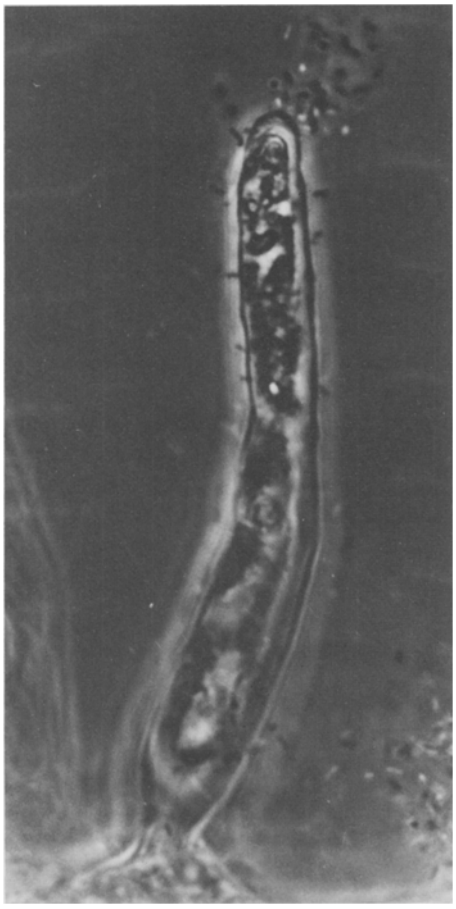


Fig. 13. Attachment of *Rhizobium trifolii* 0403 to a clover root hair after 4 hr of incubation with a high inoculum density. Note random attachment of bacteria to the root hair tip and distinct polar attachment of bacteria along the sides of the root hair (phase contrast micrograph). 2,533 ×

Why was there a delay in polar attachment of the bacteria when a high inoculum of uniformly encapsulated, lectin-binding cells was used? The answer was obtained by analyzing the effect of concentrated root exudate of clover seedlings on encapsulated cells of *R. trifolii*. We found that the lectin-binding capsule of cells was altered by enzymes released from axenically grown roots into the surrounding environment (Dazzo et al., 1982*b*). A summary of that work is described below.

Fluorescence microscopy showed that trifoliin A in clover root exudate bound uniformly to encapsulated, heat-fixed cells during 1 hr incubation on microscope slides. After 4–8 hr of incubation with root exudate, cells only had trifoliin A bound to one pole of the cell. 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-

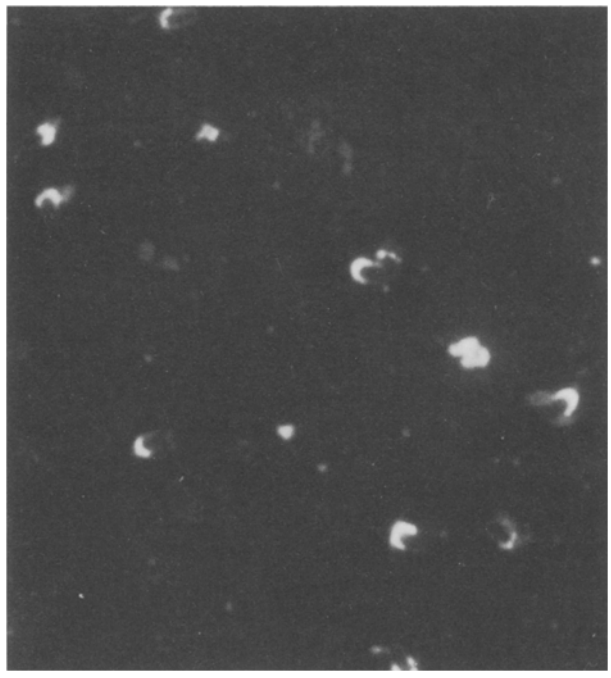


Fig. 14. Immunofluorescent detection of trifoliin A bound *in situ* to *Rhizobium trifolii* 0403 growing in the clover root environment of Fahraeus slide cultures. (From Dazzo et al. (1982*b*), and courtesy of the American Society for Microbiology.) 5,000 ×

shaped cell and then progressed towards the poles at unequal rates. Trifoliin A could no longer be detected on heat-fixed cells following 12 hr 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 Fahreaus slide cultures (Fig. 14). Inhibition studies using the hapten 2-deoxy-D-glucose showed that trifoliin A in root exudate had 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 from plants grown with 15 mM nitrate. 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 protein(s) in root exudate that induced this alteration of the capsule were 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

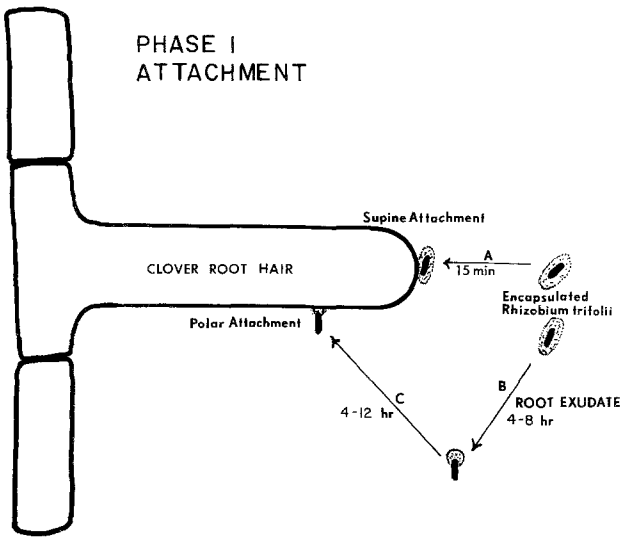


Fig. 15. Schematic diagram of the sequential events of Phase I Attachment of *Rhizobium trifolii* to clover root hairs, using an inoculum of fully encapsulated cells

suggest that enzymes, which are antigenically unrelated to trifoliin A, accumulate in root exudate and alter the trifoliin A-binding capsule in a way which would favor polar attachment of *R. trifolii* to clover root hairs.

Based on these rhizosphere studies, we have subdivided the Phase I attachment process of encapsulated *R. trifolii* to clover root hairs into the following sequential events (Fig. 15, Dazzo et al., 1981). (i) Most encapsulated cells which have trifoliin A receptors around the entire cell surface bind within minutes in a random orientation to clover root hair tips where trifoliin A accumulates. (ii) Cells which do not immediately contact the root hairs encounter enzymes in root exudate which modify their surface polysaccharides so that they become progressively less reactive with trifoliin A. This alteration proceeds less rapidly at one cell pole. Newly synthesized lectin-binding polysaccharide may also be deposited at one pole of nonencapsulated cells at a rate which may keep pace with the exudate enzyme-mediated modifications. (iii) Some cells with trifoliin A and/or its saccharide receptors bound to one pole eventually contact the cell wall along the sides of the root hair, where they then attach end-on.

Lectin is Involved in the Tip Adhesion of Root Hairs

The phenomenon of adhesion of root hair tips was recognized in early studies on the invasion of legume roots by *Rhizobium* (Fred, Baldwin & McCoy, 1932; Fahraeus, 1957). Such cell-cell adhesions are predicted by the lectin cross-bridging model (Fig. 3, Dazzo & Hubbell, 1975), where the multivalent lectin on one root hair tip would bind to complementary receptors accessible on an adjacent root hair. Quantitative microscopic studies (Dazzo, Truchet & Kijne, 1982*a*) have recently shown that root hair tip adhesions on clover seedlings grown under axenic conditions are generally restricted to a zone located 1 mm below the root hair closest to the hypocotyl and 2–3 mm above the meristem (Fig. 16*a*). Trifoliin A was localized by immunofluorescence at contact points of tip adhesions. Conditions known to reduce the levels of trifoliin A on the root surface (treatment without 2-deoxy-D-glucose or growth in medium containing 15 mM nitrate) significantly reduced the formation of root hair tip adhesions. These results suggest that trifoliin A is involved in the formation and/or stability of tip adhesions.

Root hair tip adhesions may have a physiological significance to the Rhizobium-legume symbiosis. Root hairs which sandwich rhizobia between tip adhesions frequently become infected with marked deformations (Fig. 16*b*, Napoli et al., 1975; Higashi & Abe, 1980*b*). In contrast, infected root hairs which develop separately demonstrate marked deformations such as shepherd's crooks when incubated with homologous rhizobia. In both cases, conditions optimal for rhizobial infection could occur in the microenvironment created by overlapping root hair cell walls.

We noted that some root hairs grown under axenic conditions developed marked curvatures at tip-to-tip adhesions (arrows, Fig. 16*a*). These curvatures were less tight than typical shepherd's crooks (*compare* with Fig. 1*b*), but may be a consequence of the same physiological process. Development of shepherd's crooks requires direct contact with homologous rhizobia (Yao & Vincent, 1976). It is possible that shepherd's crooks, as well as the tight curvatures of tip-to-tip root hair adhesions, are a consequence of the growth of root hairs about a fixed surface: the adherent bacterial floc or adjacent root hair tip. This process would be similar to what has been reported as the "contact guidance" system of pollen tube orientation during its passage through the style to the ovule of compatible flowering plants (Ferrari, Lee & Wallace, 1981).

Root Nodulation of Soybean Lines Lacking the 120,000-Dalton Lectin in their Seeds

Pull, Pueppke, Hymowitz, and Orf (1978) have identified five lines of soybean which lack

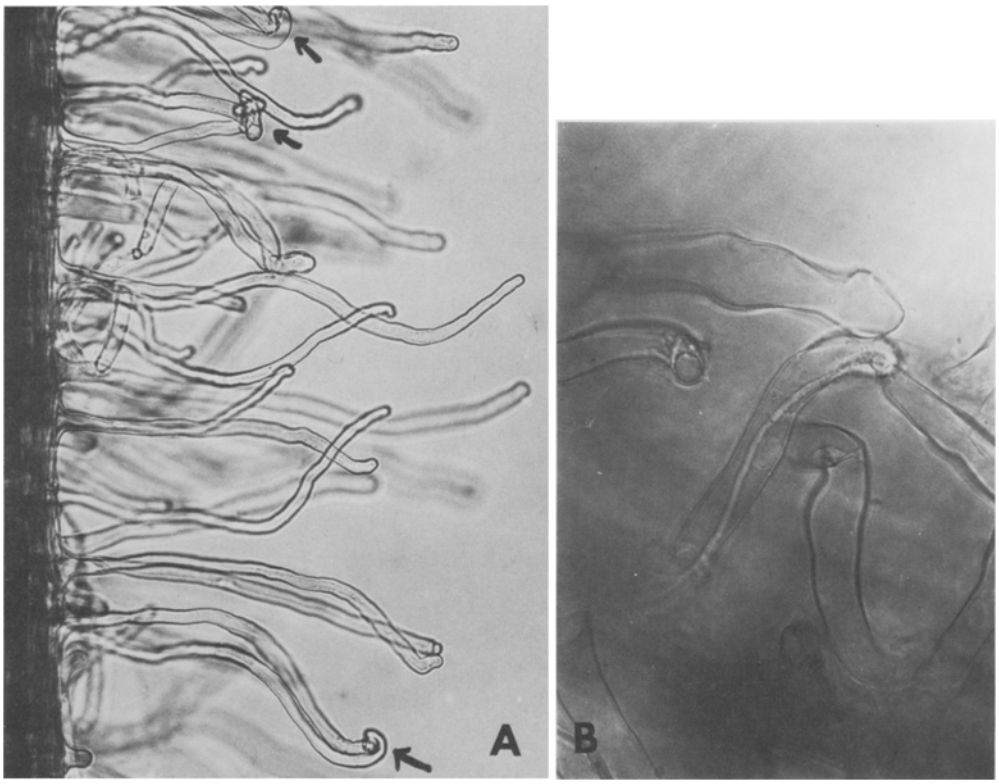


Fig. 16. (*A*): Adhesion of root hair tips (arrows) on axenically growing clover seedling. 500×. (*B*): Tip-adhered root hair infected with *Rhizobium trifolii* 0403. Note lack of marked curling of root hair tip (*compare* with Fig. 1*b*). 1,286×

the N-acetylgalactosamine-specific 120,000-dalton soybean lectin in their seeds (but *see* Schmidt, 1979). Root hairs of these lines are still infectable by *R. japonicum* and root nodulation is specific for the soybean rhizobia (Pueppke, 1982). Thus, the presence of this lectin in soybean seeds is not necessary for root nodulation by *R. japonicum*. It will be important to know whether the root hairs of these plants have this or other lectins which recognize the soybean rhizobia during infection. Another area worth investigating with these lines of soybean is to determine if the symbiont rhizobia affect *de novo* synthesis of root hair lectins.

Summary and Concluding Remarks

The specific infection of legume root hairs by the bacterial symbiont, *Rhizobium*, involves many steps of cellular recognition which culminate in the formation of a root nodule that fixes nitrogen into ammonia fertilizer for the host plant. This article deals with the analysis of lectins and their saccharide receptors in the infection process, their involvement in specific bacterial attachment to root hairs, their regulation, and a critical evaluation of the limitations of the present work relative to understanding the biochemical basis of host specificity.

It seems unlikely that acceptance or rejection of the lectin-recognition hypothesis will ever become universal until the recognition code is deciphered and mutant strains of both symbionts – bacterium and plant – with altered lectin/lectin receptors and no pleiotropic effects are available for analysis.

The *Rhizobium*-legume symbiosis can be viewed as a delicate balance of many cell-cell communications which, in coordination, culminate in the formation of root nodules that fix N_2 into ammonia fertilizer for the plant symbiont. In addition to attachment, there is curling and branching of the root hair (Yao & Vincent, 1976) tip-to-tip adhesions (Dazzo et al., 1982*a*), penetration of the root hair cell wall by the rhizobia without host cell lysis (Napoli & Hubbell, 1975; Callaham & Torrey, 1981), dome formation of the new infection thread (Callaham & Torrey, 1981), nucleus-directed growth and extension of the infection thread down the root hair shaft (Fahraeus, 1957; Nutman, Doncaster & Dart, 1973), infection thread penetration of the cell wall at the base of the root hair, host cell proliferation in the inner cortex in front of the advancing infection thread (Libbenga & Bogers, 1974), branching of the infection thread in the nodular cells, release and envelopment of the bacteria from the infection thread into peribac-

teroid membranes (Newcomb, 1981), development of the bacteroids (Sutton, Pankhurst & Craig, 1981; Urban & Dazzo, 1982), differentiation of nodular tissue (Libbenga & Bogers, 1974; Truchet, Michel & Denarie, 1980), leghemoglobin synthesis (Verma, Ball, Guerin & Wasamaker, 1979), nitrogenase synthesis and expression (Gresshoff et al., 1981), and mechanisms for exchange of metabolites and energy between the legume and the respiring *Rhizobium* bacteroids (Imsande, 1981). Each of these events provides an excellent model to study the underlying biochemical mechanisms of plant-microorganism interactions.

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