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Bacterial Attachment as Related to Cellular Recognition in the Rhizobium-Legume Symbiosis

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Bacterial attachment is viewed as a cellular recognition event during the infection of legumes by the nitrogen-fixing symbiont, Rhizobium. Studies on the biochemical basis of selective attachment are reviewed, and suggest that this recognition process is accomplished by specific glycoprotein lectin–polysaccharide interactions on the surfaces of the symbionts. An understanding of host specificity may lead to ways to broaden the host range of nitrogen-fixing symbioses.

Key words: Rhizobium, legume, nitrogen-fixing symbiosis, specificity, lectins, polysaccharides, attachment

CELLULAR RECOGNITION IN PLANT–MICROBE INTERACTIONS

Plants are constantly coming into contact with microorganisms, and the events that ensue have profound influences on plant morphogenesis, nutrition, and pathogenesis [1]. Specificity is the hallmark of most plant–microbe interactions, which implies that recognition systems function during certain stages in the development of the relationship. Recognition is most obvious when there is a restricted host range, eg, the nitrogen-fixing Rhizobium-legume symbiosis, but it also occurs in those interactions where the host range is broad, eg, Agrobacterium tumefaciens crown-gall disease. In the latter case, recognition may involve molecules which are commonly found in the cell walls of many different plants [2].

When a plant encounters an incompatible pathogen, the host cell may a) form structures that restrict invasion of the parasite; b) accumulate inhibitory substances; or c) collapse, thus effectively preventing the spread of the parasite [3]. The latter phenomenon is called the *hypersensitive response*, and makes an important contribution to the plant's resistance to disease. Thus, in many ways, survival of a higher plant species depends on its ability to participate effectively in cellular recognition.

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Most of what little is known about the underlying biochemical basis for cellular recognition in higher plants has been discovered since 1973. The fundamental working hypothesis upon which most subsequent investigations were based was that positive recognitions between cells arise from a specific union, reversible or irreversible, between chemical groupings on the surface of interacting cells [4]. This hypothesis implies that important communication events occur when cells that recognize one another come into contact, and therefore the complementary components of the cell surfaces have naturally been the center of focus for most biochemical studies to follow. Such was the case for the studies by Hamblin and Kent [5] and Bohlool and Schmidt [6], which suggested that attachment of Rhizobium to roots of its legume host may be mediated by specific, complementary lectin–polysaccharide interactions as a basis of specificity in this nitrogen-fixing symbiosis.

There are many recognition phenomena which occur when Rhizobium infects the legume host roots, and the attachment that follows the contact of the microbe with the host cell is one good example of a recognition event studied at the biochemical level. This is not to imply that accomplishment of firm attachment need be the key recognition step; rather, it is one of the keys needed to unlock the many doors blocking the way to successful infection. An understanding of the mechanism and control of host recognition would not only help elucidate the developmental events in the legume symbiosis, but also indicate ways in which Rhizobium and the host plant may be manipulated genetically to increase the range of agricultural crops which can enter efficient nitrogen-fixing symbioses.

This review updates several previous reviews [1,7–11] on the role of bacterial attachment as a cellular recognition event in the Rhizobium-legume symbiosis.

ATTACHMENT OF RHIZOBIUM TO LEGUME ROOT HAIRS

Rhizobium is a genus of Gram-negative bacteria that selectively infects legume roots, and then forms root nodules that fix atmospheric nitrogen into ammonia [1]. The infection process is very selective for certain combinations of rhizobia and legume, and this high degree of host-range specificity currently defines the taxonomy of the microorganisms at the species level. For example, R trifolii infects clover, R meliloti infects alfalfa, and R japonicum infects soybean. Successful infection of legumes by these nitrogen-fixing bacteria is of immense importance in the nitrogen cycle of terrestrial life.

Phase I Attachment

Attachment of the symbionts to the root surface constitutes one of the many, critical "recognition" events that occur before successful infection and nodular development of the symbiotic state. Quantitative microscopic assays [1,12] and numerous electron microscopic studies [1,10,15] have revealed multiple mechanisms of rhizobial attachment to clover root hairs. A nonspecific mechanism allows all rhizobia to attach in low numbers (2–4 cells per 200 μm root hair length per 12 hr). In addition, a specific mechanism that has been studied in the Rhizobium trifolii-clover symbiosis allows selective attachment in significantly ($P = 0.005$) larger numbers (22–27 cells per 200 μm root hair length per 12 hr) [12].

Electron microscopy [13] 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 fibrillar clover root hair cell wall (Fig. 1). This "docking" stage is the first step of Phase I attachment, and occurs within minutes after inoculation of encapsulated cells of R trifolii on the host clover.

What cell surface molecules are involved in Phase I attachment? We have approached this problem by examining 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 of clover epidermal cells contain a unique antigen that is immunochemically cross-reactive [13,14], suggesting its structural relatedness on both symbionts. This antigent contains receptors that bind noncovalently to a multivalent lectin called trifoliin A (originally trifoliin), which has recently been purified from clover seeds and seedling roots and is partially characterized [16]. A specific hapten inhibitor of trifoliin A is 2-deoxyglucose [13, 17]. The first clue that trifoliin A on the root may be involved in rhizobial attachment came from the observation that 2-deoxyglucose specifically inhibited the attachment of R trifolii to clover root hairs [12], reducing the high

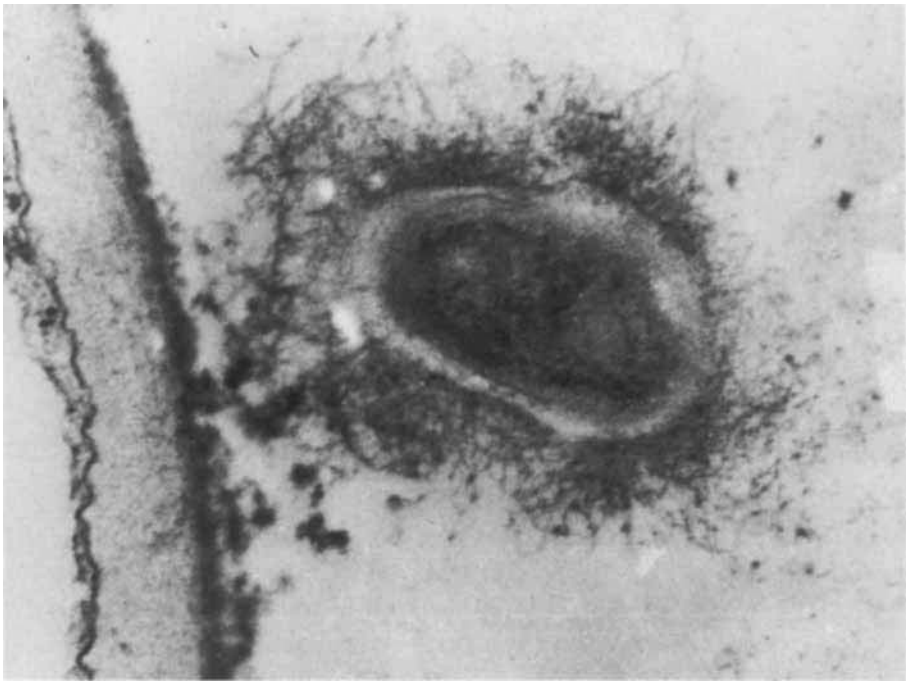

Fig. 1. Transmission electron micrograph of Rhizobium trifolii NA 30 in the docking stage of Phase I attachment to a clover root hair. The fibrillar capsule of the bacterium is in contact with electron-dense aggregates on the outer periphery of the root hair cell wall (Dazzo and Hubbell, 1975, and courtesy of the American Society for Microbiology [13]). (2,730 ×.)

level of bacterial adhesion to that characteristic of background. Subsequent studies showed that 2-deoxyglucose specifically facilitated the elution of trifoliin A from the intact clover root [16,17], and inhibited the binding of R trifolii capsular polysaccharide to clover root hairs [17]. As a negative control, 2-deoxyglucose did not inhibit adsorption of R meliloti or its capsular polysaccharide to alfalfa root hairs [12,17]. Similar hapten-facilitated elution of lectin from pea and alfalfa roots, as well as the presence of structurally related polysaccharides or antigens in these legumes and their homologous rhizobial symbionts, have also been found [17a] (Kamberger, personal communication).

We proposed a model to explain this early recognition event of Phase I attachment on the clover root hair surface prior to infection [13]. According to this hypothesis, trifoliin A recognizes similar saccharide resides on R trifolii and clover and cross-bridges them in a complementary fashion to form the correct molecular interfacial structure that initiates the preferential and specific adsorption of the bacteria to the root hair surface (Fig. 2).

This model proposes that there are host-specific receptor sites on the legume root where recognition of the rhizobial symbiont occurs. 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 fluorescent dye fluorescein isothiocyanate, incubating the conjugate with sterile seedling roots for a brief period, and then examining the

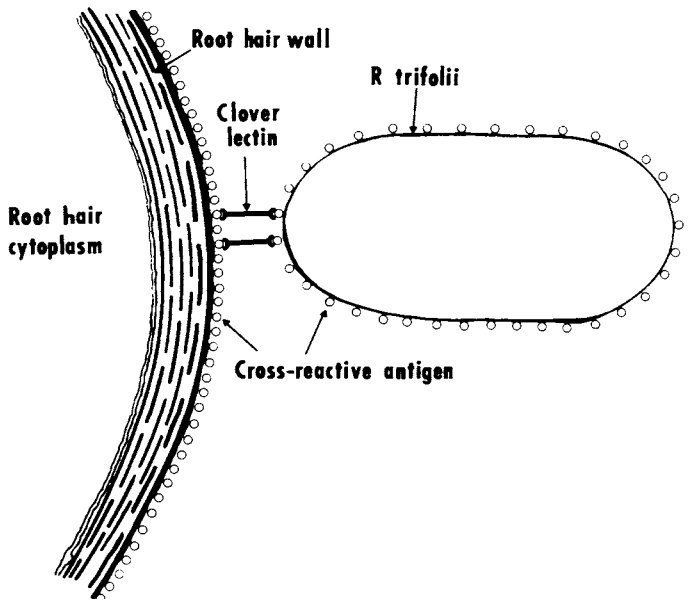

Fig. 2. Schematic diagram of a model to explain Phase I attachment. According to this model, antigenically cross-reactive saccharide receptors on Rhizobium trifolii cells and clover root hairs are specifically cross-linked with the clover lectin, trifoliin A (Dazzo and Hubbell, 1975, and courtesy of the American Society for Microbiology [13]).

root by fluorescence microscopy [17]. The results are shown in Figure 3. The receptor sites on clover roots that immediately bound the R trifolii capsular polysaccharide were located at discrete root hair sites that had differentiated on the epidermal root surface. They accumulate at root hair tips (single arrow) and diminish toward the base of the root hair [14,17]. This result highlights the importance of epidermal cell differentiation in the development of receptor sites that recognize rhizobia. Undifferentiated epidermal cells did not bind the bacterial polysaccharide, whereas epidermal root hair primordia gained this cell surface property (double arrow, Fig. 3).

Specificity of these receptor sites was demonstrated by the ability of unlabeled capsular polysaccharide from R trifolii but not from R melioti to block the binding of the labeled polysaccharide. This experiment has been reproduced with combinations of R melioti-alfalfa [17], R japonicum-soybean [18], (Hughes and Elkan, unpublished observation), and R leguminosarum-pea [18a] (Kamberger, personal communication). In each case, the specific binding of the rhizobial polysaccharides to the host was selective for the root hairs, where this recognition occurs. The sugar haptens for the corresponding host lectin specifi-

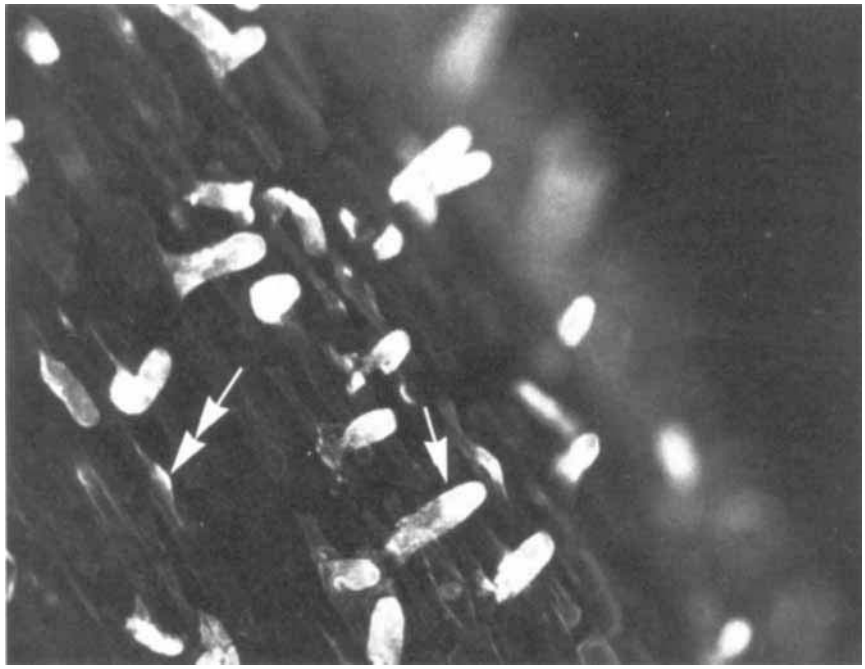

Fig. 3. One of the exclusive relationships between a legume and a nitrogen-fixing bacterium is demonstrated by the binding of Rhizobium trifolii to root hairs of its plant host clover. A fluorescein dye was first conjugated to the bacterial capsular polysaccharide. This labeled polysaccharide was then incubated with clover roots. Fluorescence of the roots showed that binding of the bacterial polysaccharide was restricted to the root hairs (single arrow) or root hair primorida (double arrow), the differentiated cells that serve as target cells for infection by R trifolii. Similar results have been obtained with other rhizobia and their corresponding specific legume hosts (Dazzo and Brill, 1977, and courtesy of the American Society for Microbiology [17]). (620 ×.)

cally inhibited the binding of the labeled rhizobial polysaccharide as evidence that the root lectins were involved in this recognition process. In addition, these receptor sites match the distribution of trifoliin A and the primary sites of rhizobial attachment [16] (Fig. 4).

Rhizobium cells precoated with their corresponding plant lectin will adhere in higher numbers than noncoated cells to their host roots [12,19], indicating the accessibility of these lectin receptors on the root surface. Quantitative adsorption studies with seedling roots of several legumes indicate that the cross-reactive antigen on R trifolii is host-specific since antibody that cross-reacts with R trifolii can be adsorbed by clover roots but not by roots of alfalfa or joint vetch, which are examples of other cross-inoculation groups [14].

Several experiments suggest that trifoliin A and the cross-reactive anticlover root antibody bind to the same or similar overlapping determinants on R trifolii [14]. First, the antibody and the lectin bind specifically to the same isolated polysaccharides from R trifolii. Second, this interaction is specifically inhibited by 2-deoxyglucose. Third, the genetic markers of R trifolii that bind trifoliin A and the antibody cotransform into Azotobacter vinelandii with 100% frequency [20]. And fourth, monovalent Fab fragments of IgG from anticlover root antiserum strongly block the binding of trifoliin A to R trifolii [14]. 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 trifoliin A.

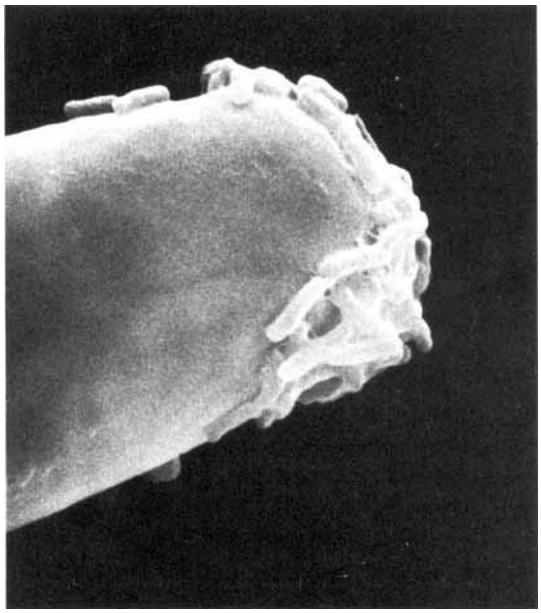

Fig. 4. Phase I attachment of Rhizobium trifolii 0403 to the tip of a clover root hair after 15 min of incubation as examined by scanning electron microscopy (Dazzo and Brill, 1977, and courtesy of the American Society for Microbiology [14]). (7,500 ×.)

The model would predict that all infective strains of R trifolii, regardless of their antigenic disparity, should possess the same saccharide sequence cross-reactive with clover. It was very important to test this prediction, since it is well known that the serology of surface polysaccharides on Rhizobium is very strain-specific because of compositional differences in the immunodominant determinants [21, 22]. Immunological tests involving passive hemagglutination and its inhibition nevertheless showed that the saccharide sequence responsible for the antigenic cross-reactivity with clover roots was conserved in otherwise immunochemically distinct surface polysaccharides of R trifolii strains [14], and therefore it is not the immunodominant structure of the polysaccharides.

The results of three experiments indicate that trifoliin A and antibody to the clover root cross-reactive antigen bind to the same R trifolii saccharide determinants that bind these bacteria to clover root hairs [14]. First, Fab fragments of anticlover root antibody adsorbed on cells of R trifolii blocked their attachment to clover root hairs. Second, only the A vinelandii hybrid transformants that carried the trifoliin A receptor bound to clover root hairs. Third, competition assays using fluorescence microscopy indicated that the R trifolii polysaccharides that carried the trifoliin A receptors had the highest affinity for clover root hairs.

Soybean root cells in culture selectively adhere to homologous R japonicum cells, and bacterial adherence is inhibited by pectinase treatment of the root cells [23]. Encapsulated cells of R japonicum or cells associated with slime specifically bind to soybean lectin [6,24–26] and to soybean root hairs [24]. The specific attachment of R japonicum to root hairs of the small soybean (Glycine soya) is specifically inhibited by N-acetylgalactosamine, the specific hapten for the soybean root lectin [26]. Similarly, the lectin from bean (Phaseolus vulgaris) binds specifically with surface polysaccharides of the bean symbiont, R phaseoli [27], and there is indirect evidence that this lectin may be on the bean root epidermis at sites where binding by rhizobia and symbiotic infection occur [5]. As with R trifolii [12], nonnodulating mutant strains of R leguminosarum or heterologous rhizobia [18a] adhere in smaller numbers to pea root hairs as compared with the wild-type nodulating strains (C.A. Napoli, personal communciation). Clearly, selectivity in rhizobial adherence to legume roots may operate in several species.

At low inoculum densities, R trifolii may be exposed to clover root exudate for a few hours before the bacterial cells contact the root hair surface. During this time, trifoliin A in root exudate may bind to the cells [28]. Recently, we have found that R trifolii growing near clover roots has trifoliin A selectively deposited in situ at one cell pole [29]. This alteration in distribution of trifoliin A receptors may possibly explain the distinctive polar attachment of the bacterial cells to the root hair surface toward the end of Phase I attachment. Under certain growth conditions, the receptor sites on R japonicum that bind soybean lectin also accumulate at one cell pole [26,30,31].

Phase II Adherence

Phase I attachment is followed by Phase II adherence, which firmly anchors the bacteria to the root hair surface during later preinfective stages [1,10,11]. During Phase II adherence, fibrillar materials visible under the scanning electron microscope are associated with the adherent bacteria after prolonged incubation with the clover root [1,10,11] (Fig. 5).

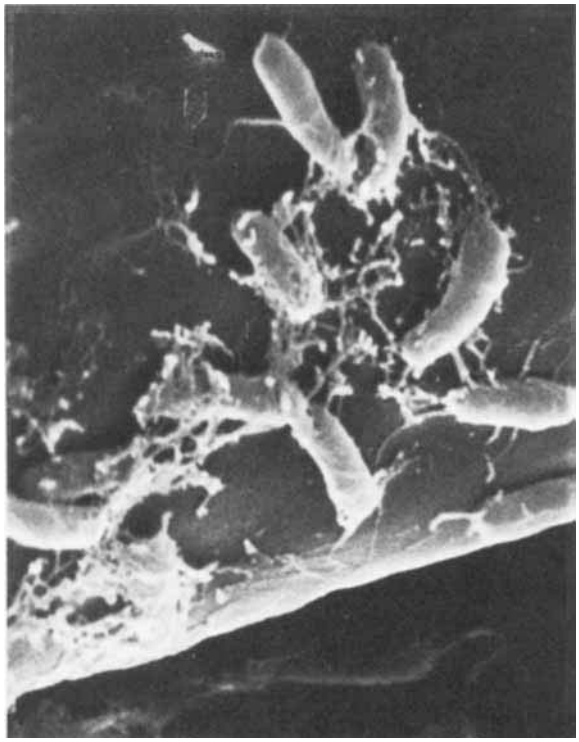

Fig. 5. Phase II adherence of Rhizobium trifolii 0403 after prolonged incubation on a clover root hair as examined by scanning electron microscopy. Note the fibrillar appendages associated with the adherent bacteria. (15,000 ×.)

The composition of aggregated microfibrils characteristic of Phase II adherence is unknown. One possibility is that they are the cellulose microfibrils produced by many rhizobial species [32,33]. These neutral exopolysaccharides tend to flocculate cells, often when they are in stationary phase of growth in broth culture. Phase II adherence may be important in maintaining the first 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 [32].

Although adherence 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. Heterologous rhizobia can adhere in small numbers to root hairs but do not infect them [1,12], and very few of the root hairs to which infective rhizobia adhere eventually become infected. Genetic hybrids of Azotobacter vinelandii (which carry the trifoliin A-binding saccharide receptor on their surface as a result of intergeneric transformation with DNA from R trifolii [20]) have acquired the ability to adhere specifically to clover root hairs [14] but do not infect them.

The latter experiment is particularly definitive, since we put into Azotobacter what it takes R trifolii to attach specifically and adhere, yet the hybrid cells lacked the ability to carry the infection process to the stage of root hair penetration. Possibilities for other genes or gene products that may have not been transferred or expressed include those controlling cell-wall hydrolytic enzymes [34,35] inducers of host polygalacturonase [36,37], root hair curling factors [38], and periplasmic extrinsic substance, ES-6000, which promotes root hair infection [39].

REGULATION OF ATTACHMENT IN THE RHIZOBIUM-LEGUME SYMBIOSIS

How is the attachment process regulated? Do both symbionts participate in regulating the components of the adhesive interface? Answers to these important questions are beginning to emerge. It has been known for many years that fixed nitrogen (eg, NH_4^+, NO_3^-) is one of the many environmental factors that limit the development and the success of the Rhizobium-legume symbiosis in nature. For instance, root hairs are resistant to infection by rhizobia when the roots are supplied with critical concentrations of NO_3^-. In order to study this phenomenon in more detail, we developed an immunocytofluorimetric assay to measure levels of trifoliin A on the surface of clover roots grown in different levels of NO_3^- [40]. We found that the immunologically detectable levels of trifoliin A and the specific binding of R trifolii to root hairs decreased in a parallel fashion as the concentration of NO_3^- increased in the rooting medium to 15 mM (Fig. 6) [40]. This experiment also supported the hypothesis that trifoliin A is involved in binding R trifolii to clover root hairs. The levels of NO_3^- that inhibited attachment were well below the levels that stunted seedling growth. Interestingly, the levels of trifoliin A and rhizobial attachment were increased at 1 mM NO_3^-. Thus, low levels of NO_3^- enhanced the recognition process, and high levels shut it off.

We have begun to search for an explanation of this nitrate effect on recognition. Ligand-binding studies using radiolabelled $^{13}NO_3^-$ have detected no direct interaction of NO_3^- with the lectin, trifoliin A, and NO_3^- did not interfere with the interaction of this lectin with its homologous antibody or R trifolii cells and clover roots after brief periods of incubation [29]. These studies indicate that the NO_3^--modulation of trifoliin A and of rhizobial accumulation on clover roots is in some way regulated by an intervening process rather than a direct interaction of receptors with this anion. Consistent with this hypothesis are two exciting new developments: the immunologically detectable levels of trifoliin A in root exudate and the accessibility of trifoliin A receptors on purified root cell walls are significantly reduced when developing clover seedlings are grown in 15 mM NO_3^- [28,29].

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 comes from data that show that the transient appearance of trifoliin A receptors on R trifolii may influence the ability of these bacteria to attach to clover root hairs [41]. 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 culture, the antigenic determinants on the bacteria that are cross-reactive with clover roots were "exposed" for only short

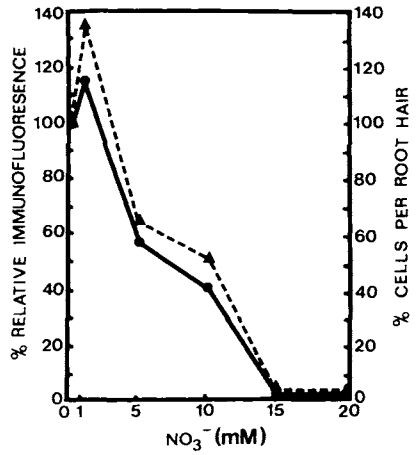

Fig. 6. The effect of NO_3^- on adsorption of Rhizobium trifolii 0403 to root hairs (solid line) and on immunologically detectable trifoliin (dotted line) in the root hair region of clover seedlings. Bacterial adsorption was measured by direct counting, and trifoliin was measured by cytofluorimetry using indirect immunofluorescence. Values from roots grown in N-free medium are taken as 100%, and represent 980 photovolts/mm^2 and 21 cells/root hair 200 μm in length. Points are corrected for nonspecific rhizobial adsorption, root autofluorescence, and nonspecific adsorption of conjugated goat antirabbit γ globulin (Dazzo and Brill, 1978, and courtesy of the American Society of Plant Physiologists [40]).

periods as cultures left their lag phase of growth and again as they entered stationary phase (Fig. 7). Clover roots adsorbed the bacteria in greatest quantity when the cells were harvested from plate culture incubated for 5 days and from broth cultures in early stationary phase. These studies draw attention to the basis of many anomalous lectin-binding results, and also indicate that the regulation of these lectin receptors may be very complex.

We recently found that growth-phase dependence for attachment is related to the appearance of a unique determinant in the surface polysaccharide of the bacteria [42]. As the culture advances from exponential to early stationary phase, changes in the immunochemistry of the surface polysaccharides were detected with antisera made specific for polysaccharides of cells in early stationary phase by exhaustive adsorption with exponentially growing cells. Culture-phase-dependent differences were found in the quantities of some monosaccharides (eg, quinovosamine, which is 2-amino-2,6-dideoxyglucose) in the polysaccharides that bound trifoliin A, and the latter aminodideoxyhexose was found to be an effective hapten inhibitor of trifoliin A when in the β-anomeric configuration. Other alterations in the polysaccharides included increases in apparent size of the R-core-O-antigen. The new immunochemical determinants that occur in the polysaccharides as cells enter stationary phase were apparently sites for trifoliin A binding, since immune monovalent Fab fragments specific for these unique determinants blocked the agglutination of cells with trifoliin A.

There is a transient appearance and disappearance on R japonicum of the receptor that specifically binds soybean lectin [7,43]. Most strains had their highest

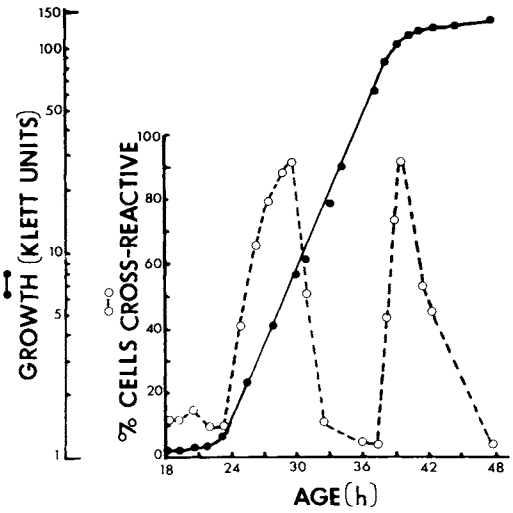

Fig. 7. The effect of culture age on the percentage of Rhizobium trifolii 0403 cells reactive with anticlover root antiserum. Cells were grown in a chemically defined broth at 30°C with shaking. Culture growth (closed circles, log scale) was measured with a Klett-Summerson colorimeter at 660 nm and the cross-reactive antigen (open circles, linear scale) by indirect immunofluorescence (Dazzo et al, 1979, and courtesy of Springer-Verlage [41]).

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 [44]. A decline in lectin-binding activity accompanying culture aging is concurrent with a decline in galactose content and a rise in 4-0-methyl galactose residues. 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.

With regard to genetic regulation, exciting evidence is beginning to emerge that genetic elements important to surface polysaccharides and symbiotic recognition are encoded on plasmids of Rhizobium [45–48]. For instance, the genes responsible for the 2-deoxyglucose inhibitable attachment of R trifolii to clover hairs are encoded on the large nodulation plasmid-designated pWZ2 [48].

In the past, a major controversy existed concerning the nature of the carbohydrate receptor for the lectin on the Rhizobium cell. Lectin receptor molecules have been identified as capsular polysaccharide [13], LPS [27], and a glycan [49]. The picture now emerging is that there are multiple types of lectin receptor molecules on the rhizobial cell. Both host-specific lectin-LPS interactions (R meliloti-alfalfa) and exopolysaccharide-lectin interactions (R japonicum-soybean) have been found [50]. Nonnodulating mutant strains of R japonicum have a defective carbohydrate antigen associated with their LPS and acidic heteropoly-

saccharide [51]. In the case of the fast-growing rhizobia that infect peas and clover, the host lectin will bind to both the LPS and capsular polysaccharides of the symbiont rhizobia at certain culture ages [18a,42,50]. Based on genetic and immunochemical studies [52], Kamberger has proposed the hypothesis that root hair attachment is mediated by lectins cross-bridging the capsular polysaccharides as a primary recognition event, followed by a more critical recognition event involving the host-specific binding of the lectin to the underlying bacterial LPS, which then triggers successful infection. One challenge of the next few years is to test the validity of this hypothesis.

The Rhizobium-legume symbiosis is a marvelous 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 [for reviews, see 1,8,9,10]. In addition to attachment, there is curling and branching of the root hair, penetration of the root hair cell wall by the Rhizobia without host cell lysis, dome formation of the new infection thread, nuclear-directed growth and extension of the infection thread down the root hair shaft, 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, branching of the infection thread in the nodular cells, release and envelopment of the bacteria from the infection thread into peribacteroid membranes, transformation of the vegetative bacteria into bacteriods, differentiation of nodular tissue, leghemoglobin synthesis and assembly, nitrogenase expression, mechanisms for transport of photosynthates, O_2, and N_2 to respiring bacteroids, and excretion of newly fixed NH_3 from bacteroids and its incorporation into amino acids. Each of these events provides an excellent model to study the underlying biochemical mechanisms of plant-microbe cell interactions.

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