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Specific Phases of Root Hair Attachment in the *Rhizobium trifolii*-Clover Symbiosis†

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The time course and orientation of attachment of *Rhizobium trifolii* 0403 to white clover root hairs was examined in slide cultures by light and electron microscopy. Inocula were grown for 5 days on defined BIII agar medium and represented the large subpopulation of fully encapsulated single cells which uniformly bind the clover lectin trifoliin A. When 10^7 cells or more were added per seedling, bacteria attached within minutes, forming randomly oriented clumps at the root hair tips. Several hours later, single cells attached polarly to the sides of the root hair. This sequence of attachment to clover root hairs was (i) selective for *R. trifolii* at inoculum sizes of 10^7 to 4×10^8 per seedling, (ii) specifically inhibited if 2-deoxy-D-glucose, a hapten for trifoliin A, was present in the inoculum, and (iii) not observed when 4×10^8 cells were added to alfalfa seedling roots or to large clover root cell wall fragments which lacked trifoliin A but still had trifoliin A receptors. Once attached, *R. trifolii* 0403 became progressively less detachable with 2-deoxy-D-glucose. At smaller inoculum sizes (10^5 to 10^6 cells per seedling), there was no immediate clumping of *R. trifolii* at clover root hair tips, although polar binding of bacteria along the root hair surface was observed after 4 h. The interface between polarly attached bacteria and the root hair cell wall was shown to contain trifoliin A by immunofluorescence microscopy. Also, this interface was shown by transmission electron microscopy to contain electron-dense granules of host origin. Scanning electron microscopy revealed an accumulation of extracellular microfibrils associated with the lateral and polar surfaces of the attached bacteria, detectable after 12 h of incubation with seedling roots. At this same time, there was a significant reduction in the effectiveness of 2-deoxy-D-glucose in dislodging bacteria already attached to root hairs and an increase in firm attachment of bacteria to the root hair surface, which withstood the hydrodynamic shear forces of high-speed vortexing. These results are interpreted as a sequence of phases in attachment, beginning with specific reversible interactions between bacterial and plant surfaces (phase I attachment), followed by production of extracellular microfibrils which firmly anchor the bacterium to the root hair (phase 2 adhesion). Thus, attachment of *R. trifolii* to clover root hairs is a specific process requiring more than just the inherent adhesiveness of the bacteria to the plant cell wall. Root hair tips were found to be sites of early attachment and colonization of *R. trifolii* 0403 in soil.

Attachment of microorganisms to surfaces is common in all natural ecosystems. The process generally begins with a reversible phase, in which the microorganism may use chemotaxis and bridging polymers to overcome charged, repulsive energy barriers and to position itself close to the surface. This is followed by a second phase, in which the attached microorganism synthesizes exocellular polymers, irreversibly anchoring the cell to the substratum. Such phases have been described for microbial attachment to nonbiological (22, 35), microbial (25), animal (17), and plant surfaces (23).

Rhizobium cells attach to legume root hairs before infection, which can be viewed as an early recognition event of the infection process. The most apparent selective mechanism of bacterial attachment to root hairs in *Rhizobium*-legume associations involves the specific binding of bacterial heteropolysaccharides to lectins on the root hair surface (7, 10, 14, 18, 19, 27, 31, 32, 34). For instance, trifoliin A, a lectin on the surface of clover root hairs, binds specifically to surface polysaccharides of the clover symbiont *Rhizobium trifolii* (7, 14).

Extracellular cellulose microfibrils have also been proposed to play a role in the attachment of certain bacteria to plant cells (23, 26). The production of extracellular microfibrils by *Agrobacterium tumefaciens* leads to a firm cell-cell adhesion after its initial attachment to its plant host (23). Cellulose microfibrils are also produced by certain *Rhizobium* strains in pure culture (15, 26). Although extracellular microfibrils associated with *R. trifolii* attached to clover root hairs have been detected by electron microscopy (4, 5, 16, 20, 26), the timing of microfibril production relative to the attachment process and to the degree of firm adhesion have not been examined.

The capsule of *R. trifolii* 0403 undergoes a marked change when encapsulated bacteria are placed into the environment of the clover root (13). Disassembly of this capsule begins at the cell center and proceeds outward towards both cell poles, but at unequal rates (13). Recently, we found that this erosion of the bacterial capsule is due to an alteration of the capsular polysaccharides by clover root enzymes (antigenically unrelated to trifoliin A) that are released into the root exudate (13). Simultaneously, the bacteria synthesize new capsular material (13), which initially accumulates at one cell pole (31). As a result of the asymmetrical erosion and new

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synthesis of the capsule, the bacterial cell binds trifoliin A in the root exudate only at one pole (13). We predicted that these surface changes on the unattached population of bacteria in the root environment should influence their eventual orientation of attachment to clover root hairs.

In a review article (11), we proposed that attachment of *R. trifolii* to clover root hairs is accomplished by a multiphase sequence of steps. In the present study, we present the experimental evidence in support of this hypothesis, based on an examination of the time course, cellular orientation, hapten reversibility, ultrastructure, and relative strength of adhesion of rhizobia to root hairs on the primary root of white clover seedlings. The existence of different phases of attachment was confirmed, and this new information was integrated with data from previous studies into a unified conceptual model that is consistent with the general scheme of microbial attachment to surfaces. In addition, we assessed the extent to which *R. trifolii* attaches to isolated cell wall fragments of clover root hairs and other root cells without the intervention of the host lectin. Preliminary reports of this work have been presented (F. B. Dazzo and E. M. Hrabak, Abstr. Conf. Microb. Adhesion Surfaces, University of Reading, United Kingdom, 1980; 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, 0435, and TA1 and *Rhizobium leguminosarum* 1020 were obtained from the Rothamsted Experimental Station, United Kingdom; *Rhizobium japonicum* 61A89 (3I1b110), 61A137, 61A24, and 61A76, *Rhizobium meliloti* 102F51 and 102F28, *Rhizobium phaseoli* 127K5, 127K14, 127K15, and 127K26, and *Rhizobium lupini* 9CE3 were obtained from J. Burton, Nitragin Co., Milwaukee, Wis. *R. trifolii* 2S-2, T37, WU290, and *Rhizobium* sp. (cowpea 227) were obtained from D. Hubbell, University of Florida, Gainesville. *R. meliloti* L5-30 was obtained from J. Dénarié, Castanet-Tolosan, France. White clover (*Trifolium repens* L. cv. Louisiana Nolin and cv. Certified Ladino) and alfalfa (*Medicago sativa* L. cv. Vernal) were obtained commercially. The inocula for all experiments were grown for 5 days on plates of BIII agar (6) at 30°C, harvested in phosphate-buffered saline (pH 7.2; 6), and then washed twice in phosphate-buffered saline and once in nitrogen-free Fahraeus medium (6) by centrifugation at 6,000 × *g* for 15 min at 4°C. *R. trifolii* 0403 cells processed this way represent the large subpopulation of fully encapsulated cells grown on agar surfaces which binds trifoliin A uniformly around the cell surface (31). Cells were resuspended in Fahraeus medium, filtered through sterilized glass wool columns to remove nondispersable flocs and produce a suspension of evenly dispersed single cells (6), and diluted with Fahraeus medium to the desired cell density. Seedlings for root attachment assays were germinated for 36 h from surface-sterilized seeds (6) on sterile water agar plates (1.2% [wt/vol] purified agar) into humid air. All solutions were prepared with distilled deionized water (18 Mohms · cm) and were filter sterilized through 0.2-μm Acrodisc filters (Gelman Sciences, Inc., Ann Arbor, Mich.).

Bacterial attachment assays. Bacterial attachment to root hairs on primary seedling roots was examined in undisturbed hydroponic slide cultures without agar and having a physiological electrolyte concentration (6). Six replicate seedlings received inocula at various increments between 10^5 and 10^9 cells per plant. After incubation at 22°C, the slide cultures were disassembled. The roots were rinsed gently with a stream of Fahraeus medium while still on the slides and then were covered with a glass cover slip and examined by phase-contrast microscopy at ×500 along the optical median planes of the root (6, 10). In addition, clover roots incubated with *R. trifolii* 0403 were examined by indirect immunofluorescence microscopy after treatment with rabbit anti-trifoliin A immunoglobulin G (IgG) (14), by transmission electron microscopy of ultrathin sections (6) with a Philips 300 transmission electron microscope, and by scanning electron microscopy (SEM) of root segments (6, 7) with an ISI Super III scanning electron microscope.

For hapten inhibition studies, slide cultures of white clover were incubated for 1 h with *R. trifolii* 0403 suspended in Fahraeus medium as described above but containing 30 mM 2-deoxy-D-glucose or α-D-glucose. In addition, slide cultures of the seedlings and bacteria were incubated first for 0.25, 1, 4, 8, 12, and 24 h in Fahraeus medium alone and then flushed with Fahraeus medium containing either 2-deoxy-D-glucose or α-D-glucose at 30 mM. This flushing with Fahraeus medium plus hapten was accomplished by gently flowing 10 to 15 volumes of this solution between the cover slip and the slide while the slide was held in a vertical position (12) to dislodge preattached bacteria from the root. In either case, the slide cultures were then immediately examined by phase-contrast microscopy for residual bacteria attached to each root hair (all degrees of maturity) along both optical median planes of the root.

Vortexing experiments. To determine whether rhizobial attachment to clover root hairs becomes very firm and essentially irreversible, seedlings from slide cultures were exposed to the hydrodynamic shear forces of high-speed vortexing. Seedlings of Louisiana Nolin white clover were inoculated with 10^8 cells of *R. trifolii* 0403 in slide cultures. After 0.25, 1, 4, 8, 12, and 24 h, the cover slips of two slide cultures (each containing two seedlings) were removed. The four seedling replicates were collected by grasping the cotyledons with sterile forceps and transferred to tubes containing 2 ml of sterile Fahraeus medium. This volume was removed and replaced twice by using a Pasteur pipette to rinse the seedling roots. The rinsed seedlings were transferred to new tubes containing 1 ml of sterile Fahraeus medium and then vigorously agitated with a vortex mixer (Fisher Vortex-Genie) at maximum speed (1,800 rpm) for 1 min. The seedling roots were then transferred, along with a drop of the suspended medium, to microscope slides and examined by phase-contrast microscopy.

Interactions between *R. trifolii* 0403 and isolated clover root cell walls. To determine whether the plant contributes more than just a cell wall substratum for bacterial attachment, we examined the inherent adhesiveness of encapsulated *R. trifolii* 0403 to isolated clover root cell wall fragments. Axenic seedlings were grown for 5 days through wire mesh (6) into sterile Fahraeus medium in a plant growth chamber programmed for a 22°C-20°C day-night cycle and a 16-h photoperiod at 21,600 lx. To isolate large fragments of root cell walls, the roots beneath the wire mesh were excised, frozen in liquid N_2, ground to a powder, thawed, and then successively extracted and pelleted at 9,000 × *g* for 15 min in 1 M NaCl (three times), methanol (two times), and water (three times) (D. Lamport, personal communication). The final wash lacked 280 nm-absorbing material. Phase-contrast microscopy revealed no cytoplasmic organelles in the isolated cell wall preparation, and the characteristic elongate root hair cell walls could be distinguished from other cell walls. This method of wall isolation removed trifoliin A

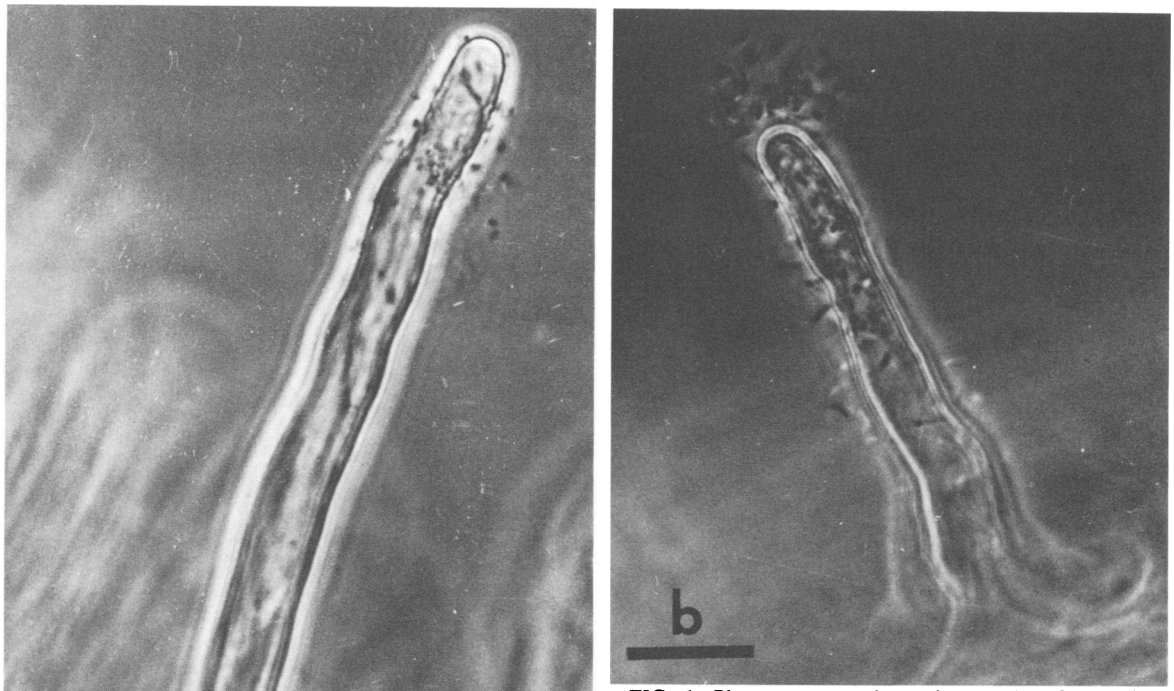

FIG. 1. Phase-contrast photomicrographs of *R. trifolii* 0403 attached to Louisiana Nolin white clover root hairs with a large inoculum (10^7 to 10^8 cells per seedling). (a) Random orientation of clumped cells near a root hair tip after 15 min of incubation. (b) 4 h later, bacteria are clumped in random orientation to the root hair tips and in polar orientation along the sides of the same root hair. Bar, 20 μm.

associated with the root hair surface, as indicated by a lack of indirect immunofluorescence staining with anti-trifoliin A IgG (unpublished data). Nevertheless, these wall fragments had wall receptors which could bind exogenously added trifoliin A and contained other characteristic wall polymers, such as pectins, hemicelluloses, cellulose, and hydroxyproline-rich glycoproteins (8; E. M. Hrabak and F. B. Dazzo, unpublished data). The lyophilized walls (1 mg) were mixed in a tube with 2×10^8 encapsulated cells of *R. trifolii* 0403 in 1 ml of Fahraeus medium, occasionally shaken for 4 h at room temperature, collected on 5-μm Mitex filters (Millipore Corp., Bedford, Mass.), rinsed with 10 ml of Fahraeus medium, mounted in Fahraeus medium on microscope slides, and examined by phase-contrast microscopy.

Attachment of *R. trifolii* 0403 to white clover root hairs in soil. A sandy soil (pH 6.6; CEC = 3.9) was dispensed in 2-g amounts into enclosed tubes (13 by 100 mm) and autoclaved for 1 h on 3 consecutive days. Each tube of soil was inoculated with 10^7 *R. trifolii* 0403 cells in 0.5 ml of Fahraeus medium. Twenty-four hours later, 36-h-old seedlings of Louisiana Nolin white clover germinated from surface-sterilized seeds were gently inserted vertically into a space in the soil created by a surface-sterilized spatula (one seedling per tube). The soil was pushed against the seedling root, and the tube was recapped. After 6 h at room temperature, the seedlings were removed from the soil and rinsed three times with 2 ml of Fahraeus medium. The seedlings were then treated with rabbit anti-*R. trifolii* 0403 antiserum (1 mg/ml) and examined by indirect immunofluorescence microscopy (14).

RESULTS

Phase 1 attachment. The time courses and cellular orientations of attachment of *R. trifolii* 0403 and *R. trifolii* TA1 to clover root hairs in slide cultures were essentially the same. After incubation of the seedlings for 15 min to 1 h with 10^7 to 4×10^8 encapsulated cells, a clump of randomly oriented bacteria was seen close to the root hair tip (Fig. 1a). Few bacteria attached to undifferentiated epidermal cells during the first hour. After 4 h, cell clumps remained attached to root hair tips, and individual bacteria began to attach polarly to the sides of the root hairs (Fig. 1b). When 68 Louisiana Nolin clover seedlings were inoculated with *R. trifolii* 0403 (10^8 cells per seedling) and examined 4 days later, 93% of the infected root hairs (982 total infections) had bacteria clumped to the root hair tips and polarly attached along the sides of the root hair. The remaining 7% of the infected root hairs counted on the same roots had bacteria attached individually and predominantly polarly oriented. To simplify the discussion of this sequence of bacterial attachment, we shall call the rapid attachment of randomly oriented bacteria into clumps at root hair tips the 1A attachment, the 4-h delay in polar attachment the 1B transition period, and the predominantly polar attachment of individual rather than aggregated cells to root hairs the 1C attachment.

This sequence of bacterial attachment was reexamined quantitatively with *R. trifolii* 0403 and TA1 at 4×10^7 cells per seedling on two varieties of white clover (Louisiana Nolin and Ladino) and found to be very similar (Table 1). Their ability to attach to alfalfa root hairs during 4 h of

TABLE 1. Time course and orientation of attachment of *Rhizobium* spp. to legume root hairs[a]

Rhizobium strain/plant[b]	% Root hairs with bacterial attachments[c] after incubation for:				
	1 h		4 h		
	Phase 1A	Phase 1C	Phase 1A	Phase 1C	Phases 1A and 1C
R. trifolii 0403/LN	63.2	2.6	11.9	11.6	57.5
R. trifolii TA1/LN	59.7	7.2	19.1	25.4	46.3
R. trifolii 0403/LAD	47.4	5.6	21.6	11.8	59.0
R. trifolii TA1/LAD	48.8	6.4	18.6	23.8	51.0
R. meliloti L5-30/LN	0	0	0	2.3	0
R. meliloti 102F51/LN	0	1.0	0	2.8	0
R. phaseoli 127K26/LN	0	6.5	0	0.9	0
R. leguminosarum 1020/LN	0	2.4	0	4.9	0
R. japonicum 61A137/LN	0	0	0	3.1	0
R. japonicum 61A89/LN	0	6.5	0	1.4	0
R. trifolii 0403/MSV	2.5	3.8	1.5	9.8	0

[a] The inoculum was 4×10^7 bacteria per seedling; ca. 50 root hairs were counted per treatment, representing those in focus along the optical median planes (6).

[b] LN and LAD, Louisiana Nolin and Ladino varieties of white clover, respectively, incubated in modified slide cultures; MSV, *Medicago sativa* var. Vernal (alfalfa) incubated in agar slant cultures.

[c] 1A attachments represent randomly oriented cells clumped to root hair tips, and 1C attachments represent single cells polarly attached to root hairs. Root hairs with both 1A and 1C attachments contained randomly oriented clumps of bacteria at their tips and polarly attached bacteria along the sides of the root hairs. The remaining percentage of root hairs in each case did not have bacteria attached.

incubation either in slide cultures or on agar slopes of the same medium was negligible (Table 1). For comparison, heterologous rhizobia (which infect legumes other than clover) were incubated with Louisiana Nolin white clover roots in slide cultures under the same conditions. Within this inoculum range, the frequency of 1A attachment to clover root hairs after 1 h and the combination of 1A and 1C attachment to the same root hairs after 4 h were only observed in the homologous association of *R. trifolii* and clover (Table 1). The attachment of single rather than clumped cells (1C attachment only) was not restricted to *R. trifolii* after 1 h, but it was significantly more frequent with *R. trifolii* than with heterologous rhizobia after 4 h of incubation (Table 1). This level of symbiont specificity in 1A attachment was not found at excessively large inoculum sizes, since *R. meliloti* 102F28 clumped to clover root hairs after 1 h with an inoculum of 10^9 cells per seedling.

The main difference observed when the inoculum of *R. trifolii* 0403 was smaller (10^5 to 10^6 cells per seedling) was the lack of large clumps of bacteria at the root hair tips during the first hour. Instead, a few bacteria would bind to the root hair tips during the first hour. Observations between 4 and 12 h showed several bacteria displaying the 1C polar attachment along the root hair surface without preference for root hair tips (Fig. 2; contrast with Fig. 1b). After 12 h, attachment of bacteria to clover root hairs of constant length (200 μm) was quantitatively greater with infective strains of *R. trifolii* than with heterologous rhizobia (Table 2).

The sugar 2-deoxy-D-glucose is a specific hapten inhibitor of the interaction between trifoliin A and *R. trifolii*. Microscopic observations showed that when added with the bacterial inoculum, 2-deoxy-D-glucose inhibited the formation of 1A clumps during the first hour of incubation. The frequency of 1A attachments on root hairs along the optical median planes was 58% in uninhibited controls, 1% with 2-deoxy-D-glucose, and 55% with α-D-glucose. Microscopic

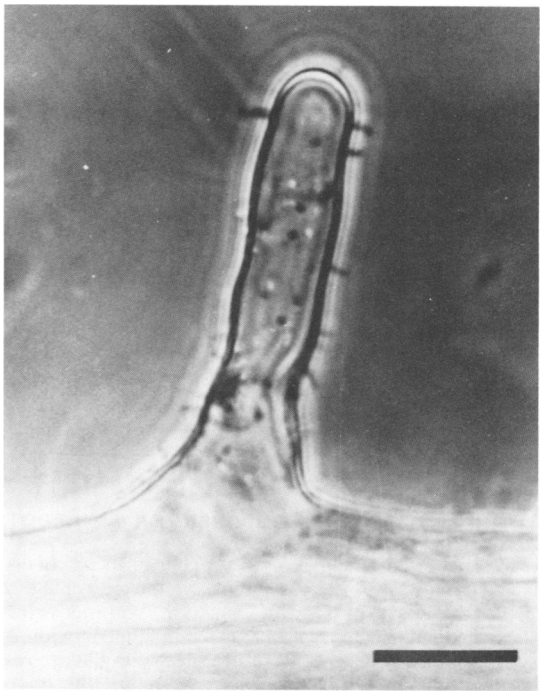

FIG. 2. Phase-contrast photomicrograph of *R. trifolii* 0403 attached to a Louisiana Nolin white clover root hair after 12 h of incubation with a small inoculum (10^5 to 10^6 cells per seedling). Note polarly attached bacteria distributed along the root hair surface. Bar, 20 μm.

observations also showed that 2-deoxy-D-glucose could effectively remove from root hair tips 1A attachments which were allowed to form in slide cultures in the absence of this inhibitor (Fig. 3). When applied to slide cultures after 4 h of preincubation, 2-deoxy-D-glucose dislodged both 1A and 1C attachments on the same root hair (Fig. 3). However, 2-deoxy-D-glucose became progressively less effective in dislodging preattached cells from root hairs when added at 8 h and was completely ineffective after 12 h (Fig. 3). The same concentration of α-D-glucose was not effective in inhibiting attachment or dislodging preattached cells from root hairs.

Trifoliin A was detected at one pole of many unattached *R. trifolii* 0403 cells in situ in the slide cultures beginning at 4 h after inoculation (data not shown). The immunofluores-

TABLE 2. Adsorption of *Rhizobium* spp. to root hairs of white clover (Louisiana Nolin)[a]

Species	No. of strains tested[b]	No. of adsorbed cells per root hair (mean ± SD)
R. trifolii	5	23.5 ± 2.0
R. meliloti	3	2.6 ± 0.2
R. japonicum	3	2.2 ± 0.7
R. leguminosarum	2	1.9 ± 0.9
R. phaseoli	4	3.6 ± 0.8
R. lupini	1	3.8 ± 0.3
Rhizobium sp. (cowpea)	1	2.5 ± 0.6

[a] From 15 to 20 root hairs (ca. 200 μm in length) were examined per strain, 12 h after inoculation of 2×10^6 cells per seedling in slide cultures.

[b] Strains were *R. trifolii* 0403, T37, WU290, 2S-2, and 0435; *R. meliloti* 102F26, 102F28, and 102F51; *R. japonicum* 61A24, 61A76, and 505W; *R. leguminosarum* 3H0Q51 and 1020; *R. phaseoli* 127K5, 127K14, 127K15, and 127K17; *R. lupini* 9CE3; and *Rhizobium* sp. cowpea 227.

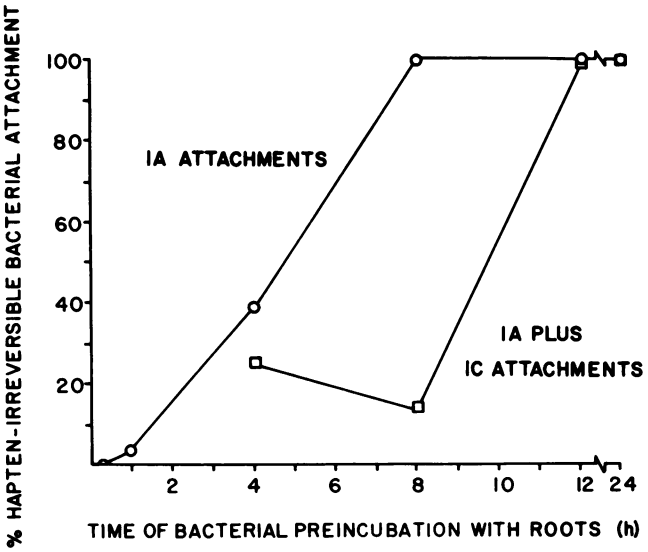

FIG. 3. Effect of preincubation of 10^8 cells of *R. trifolii* 0403 per Louisiana Nolin white clover seedling on the percentage of 1A attachment (○) and 1A plus 1C attachment (□) on root hairs along the optical median planes which resisted treatment with 30 mM 2-deoxy-D-glucose. The percentage of 2-deoxy-D-glucose-resistant attachment (ordinate) is calculated as 1.00 minus [the fraction of root hairs with attached bacteria after irrigation with α-D-glucose (as the uninhibited control) minus the fraction of root hairs with attached bacteria after irrigation with 2-deoxy-D-glucose, divided by the fraction of root hairs with attached bacteria after irrigation with α-D-glucose] × 100. Each data point is the average of four replicates.

ence microscopy of polarly attached bacteria with a ×100 oil immersion lens made it possible to examine the bacteria-root hair interface with most of the typical yellow-green immunofluorescence associated with anti-trifoliin A IgG on the root hair surface itself out of the focal plane. This method detected trifoliin A at the bacterial cell pole attached to the root hair surface (Fig. 4). Similar studies with preimmune IgG instead of anti-trifoliin A IgG showed no yellow-green immunofluorescence (data not shown).

The attachment of *R. trifolii* 0403 to clover root hairs was sufficiently firm to withstand the various steps of specimen preparation for ultrathin sectioning and transmission electron microscopy (Fig. 5a through c). A feature common to both the 1A attachments near the root hair tip (Fig. 5a) and the 1C attachments along the sides of the root hair (Fig. 5b and c) was the presence of electron-dense globular aggregates deposited on the outer face of the fibrillar root hair cell wall. These excreted particles were also found on root hairs of uninoculated seedlings (Fig. 5d).

Phase 2 adhesion. SEM revealed a progressive increase in accumulation of extracellular microfibrils of unknown origin associated with the attached rhizobia (Fig. 6a and b), beginning after about 12 h of incubation with the root (Table 3). These microfibrils were not apparent on uninoculated root hairs (Fig. 6c). These SEM results, and the studies described above which showed a temporal increase in 2-deoxyglucose-irreversible attachment, prompted us to determine whether rhizobial attachment to root hairs eventually becomes very firm and essentially irreversible. To test this hypothesis, seedlings in slide cultures were inoculated with *R. trifolii* 0403, incubated for various lengths of time, rinsed, vortexed, and examined by phase-contrast microscopy. Two measurements were made with these preparations in an effort to assess whether there was an increase in degree of adhesion of the bacteria to the root hairs with time (Table 3). In one case, counts were made of root hairs located along the optical median planes of the roots which still had bacteria attached to them after vortexing (Fig. 7a). This measurement showed a significant increase in the percentage of root hairs with attached bacteria which withstood this shear force after the bacteria had been incubated with the root for 12 h or longer (Table 3). In the second case, a significant increase in the percentage of the detached root hairs which still had bacteria attached to them (Fig. 7b) occurred after the bacteria had been incubated with the root for 12 h (Table 3). Both results show that the degree of adhesion between the attached bacteria and the root hairs increased significantly between 8 and 12 h of incubation.

Interaction of *R. trifolii* 0403 with isolated cell walls of clover seedling roots. We reasoned that large, isolated cell wall fragments could be used to assess the degree of inherent adhesiveness of the encapsulated bacteria to this root surface without the intervention of the host lectin and other less firmly associated surface materials. After 4 h of incubation, only a few bacteria (and no clumps) attached to isolated cell walls of root hairs or of undifferentiated epidermal cells (Fig. 8). This contrasts with the combination of the 1A clumping and 1C polar attachment to root hairs of intact seedlings that occurs during 4 h of incubation with the same size inoculum (Fig. 1b).

Attachment of *R. trifolii* 0403 to clover root hairs in soil. Use of indirect immunofluorescence with the homologous antibody at a low concentration made it possible to detect *R. trifolii* 0403 cells against a weakly fluorescent background of clover root hairs. This technique demonstrated that root hair tips are sites of early attachment and colonization of *R. trifolii* 0403 in soil (Fig. 9).

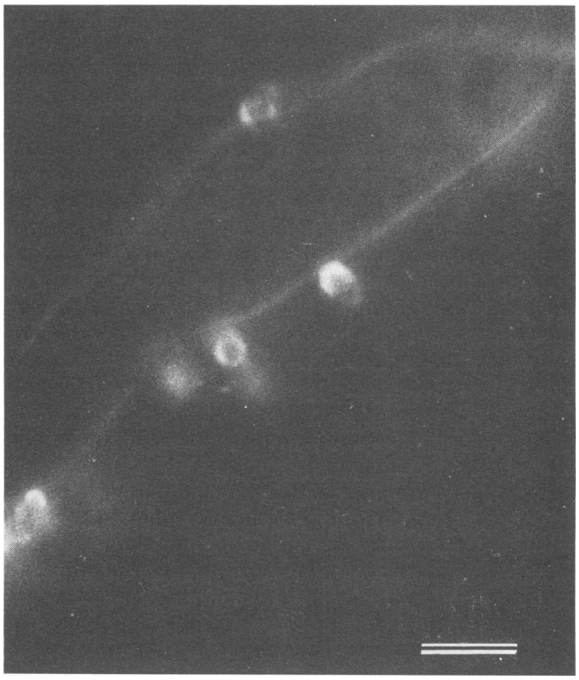

FIG. 4. Indirect immunofluorescence detection of trifoliin A at one pole of *R. trifolii* 0403 cells attached to the clover root hair. Photographed at ×1,250, so the depth of field is very small. Preimmune IgG was negative. Bar, 2 μm.

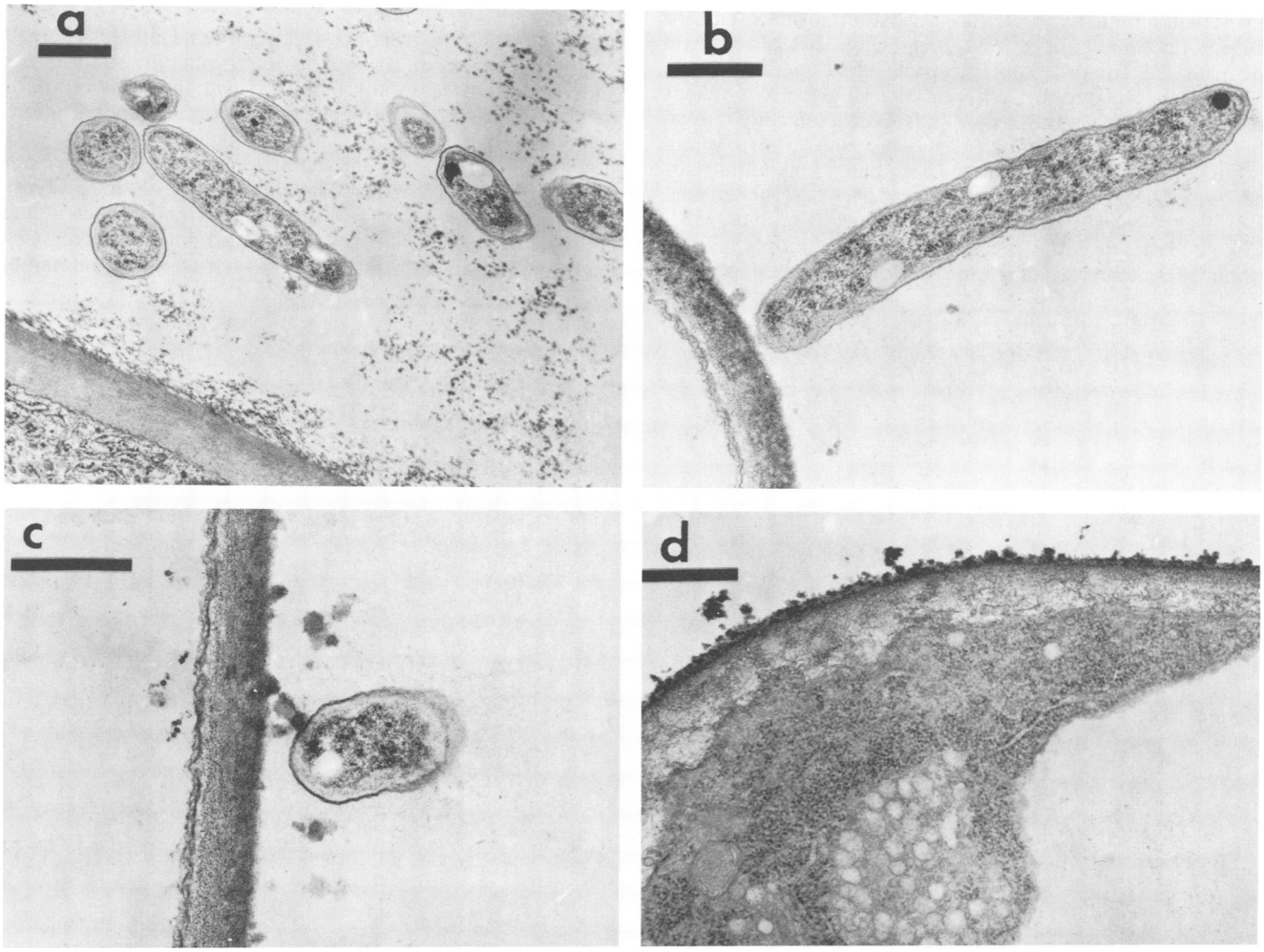

FIG. 5. Transmission electron micrographs of *R. trifolii* 0403 attached to clover root hairs. Note the electron-dense globular aggregates associated near the attached bacteria. (a) Section through a clump of bacteria near the root hair tip. (b) Longitudinal section through a bacterium polarly attached to the side of the root hair. This cell may have been dividing. (c) An oblique section through the contact interface of a polarly attached cell. (d) Near longitudinal section close to the tip of a root hair on an uninoculated seedling. Bar, 0.5 μm.

DISCUSSION

The attachment of *R. trifolii* to clover root hairs in Fahraeus slide cultures with an inoculum of fully encapsulated single cells which uniformly bind trifoliin A occurs in distinct phases. In accord with our results and with the kinetics of the polar alteration in the lectin-binding capsular polysaccharides by enzymes in root exudate (13), we propose the following phases of attachment of *R. trifolii* to the surface of maturing clover root hairs on the primary seedling root (Fig. 10). The extent and selectivity of the first stage will depend on the inoculum size. Beginning within a few minutes and continuing for the first hour with an inoculum of 10^7 to 4×10^8 cells per seedling, many of the bacteria will specifically attach in random orientation to clover root hair tips (phase 1A), the principal location of trifoliin A on the root surface (14). The gradient of trifoliin A is greatest at the root hair tip and decreases toward the base of the root hair (14). At $\geq 10^9$ cells per seedling, binding of the bacteria to root hair tips is not restricted to the homologous *R. trifolii* symbiont (3; this report). A second stage in the sequence (transition phase 1B) involves the alteration of the capsule of unattached cells in the external clover root environment, resulting in a population of bacteria with lectin-binding polar capsules (13) beginning ca. 4 h after inoculation. Trifoliin A in the root exudate of the slide culture binds to these cells in situ preferentially at one cell pole. A third stage in the first phase of attachment after completion of phase 1B involves the preferential end-on attachment of cells to other sites on the root hair surface (phase 1C). Beginning 12 h after inoculation, cell adhesion to the root hair surface becomes essentially irreversible, concurrent with the detection of extracellular microfibrils (phase 2).

The "docking" stage of phase 1A attachment in the *R. trifolii*-clover symbiosis was shown in earlier transmission electron microscopy studies to consist of an initial contact between the bacterial capsule and electron-dense globular aggregates which lie on the outer periphery of the root hair cell wall (9). These aggregated particles surround *R. trifolii* 0403 cells in clumps near the root hair tips (Fig. 5a) and also are seen near points of contact between individual bacteria and the sides of root hairs (Fig. 5b and c). Particles with ultrastructure similar to that of those particles on the root hair surface bound to the periphery of encapsulated *R. trifolii* 0403 cells shortly after incubation with clover root exudate or when inoculated into the clover root environment

(13). These globular particles are of host origin since they are present on uninoculated root hairs (Fig. 5d) but not on encapsulated bacteria kept in sterile plant medium alone (13). When isolated by differential and density gradient centrifugation from the root exudate of axenically grown clover seedlings, these particles were shown to contain trifoliin A, among other proteins, and had a high affinity for the bacterial capsule (J. E. Sherwood, G. L. Truchet, and F. B. Dazzo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K79, p. 149). Trifoliin A is also present at the interface of *R. trifolii* 0403 polarly attached to the clover root hair surface.

The negatively charged bacterial cell must overcome repulsive energy barriers to attach to the negatively charged root hair surface in an aqueous environment, such as the slide culture used in this study. Since the plant nutrient medium is of an intermediate electrolyte concentration, these repulsive energy barriers will include both the structured water layers and the electrical double layers around the interacting cells (29). The dimensions of these layers depend on the electrolyte concentration and represent the combined distances exerted by the primary and seconary energy minima which successively take effect as the bacte-

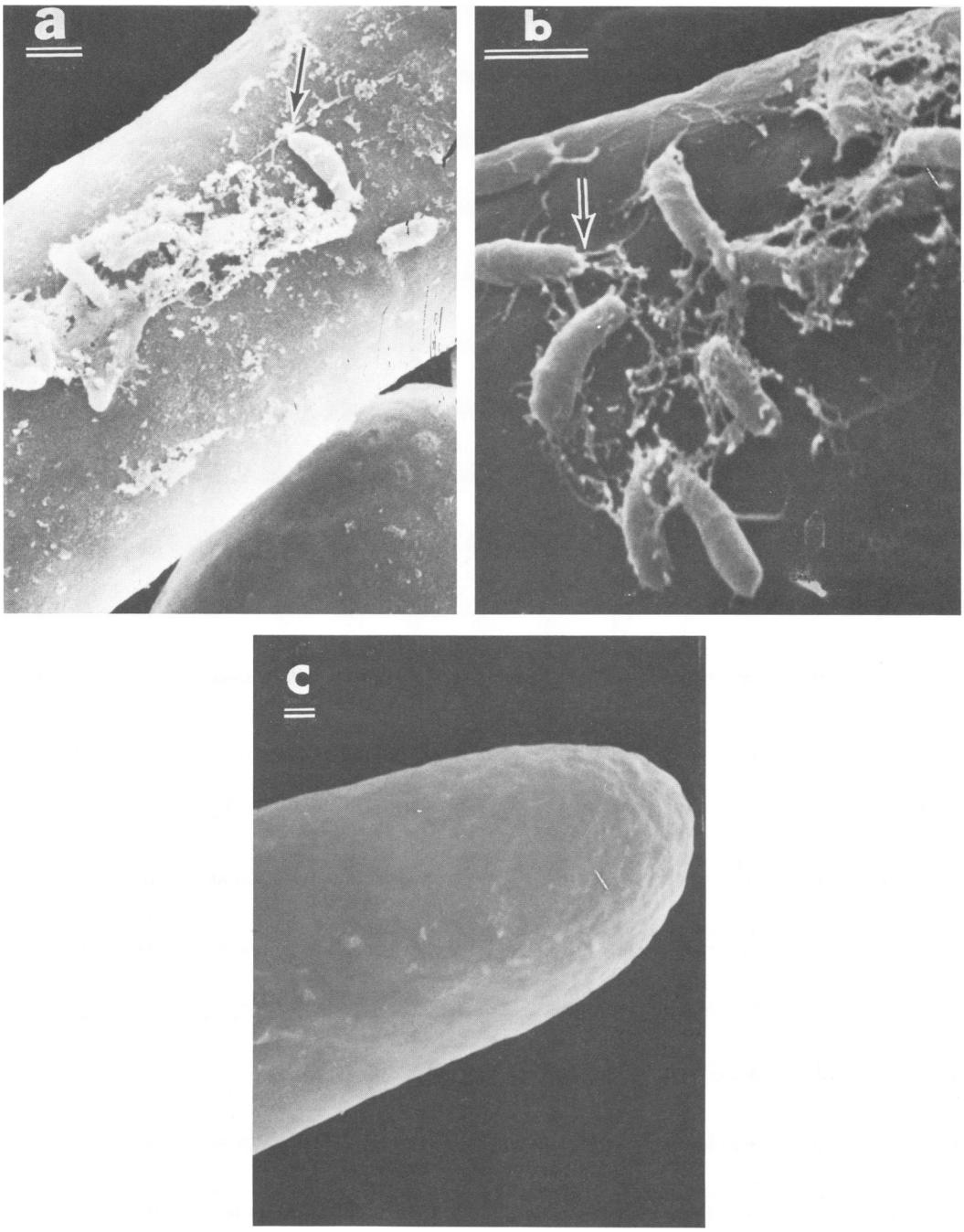

FIG. 6. Scanning electron micrographs of *R. trifolii* 0403 attached along the sides of clover root hairs. Note the progressive increase in associated extracellular microfibrils (arrows) after incubation of bacteria with the root for 12 h (a) and 4 days (b). (c) Surface of uninoculated clover root hair control. Bar, 1 μm.

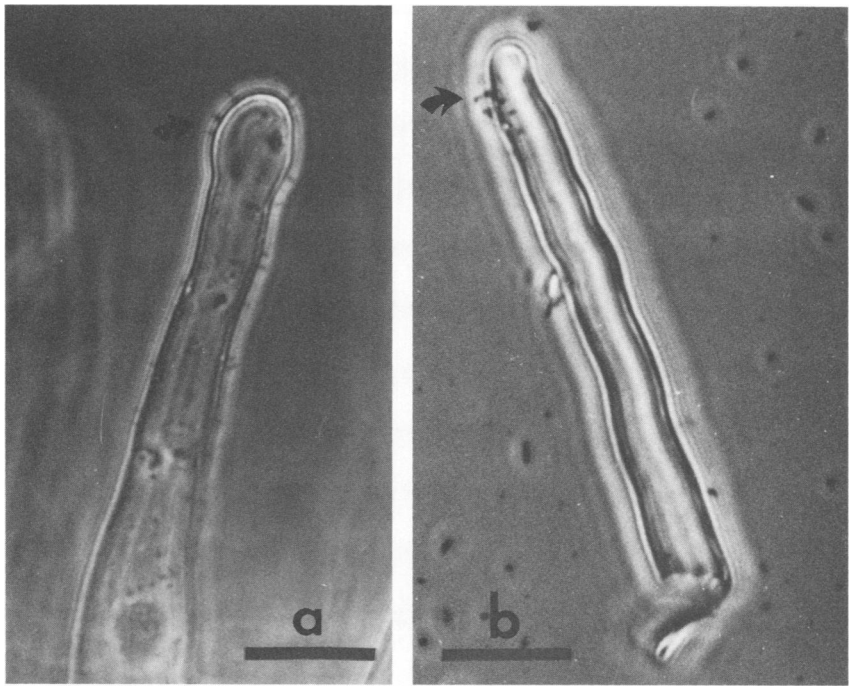

FIG. 7. Phase-contrast photomicrographs of *R. trifolii* 0403 (arrows) remaining attached to clover root hairs after vortexing at high speed for 1 min. Root hair is attached to the root in (a) and detached from the root in (b). Bar, 20 μm.

rial cell approaches the root hair before contact (29). The fibrillar appendages of the acidic capsule of *R. trifolii* 0403, as prepared in this study, extend 1 to 2 μm from the outer membrane of the bacterial cell (7, 9, 13, 31). It should take less energy for a bacterial cell with appendages of this length than for a cell lacking appendages to overcome these repulsive energy barriers. This is because these appendages are of sufficient length to cross the combined distances exerted by the primary and secondary energy minima to accomplish attachment in this environment (29).

The combination of phase 1A and 1C attachments to the same root hair (Fig. 1b) occurs most frequently with the homologous clover rhizobia (Table 1). This unique combination of bacterial attachments is consistent with the lectin cross-bridging model (9). When 2-deoxy-D-glucose is added with *R. trifolii* to clover seedling roots, the level of bacterial attachment to root hairs examined 12 h later is specifically and significantly inhibited (10, 34). The specific detachment of bound cells by 2-deoxy-D-glucose suggests that phase 1A attachment involves an interaction between the uniformly distributed lectin receptors of the capsule at this culture age and trifoliin A accumulated in relatively large quantity on the root hair tips. This hapten-reversible clumping would occur as other bacteria come into contact with bacteria or plant material at the root tip, or both. Other remaining bacteria

TABLE 3. Phase 2 adhesion of *R. trifolii* 0403 to clover root hairs

Observation	% Root hairs with attached bacteria[a] at time (h) after inoculation on roots:					
	0.25	1	4	8	12	24
Microfibrils on attached bacteria	−	−	−	−	+	++
Vortex expt 1	0	3	7	5	50	49
Vortex expt 2	0	0	0	0	73	85

[a] For microfibrils: −, microfibrils not found on root hairs having attached bacteria; + and ++, increasing amounts of microfibrils were found on root hairs having attached bacteria (see Fig. 6a and b). For vortex experiment 1, values are the percentage of root hairs along the optical median planes of four seedlings which had attached bacteria and which remained on the root after vortexing at 1,800 rpm for 1 min. For vortex experiment 2, values are the percentage of root hairs which had attached bacteria and which were detached from the root by vortexing at 1,800 rpm for 1 min.

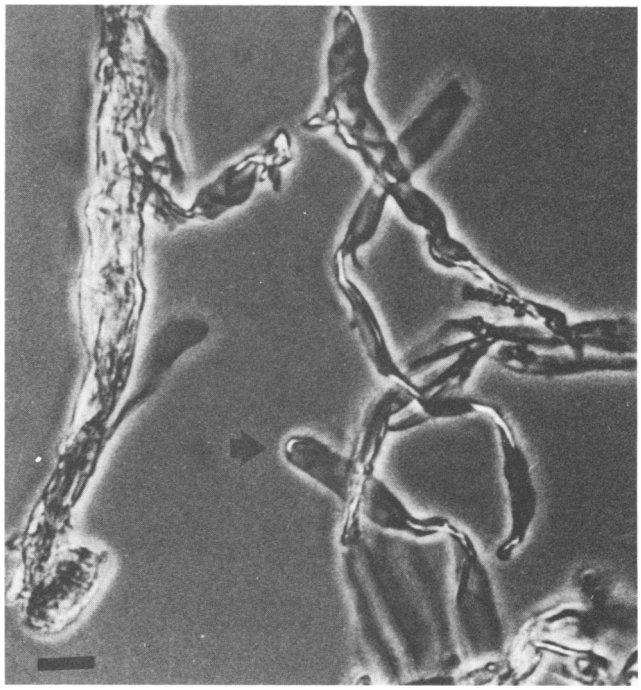

FIG. 8. Isolated clover root cell wall fragments incubated with 10^8 cells of *R. trifolii* 0403 for 4 h. Cell walls of root hairs (arrow) and other cells are shown. Bar, 20 μm.

which undergo the phase 1B transition could participate in the specific phase 1C attachment on the same root hair by two different mechanisms. In one case, lectin in the root exudate would bind to the bacterial pole in the external root environment (13) and then cross-bridge the cell to accessible lectin receptors (12) on the root hair and other epidermal cells. This first mechanism is consistent with the observation that pretreatment of *R. trifolii* with clover lectin will enhance specific attachment of the bacteria to root hairs and other epidermal cells of clover (10), even though these other epidermal cells do not accumulate trifoliin A on their surfaces (14). In the second case, the bacterium would bind, via its lectin receptors concentrated at one pole, to trifoliin A located along the sides of the root hair (14).

It has been suggested that rhizobia are inherently adhesive and therefore display rapid, nonspecific attachment to many different surfaces. This nonspecific mechanism could contribute to the attachment of *R. trifolii* to isolated clover root cell walls where trifoliin A was not present and to root hairs of heterologous legumes (3, 10, 21, 24, 33). Apparently, any *Rhizobium* cell can come into random contact with clover root hairs, but symbiont specificity is displayed in formation of phase 1A and 1C attachments of the bacteria on the same root hair. Since very few bacteria attached to isolated cell walls of clover roots without preference for root hair tips, the root contributes more than just a cell wall substratum for the phase 1 attachment sequence of fully encapsulated cells as described here. Thus, this phase 1 attachment sequence is a symbiont-specific, hapten-reversible mechanism resulting in significantly more clover rhizobia attached to clover root hairs than can be accounted for by the inherent adhesiveness of the bacterium alone. We propose that the gain in physicochemical forces of adhesion due to additional, specific, complementary lectin-polysaccharide interactions enhances the probability that the adhesion events advance to secondary irreversible stages to account for the selective accumulation of *R. trifolii* on clover root hairs.

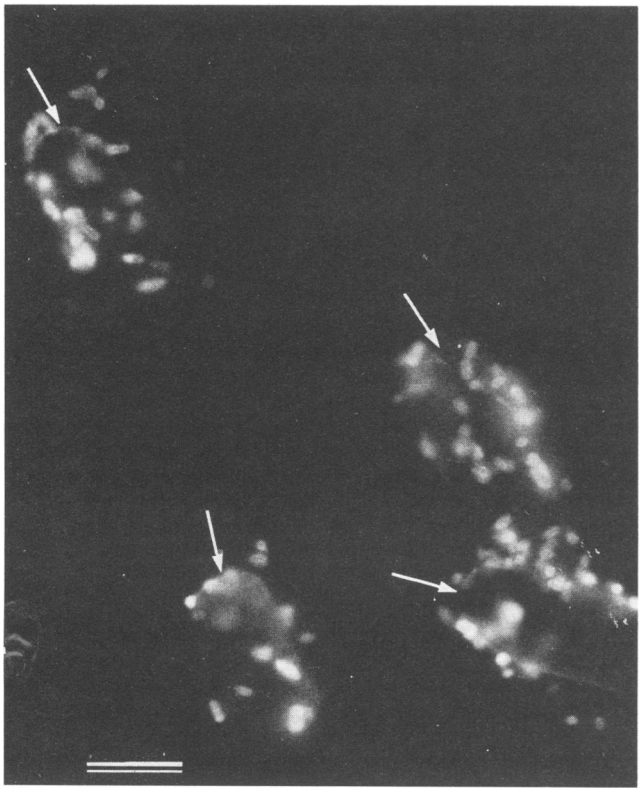

FIG. 9. Immunofluorescent detection of *R. trifolii* 0403 cells attached to root hair tips (arrows) of white clover seedlings in soil. Bar, 5 μm.

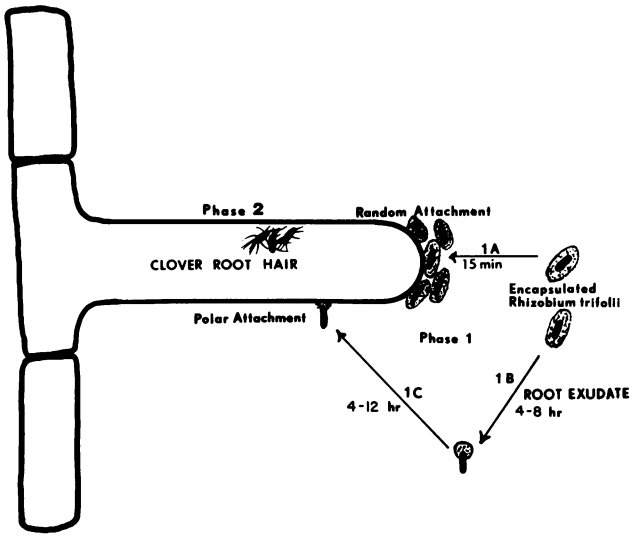

FIG. 10. Proposed sequential steps of phase 1 and 2 attachment of *R. trifolii* to the surface of clover root hairs. The inoculum consists of fully encapsulated cells which bind trifoliin A uniformly. The sequence is phase 1A, 1B, and 1C and then phase 2.

Extracellular microfibrils associated with certain strains of rhizobia attached to clover root hairs have been reported previously (4, 5, 16, 20, 21) and are confirmed here with *R. trifolii* 0403. These microfibrils were detected on attached cells of *R. trifolii* 0403 only after at least 12 h of incubation of the standardized inoculum with the seedling. Thus, these bacteria can accomplish phase 1A and 1C attachments on maturing clover root hairs before the microfibrils are detected by SEM. Extracellular polymer synthesis by cowpea rhizobia is stimulated by cowpea root exudate (1), and therefore a component in the root exudate may stimulate the formation of these microfibrils, as was suggested by transmission electron microscopy studies of *R. trifolii* grown in clover root exudate (C. A. Napoli, Ph.D. thesis, University of Florida, Gainesville, 1976).

The lack of extracellular microfibril synthesis in certain nonnodulating mutant strains of *R. trifolii* (28) suggests that these extracellular fibrils are somehow involved in root nodulation. It has been proposed that production of extracellular microfibrils contributes to the irreversible adhesion of plant-infecting bacteria onto the host cell after initial reversible stages of attachment are completed (23, 26). Our results are consistent with this hypothesis, since the extracellular microfibrils associated with the attached cells of *R. trifolii* 0403 are first detected by SEM at the same time that the bacterial adhesion to the clover root hair becomes very firm (phase 2 adhesion) and 2-deoxy-D-glucose resistant. These extracellular microfibrils could serve as bridging polymers which increase the surface area of contact between the bacteria and the root hair once they are made. Firm adhesion of *R. trifolii* and *R. meliloti* to clover and alfalfa roots after several hours of incubation is host specific (33) and positively correlated with the success of rhizobial strains in interstrain competition for nodule sites on the host root (33;

L. Bordeleau and N. Amarger, personal communication). Based on the length of incubation, *R. trifolii* cells attached to clover root hairs in other studies (4, 16, 20, 26, 30) would have already been in the phase 2 adhesion stage.

The phenotype of certain genetically altered strains of *R. trifolii* can be more accurately defined in terms of this phase 1 attachment process. Certain nonnodulating mutant strains lack the ability to accomplish phase 1A attachment and are significantly restricted in phase 1C attachment (10, 34). The selective phase 1B binding of trifoliin A to one cell pole of *R. trifolii* in the simulated rhizosphere of the slide culture, as displayed by wild-type strains, does not occur with a noninfective mutant with a Tn*5* insertion in a conserved plasmid gene essential for nodulation (F. Dazzo and B. Rolfe, manuscript in preparation). Genes which govern the 2-deoxy-D-glucose inhibitable steps of phase 1 attachment are located on large, indigenous plasmids (34) and can be transferred to and expressed in plasmidless recipients of *R. trifolii* (34) and a Ti plasmid-cured *Agrobacterium tumefaciens* strain (F. B. Dazzo, G. L. Truchet, and P. J. Hooykaas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K9, p. 178). However, the trifoliin A-binding hybrids of *Azotobacter vinelandii* obtained by intergeneric transformation with *R. trifolii* DNA (2), which displayed 2-deoxy-D-glucose inhibitable phase 1A attachment (7), did not subsequently advance the infection process to the stage of clover root hair penetration. Furthermore, *R. trifolii* will specifically attach to far more root hairs than it will infect. These results place the events of specific, host lectin-mediated, phase 1 attachment into their proper perspective relative to the overall infection process and indicate that crucial events other than attachment are also needed to ensure successful infection of clover root hairs in this nitrogen-fixing symbiosis.

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