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Rhizobium Lipopolysaccharide Modulates Infection Thread Development in White Clover Root Hairs

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The interaction between *Rhizobium* lipopolysaccharide (LPS) and white clover roots was examined. The *Limulus* lysate assay indicated that *Rhizobium leguminosarum* bv. trifolii (hereafter called *R. trifolii*) released LPS into the external root environment of slide cultures. Immunofluorescence and immunoelectron microscopy showed that purified LPS from *R. trifolii* 0403 bound rapidly to root hair tips and infiltrated across the root hair wall. Infection thread formation in root hairs was promoted by preinoculation treatment of roots with *R. trifolii* LPS at a low dose (up to 5 μg per plant) but inhibited at a higher dose. This biological activity of LPS was restricted to the region of the root present at the time of exposure to LPS, higher with LPS from cells in the early stationary phase than in the mid-exponential phase, incubation time dependent, incapable of reversing inhibition of infection by NO_3^- or NH_4^+, and conserved among serologically distinct LPSs from several wild-type *R. trifolii* strains (0403, 2S-2, and ANU843). In contrast, infections were not increased by preinoculation treatment of roots with LPSs from *R. leguminosarum* bv. viciae strain 300, *R. meliloti* 102F28, or members of the family *Enterobacteriaceae*. Most infection threads developed successfully in root hairs pretreated with *R. trifolii* LPS, whereas many infections aborted near their origins and accumulated brown deposits if pretreated with LPS from *R. meliloti* 102F28. LPS from *R. leguminosarum* 300 also caused most infection threads to abort. Other specific responses of root hairs to infection-stimulating LPS from *R. trifolii* included acceleration of cytoplasmic streaming and production of novel proteins. Combined gas chromatography-mass spectroscopy and proton nuclear magnetic resonance analyses indicated that biologically active LPS from *R. trifolii* 0403 in the early stationary phase had less fucose but more 2-*O*-methylfucose, quinovosamine, 3,6-dideoxy-3-(methylamino)galactose, and noncarbohydrate substituents (*O*-methyl, *N*-methyl, and acetyl groups) on glycosyl components than did inactive LPS in the mid-exponential phase. We conclude that LPS-root hair interactions trigger metabolic events that have a significant impact on successful development of infection threads in this *Rhizobium*-legume symbiosis.

Establishment of an effective *Rhizobium*-legume symbiosis can be viewed as a process of cellular recognition and compatibility between bacterial and plant cells. The infection process involves bacterial attachment, root hair deformation, bacterial penetration of the root hair wall, formation and sustained development of the infection thread, bacterial release from infection threads within emerging root nodule cells, and bacterial differentiation into nitrogen-fixing bacteroids.

The lipopolysaccharides (LPS) of rhizobia are likely to be involved in the infection process. They are major glycoconjugates on the surface of *Rhizobium leguminosarum* biovars viciae, trifolii, and phaseoli (hereafter called *R. leguminosarum*, *R. trifolii*, and *R. phaseoli*, respectively), *R. meliloti*, and peanut rhizobia which bind host lectins (36, 38, 40, 44, 50, 69). Certain symbiotically defective mutant *Rhizobium* strains have altered LPS (4, 6, 9, 10, 10a, 20, 28, 39, 49, 54, 57–61). In general, rough mutants lacking the O antigen and unknown amounts of the R core are often unable to sustain development of infection threads in host root hairs and multiply and fix nitrogen (Fix^-) inside the nodule. Some rough LPS mutant strains of *R. trifolii* can be restored to the Fix^+ phenotype on alsike clover by complementation with cosmids containing *lps* genes from *R. phaseoli* (6). These recombinant hybrid strains produce an O antigen which is immunologically similar to that of the *R. phaseoli* donor (6). This finding suggests that the intact O antigen portion of LPS is not essential for bacterial penetration of cell walls of the host root hair but rather is required to sustain growth of infection threads within these target host cells and effectively colonize host nodule cells. Also, various rhizobial strains differing in LPS composition and serology can nodulate the same host (8). Thus, if LPS contains specific features required for effective nodulation, these LPS domains must be conserved in various strains of *R. leguminosarum* biovars and/or be poorly immunogenic.

A complication of the above-described studies is the possibility that the observed phenotypes are due to pleiotropic effects of LPS alterations on outer membrane biogenesis,

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structure, and function. This problem is exemplified in *Salmonella typhimurium*, where mutations causing rough and deep rough alterations in LPS lead to pleiotropic loss of intrinsic outer membrane proteins (2), failure of porin polypeptides to trimerize (63), and redistribution of phospholipids (53), all of which alter outer membrane permeability. In *R. leguminosarum*, mutations causing altered LPSs also eliminated true motility and changed the levels of certain outer membrane proteins (20).

An alternative approach to study of the role of LPS in symbiotic development has been to examine the biological activity of *Rhizobium* LPS applied directly to legume roots, but in most cases the purity of the LPS was not established. Examples include reports of cytokininlike activity of LPS preparations from *R. leguminosarum* on pea root explants (41) and deceleration of symplastic communication between adjacent soybean cells in suspension culture by LPS from *Bradyrhizobium japonicum* (26). Partially purified LPS from *R. trifolii* was assayed for the ability to deform clover root hairs but found to be inactive (23, 70). LPS from *R. leguminosarum* and *R. meliloti* could block attachment of the bacteria to host root surfaces (40, 45) but not to root hair tips (64).

Involvement of LPS in plant pathogenesis has also been reported. LPS treatment of hosts, followed by inoculation with the pathogen, leads to tumor inhibition (67), induction of disease resistance (29), and modulation of hypersensitive necrosis (52).

In this report, we describe interactions between purified LPS from rhizobia and root hairs from white clover. We establish that *Rhizobium* LPS (i) is released from the bacteria in the root environment, (ii) binds to root hair tips and infiltrates their cell wall, and (iii) elicits cellular responses in root hairs, including concentration-dependent stimulation of infection thread development and changes in cytoplasmic streaming and protein composition.

(A preliminary summary of this work was presented at the 7th International Congress on Nitrogen Fixation, August 1983, Noordwijkerhout, The Netherlands [16].)

MATERIALS AND METHODS

Bacterial and plant cultures. The bacterial strains used and their sources were as follows: *R. trifolii* 0403, Rothamsted Experimental Station, Harpenden, United Kingdom; *R. trifolii* 2S-2, D. Hubbell, University of Florida; *R. trifolii* ANU843 and *R. leguminosarum* 300, B. Rolfe, Australian National University; and *R. meliloti* 102F28, J. Burton, The Nitragin Co., Milwaukee, Wis. Bacteria were maintained on BIII agar slants (12). The legume host was white clover (*Trifolium repens* L.) variety Louisiana Nolin. All assays with seedlings were performed under microbiologically controlled conditions using nitrogen-free Fahraeus medium (−NF) (24) as the plant growth medium. Slide cultures without agar supports (12, 24) were incubated in a plant growth chamber delivering 26,900 lux of mixed incandescent and fluorescent lighting for a 14-h photoperiod at 22°C at daytime and 20°C at night.

Isolation and purification of LPS. Bacteria were cultured at 30°C in shaken flasks containing BIII broth and monitored for growth by turbidity with a Klett-Summerson colorimeter with a no. 66 red filter. Cultures of *R. trifolii* 0403 were harvested by centrifugation in the mid-exponential (50 Klett units) or early stationary (90 Klett units) phase. The LPSs isolated from *R. trifolii* 0403 in these growth phases are hereafter referred to as K50 LPS and K90 LPS, respectively (36). Cultures from other rhizobia were harvested in the early stationary phase. Pelleted cells were suspended in 0.5 M NaCl and stirred rapidly for 1 h to remove capsular polysaccharide. LPS was extracted from cells by hot phenol-water, dialyzed against water, and purified by a combination of nuclease digestion, ultracentrifugation, ion-exchange chromatography, and gel filtration chromatography to yield an LPS peak with a constant ratio of total carbohydrate, uronic acid, 2-keto-3-deoxyoctulonic acid, and heptose (8, 36). LPS peak fractions were pooled, dialyzed against deionized water, and lyophilized. For fatty acid analysis, the LPS sample obtained after nuclease digestion was fractionated directly on a Sepharose 4B column (33). The carbohydrate peak containing the same marker LPS sugars as LPS purified by the original method (as determined by gas chromatography [GC]-mass spectroscopy [MS]) was then dialyzed and lyophilized. LPSs from *Escherichia coli* O127:B8 and *Salmonella typhimurium* W were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Difco Laboratories (Detroit, Mich.), respectively, and used without further purification.

***Limulus* lysate assay for free LPS.** The presence of *Limulus* lysate-reactive material as an indicator of extracellular LPS in seedling slide cultures was measured by using pyrogen-free reagents and glassware as recommended by the supplier (Sigma Chemical Co.). Slide cultures were inoculated with *R. trifolii* 0403 (2×10^7 cells per seedling). After 2 days of incubation, the culture medium under the coverslip was diluted with pyrogen-free water, filter sterilized through 0.2-μm-pore-size filters, and assayed. The lower limit of cell-free LPS detection by the *Limulus* lysate assay was established by determining the minimum quantity of purified *R. trifolii* 0403 K90 LPS required to cause a positive gel reaction within 2 h of incubation.

Localization of LPS-root hair interactions. Rabbit antiserum to *R. trifolii* 0403 K90 LPS was prepared as previously described (36), by using a passive hemadsorption immunogen (18). Immunofluorescence microscopy was used to establish the dilution of anti-K90 LPS serum which would minimize its cross-reactivity with clover roots. In subsequent experiments, seedlings were incubated for 30 min with gentle shaking in −NF medium containing K90 LPS (1 μg/ml, 1 μg per seedling). Seedlings were then rinsed with −NF medium, and bound LPS was detected on the root surface by immunofluorescence microscopy and immunocytofluorimetry (17, 36) by using rabbit anti-K90 LPS serum (3 mg/ml diluted in phosphate-buffered saline [10 mM KH_2PO_4-K_2HPO_4 and 145 mM NaCl, pH 7.2; PBS]) and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG; Sigma Chemical Co.). Controls included incubation of LPS-treated roots with preimmune rabbit IgG and incubation of nontreated roots with immune rabbit anti-K90 LPS serum.

For electron microscopy, seedlings were incubated with filter-sterilized K90 LPS in −NF (1 μg per seedling) for 2 h and then rinsed in −NF. The roots were excised in 2% glutaraldehyde in buffer A of Maupin and Pollard (51) containing saponin and tannic acid at pH 7.0. After 1 h of fixation, the roots were rinsed in buffer A at pH 6.2, postfixed for 1 h in 1% OsO_4, dehydrated through a graded ethanol series, and then infiltrated with and embedded in vinylcyclohexene dioxide/hexenyl succinic anhydride (VCD/HXSA) (Ladd Research Industries, Burlington, Vt.). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM-10 transmission electron microscope.

For immunoelectron microscopy, roots were treated for

30 min with filter-sterilized K90 LPS (5 μg in 1 ml of −NF), rinsed in −NF, fixed in glutaraldehyde, dehydrated through an ethanol series, and then infiltrated with and embedded in Lowicryl K4M resin (3). Ultrathin sections were collected on nickel grids coated with Butvar B-98 (Electron Microscopy Sciences, Fort Washington, Pa.). Sections were floated on PBS containing 0.05% Tween 20 (PBST) for 5 min and transferred to 1% bovine serum albumin in PBST for 15 min to block nonspecific sites. The grids were transferred to rabbit anti-K90 LPS serum (diluted to 3 mg/ml in PBST) for 1 h, washed three times for 5 to 15 min each time in PBST, and floated for 1 h on a suspension of colloidal gold particles (15-nm diameter) coated with goat anti-rabbit IgG (Janssen Life Sciences, Olen, Belgium) diluted 1:20 in PBST. Two controls included incubation of LPS-treated roots with preimmune serum and untreated roots with immune anti-LPS serum (diluted to 3 mg/ml in PBST). After being washed with PBST and distilled water, the sections were treated and examined as described above. Gold particle density was calculated from several randomly selected electron micrographs by using the point count and area modes of a Bioquant System IV Image Analyzer (15).

Plant bioassay for infection-related biological activity of LPS. A bacterial inoculum was grown on BIII plates for 5 days at 30°C, suspended in PBS, centrifuged at 34,500 × *g* for 20 min, suspended in −NF medium, filtered through glass wool to remove flocs, and adjusted to 2.5×10^8 bacteria per ml. Axenically grown seedlings (12) were incubated for 1 h with 0.2-μm-pore-size filter-sterilized solutions of purified LPS in −NF medium. They were then rinsed with −NF medium to remove unadsorbed LPS and inoculated with *R. trifolii* (2.5×10^7 cells per seedling) in slide cultures without agar supports (two seedlings per slide). The locations of the root tips at the time of inoculation were marked on the back of the microscope slide. Slide cultures were incubated for 4 days. During this period, root hair infections complete a phase of exponential increase and the rate abruptly decreases as primordial nodules begin to develop (55). Roots were examined by phase-contrast microscopy on the upper side of the slide culture relative to the root tip mark. In some experiments, infected root hairs were counted regardless of the stage of infection thread development. In other experiments, root hair infections were categorized as successful or aborted on the basis of whether the infection thread grew through the root hair and penetrated the underlying root cortex during the 4-day incubation period. Infected root hairs on the underside of the root were not counted, and no allowance for unobserved infections was made in the figures or tables. This is not a major problem, however, since 66 to 80% of all infections can be seen from one side of the clover root (55).

The following parameters of this plant infection assay were varied: LPS source and concentration, duration of preinoculation exposure of the seedlings to LPS, the *R. trifolii* strain used as the inoculant, and addition of 15 mM KNO_3, 15 mM KCl, or 1 mM NH_4Cl to the −NF medium. These concentrations of combined nitrogen ions block infection of white clover root hairs by *R. trifolii* 0403 (13).

Immunoblot and SDS-PAGE analyses of LPS. One-microgram samples of LPSs from *R. trifolii* 0403, 2S-2, and ANU843 were spotted onto nitrocellulose paper and immunoblotted with rabbit anti-LPS as described by Tsang et al. (66) and modified by Darr et al. (11), except that 3% nonfat dry milk was the blocking agent, 0.05% (vol/vol) Tween 20 was in the wash buffer, and bound immunoglobulin was detected by using a 1-μg/ml solution of *Staphylococcus aureus* protein A-alkaline phosphatase conjugate (46) obtained from Sigma Chemical Co. Purified LPS samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and detected by periodate oxidation, followed by silver staining (31). The separating gel contained 15% acrylamide and was 50 cm long.

Root hair growth. Two-day-old seedlings were placed flat on −NF agar plates. One-microliter volumes of a filter-sterilized solution of LPS in −NF medium (4 μg/μl) were spotted on the youngest emerging root hairs (four plants per treatment visualized by darkfield stereomicroscopy), and the position of LPS application was marked on the back of the plate. The plates were then covered and incubated vertically in a growth chamber. Within 24 h, root hairs mature to their fully elongated length (15). After 24 h, 1- to 2-mm root segments were excised at the position of LPS application, mounted in −NF, and examined by bright-field microscopy. The lengths of root hairs at the optical medium plane of the root segment were measured by digitizing morphometry image analysis (15).

Cytoplasmic streaming in root hairs. One-day-old slide cultures of axenic white clover seedlings were examined by phase-contrast microscopy using a 536-nm interference contrast filter to locate root hairs displaying cytoplasmic streaming that could easily be recognized by a constant flow of refractile organelles. Then, while being viewed through the microscope, the needle of a 5-μl Hamilton syringe was carefully inserted underneath the coverslip to introduce 1 μl of filter-sterilized −NF solution containing 0 or 4 μg of LPS extracellularly next to these root hairs. After 20 min of incubation, these root hairs were video recorded by the time-lapse method with 6:1 time compression (10 video frames per s). Cytoplasmic streaming was measured from tract reconstruction of constantly flowing, refractile organelles in 1-s segments, and its velocity was calculated from 10 to 20 consecutive length measurements per tract by using the digitizing image analysis system (15).

Protein composition of root hairs. Approximately 800 2-day-old seedlings were incubated with 12 ml of a 5-μg/ml filter-sterilized solution of LPS for 1 h at room temperature with 100-rpm gyratory shaking. Treatments included *R. trifolii* 0403 K50 LPS, *R. trifolii* 0403 K90 LPS, *R. meliloti* 102F28 LPS, or −NF medium as a control. The bathing solution was subsequently discarded. The seedlings were placed on moistened sterile filter paper, grown in the dark for 24 h at 20°C, and then transferred to liquid N_2 to fracture and harvest the root hairs selectively (25). Root hair proteins were processed for two-dimensional polyacrylamide gel electrophoresis by protocol PP6 with Kit 1004 from Protein Databases Inc. (Huntington Station, N.Y.) and submitted to that same company for analysis. The first-dimensional isoelectric-focusing gel had ampholines covering a pH range of 3 to 10. The acrylamide concentration in the second dimension was 12.5%. After electrophoresis, gels were silver stained and the protein spots were analyzed by quantitative densitometry using PDQUEST image analysis software featuring editing of gel images, automatic protein spot matching in quadrants, and quantitative fold change analysis.

Chemical analysis of LPS from *R. trifolii* 0403 in the mid-exponential and early stationary phases. LPS (0.5 mg) was hydrolyzed with 0.5 ml of 1% acetic acid at 100°C for 2 h and then cooled to room temperature and extracted with chloroform (2 × 0.5 ml). The organic layer was removed, and the aqueous portion was evaporated to dryness under nitrogen. The lipid-free residue was analyzed by ^1H nuclear magnetic resonance (NMR) and by GC-MS after conversion

of glycosyl components to alditol acetates (including isotope labelling and selected ion monitoring for uronic acid derivatives) as described elsewhere (35). For NMR analysis, the samples were dissolved in 0.5 ml of D_2O (99.8% deuterium), lyophilized, and redissolved in 0.4 ml of D_2O (99.97% deuterium). ^1H NMR spectra were recorded with a Bruker WM250 spectrometer operating at 250 MHz for protons. The chemical shifts were measured relative to internal tetramethylsilane. Fatty acid composition of LPS was determined by flame ionization detection-GC and GC-MS as described elsewhere (33, 34).

Glycosyl standards not commercially available were prepared by synthetic methods. Quinovosamine was a gift from L. Anderson, University of Wisconsin-Madison. The gluco, galacto, and manno isomers of 3-amino-3,6-dideoxy-3-*N*-methylhexose were synthesized as previously described (32). To prepare 2-*O*-methyldeoxyhexoses, 10 mg each of rhamnose, fucose, and quinovose was treated separately with silver (I) oxide (100 mg), methyl iodide (50 μl), and dimethyl formamide (100 μl) and the reaction mixture was stirred at room temperature overnight. The solutions were filtered and evaporated to dryness. Alditol acetate derivatives of the partially methylated deoxysugars were then prepared by trifluoroacetic acid hydrolysis, reduction with $NaBH_4$, peracetylation using acetic anhydride-pyridine, identification by GC-MS, and quantitation by flame ionization detection-GC.

To detect contaminating protein, 40- and 50-μg samples of K90 LPS from *R. trifolii* 0403 were analyzed by the Folin method (48) and compared to a standard curve based on bovine serum albumin.

RESULTS

Purity of LPS isolated from *R. trifolii* 0403. Chemical analyses of isolated LPS detected no contaminating marker components of acidic heteropolysaccharide, glucans, phospholipid, or protein. A detailed description of the composition of LPS isolated from *R. trifolii* 0403 is presented below.

Extracellular *Limulus* lysate-reactive material in slide cultures of *R. trifolii* and white clover roots. With purified *R. trifolii* 0403 K90 LPS as the standard, the lower limit of detection by the *Limulus* lysate assay was 6 pg of cell-free LPS per 100-μl sample (defined as 1 U of activity). Assay of the filter-sterilized medium in 2-day-old slide cultures of *R. trifolii* 0403 and white clover indicated that the roots are exposed to cell-free LPS. The units of *Limulus* lysate activity per milliliter of −NF medium without bacteria or seedlings, with seedling only, with bacteria only, and with bacteria plus seedlings were 10, 10, 1,000, and 36,390, respectively (±3% standard error of the mean [SEM]).

Interaction of *R. trifolii* 0403 K90 LPS with white clover roots. Roots incubated with LPS, preimmune rabbit serum, and fluorescein isothiocyanate-conjugated anti-rabbit IgG showed no immunofluorescence reaction (Fig. 1A and B). Roots incubated with diluted anti-0403 K90 LPS serum, followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG (no LPS pretreatment) had a low level of fluorescence (Fig. 1C and D). In contrast, roots treated with K90 LPS, diluted anti-0403 K90 LPS serum, and fluorescein isothiocyanate-conjugated anti-rabbit IgG yielded positive immunofluorescence (Fig. 1E to I) which was 55-fold brighter than the level of cross-reactive fluorescence obtained without LPS pretreatment (6,660 ± 476 versus 122 ± 15 photovolts/mm^2 [$\bar{x}$ ± SEM]). Microscopic examination of four replicate plants indicated that 78% (±4% [SEM]) of the root hairs had their brightest fluorescence at the tip (Fig. 1I), in contrast to 14% (±2%) with uniform fluorescence and 8% (±4%) with no fluorescence.

Comparison of several fixation protocols showed that the procedure of Maupin and Pollard (51) enhanced the preservation of cytoplasmic elements in root hairs. Transmission electron microscopy of longitudinal sections showed a variety of organelles near the tip, indicative of active growth (Fig. 2A). Median sections at the tips of root hairs showed a clearly defined plasmalemma with occasional invaginations facing the periplasmic space near the inner face of the cell wall. The most striking change in the ultrastructure of root hairs upon treatment of roots with *R. trifolii* 0403 K90 LPS was accumulation of electron-dense material deposited on the outer face of root hair tips, within the wall, and within these plasmalemma invaginations (Fig. 2B to D).

These findings prompted us to examine whether this new electron-dense material on the root hair was LPS and, if so, whether it could traverse the hair wall. Immunoelectron microscopy of ultrathin sections from root hairs on LPS-treated roots revealed extensive, specific binding of anti-LPS immunoglobulins to electron-dense material bound to the external surface of the root hair, within and on the inner face of the fibrillar root hair wall (Fig. 3A). Marginal negative reactions with the two controls (preimmune serum on LPS-treated roots and anti-K90 LPS on untreated roots) are presented in Fig. 3B and C, respectively. Quantitation of immunogold particle density in multiple, randomly selected micrographs indicated that the immunogold localization of LPS associated with electron-dense material on LPS-treated root hairs was 29-fold higher using homologous anti-LPS antibody than the background levels obtained in negative controls using preimmune serum or lacking LPS pretreatment (Table 1; also, compare Fig. 3A with B and C).

Infection-related biological activity of *R. trifolii* LPS on white clover root hairs. During the 4-day period of this plant infection bioassay, *R. trifolii* infected more root hairs above than below the root tip mark (Fig. 4), despite development of root hairs in both regions. Infections occurred throughout the entire length of the root hair region present at the time of inoculation.

The number of infected root hairs per seedling above the root tip mark increased four- to five-fold when seedlings were pretreated with K90 LPS from *R. trifolii* 0403 before inoculation (Fig. 4). The optimum response with K90 LPS occurred at 5 μg/ml (5 μg per seedling), above which the number of infections declined. Since the approximate density of root hairs ranged from 400 to 800 per average root hair region at the time of inoculation, a dose of 5 μg of K90 LPS was no more than 6 to 12 ng per root hair. In contrast, K50 LPS had little effect on the number of infected root hairs over the 0.1- to 10-μg/ml range. Neither K50 LPS nor K90 LPS significantly increased the number of infected root hairs developing below the root tip mark during the 4 days of incubation. As little as 1 min of exposure of seedlings to a solution of 5 μg of K90 LPS per ml led to an increase in root hair infections (Fig. 5), and 30 min of exposure was sufficient to achieve the optimal stimulatory effect. Of course, adsorbed and/or absorbed LPS remaining on the root after washing had the 4-day duration of the bioassay to exert its influence on root hair infection. The stimulatory effect of K90 LPS from *R. trifolii* 0403 was unable to nullify the inhibitory effect of 1 mM NH_4^+ or 15 mM NO_3^- on root hair infection (Table 2).

Infection-related biological activity is conserved among serologically distinct LPSs of different *R. trifolii* strains. Im-

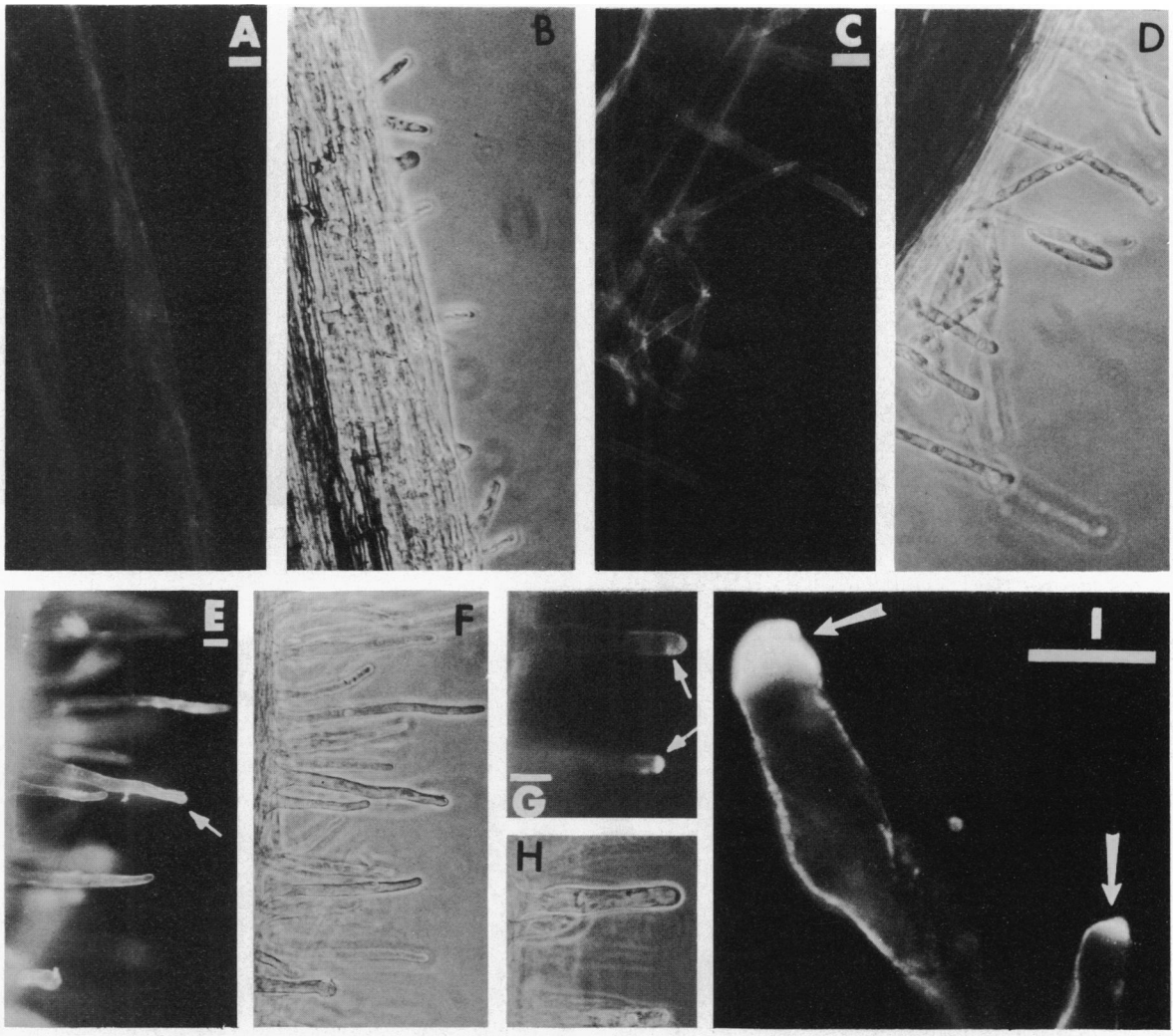

FIG. 1. Immunofluorescence (A, C, E, G, and I) and phase-contrast (B, D, F, and H) microscopy of matched regions of white clover roots incubated with *R. trifolii* 0403 K90 LPS. (A and B) Negative control using LPS-treated roots and preimmune serum. (C and D) Negative control using untreated roots and anti-LPS serum. (E to I) Roots treated with LPS and anti-LPS serum. Note binding of LPS to root hair tips (arrows). Bars, 20 μm.

munoblot analysis indicated that the immunodominant epitopes of LPSs from *R. trifolii* 0403, 2S-2, and ANU843 are distinct, with little or no cross-reactivity (Fig. 6). SDS-PAGE separated the LPSs from these *R. trifolii* strains into two bands. According to Carlson et al. (9, 10), the slower-migrating band (LPS I) consists of complete LPS and the faster-migrating band (LPS II) consists of LPS lacking the O antigen. SDS-PAGE analysis of the LPSs indicated that LPS I bands of strains 0403, 2S-2, and ANU843 had different mobilities but the LPS II bands had very similar electrophoretic mobilities (data not shown).

Two experiments showed that the infection-modulating activity is conserved among the LPSs of these different *R. trifolii* strains, despite their serological and structural diversity (Table 3). (i) The number of root hairs infected by *R. trifolii* 0403 above the root tip mark was significantly affected by preinoculation treatment of seedlings with LPS from *R. trifolii* 2S-2. This LPS produced a narrower dose response than did K90 LPS from strain 0403, with optimal stimulation of infection at 1 μg of LPS per seedling and significant inhibition of infection at 10 μg of LPS per seedling. (ii) The number of white clover root hairs infected by *R. trifolii* ANU843 was increased by pretreating seedlings for 1 h with either 0403 K90 or ANU843 LPS. This stimulation of root hair infection by LPS was observed only above the root tip mark.

In contrast, pretreatment of white clover seedlings with LPS from heterologous rhizobia or members of the family *Enterobacteriaceae* did not enhance infection of root hairs by *R. trifolii* (Table 3). LPS from *R. meliloti* 102F28 had little effect on the number of root hairs infected by *R. trifolii* 0403 and decreased infections by *R. trifolii* ANU843. These infection threads were often aborted, appearing short, thickened, and with distinct brown deposits at the site of incipient penetration by *R. trifolii* (Fig. 7A and B). Localized browning did not occur on infected root hairs of plants pretreated with LPS from *R. trifolii* 0403, regardless of whether the infection thread aborted early within the root hair or grew successfully to its base (Fig. 7C and D). Commercially available LPS preparations from *E. coli* and *S. typhimurium*

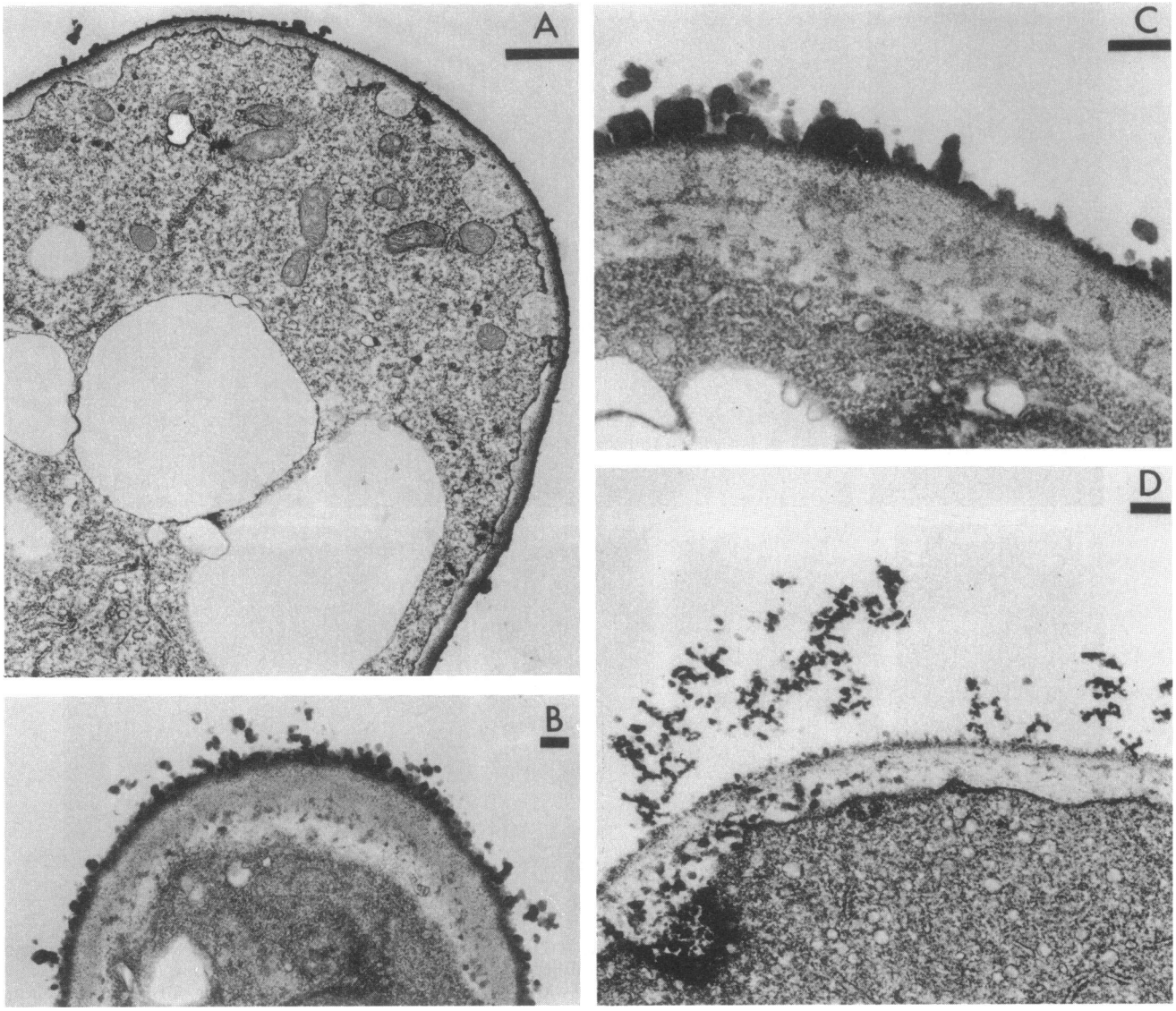

FIG. 2. Transmission electron microscopy near the tips of root hairs on axenically grown white clover roots. (A) Control, untreated root hair. (B to D) Root hairs incubated with K90 LPS from *R. trifolii* 0403. Bars, 1 μm in panel A and 0.25 μm in panels B to D.

either had no effect or reduced the number of root hair infections induced by *R. trifolii* 0403.

The relationship between LPS pretreatment and development of successful versus aborted infection threads in root hairs was examined further by using LPSs from *R. trifolii* 0403 and *R. leguminosarum* 300 and an inoculum of *R. trifolii* 0403 or ANU843. Results of the infection bioassay indicated a clear distinction in the proportion of successful versus aborted infections within root hairs, depending on the LPS source (Table 4). This ratio remained relatively constant (2.5:1) in seedlings pretreated with no LPS or with *R. trifolii* 0403 K90 LPS, despite the increased number of infected root hairs in the latter case. In contrast, pretreatment of roots with *R. leguminosarum* 300 LPS before inoculation with either *R. trifolii* strain produced a higher proportion of aborted infection threads without affecting the number of infected root hairs or inducing a localized browning response.

Effects of *R. trifolii* LPS on clover root hair elongation and cytoplasmic streaming. The average length (±SEM) of mature root hairs after 24 h of incubation with −NF medium was 375 ± 16 μm and was not significantly different from lengths of root hairs grown for the same period with *R. trifolii* 0403 K50 (385 ± 8 μm) or K90 (371 ± 10 μm) LPS. Thus, the amount of *R. trifolii* LPS used in these assays caused no obvious inhibition of clover root hair growth. By using time-lapse video microscopy, we detected a 63% increase in the rate of cytoplasmic streaming in clover root hairs upon 20 min of exposure to K90 LPS (Table 5). In contrast, the rate of cytoplasmic streaming in root hairs was unaffected by treatment with K50 LPS from *R. trifolii* 0403 or LPS from *R. meliloti* 102F28 or *R. leguminosarum* 300 (Table 5).

LPS-induced changes in the protein composition of white clover root hairs. The *x-y* coordinate positions of root hair proteins separated by two-dimensional PAGE and derived

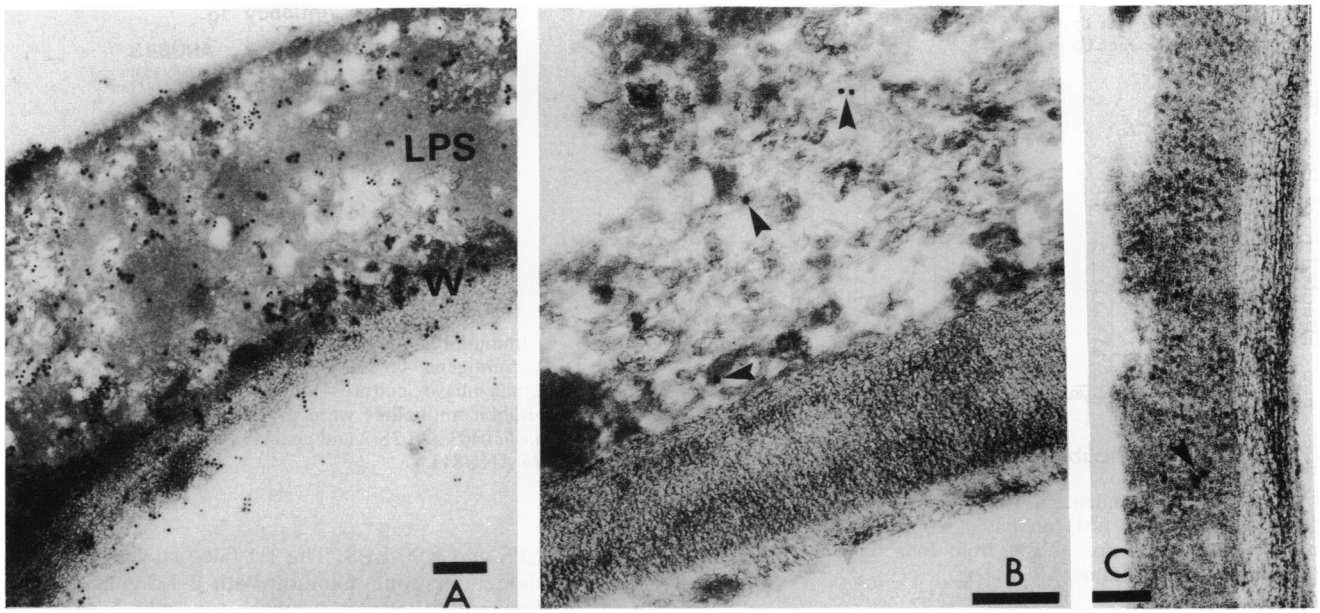

FIG. 3. Immunoelectron microscopy of K90 LPS from *R. trifolii* 0403 binding to and traversing the wall of clover root hairs. (A) Specific immune reactions (anti-LPS and 15-nm-diameter gold conjugates of anti-IgG) localizing LPS antigen in granular material deposited outside of the root hair wall (LPS) and within and on the inner face of the fibrillar root hair wall (W). Control reactions using preimmune serum on LPS-treated root hairs and anti-LPS serum on root hairs without LPS pretreatment are shown in panels B and C, respectively. Arrowheads point to marginal binding of gold conjugates in the external LPS layer in panel B and the cytoplasm in panel C. Bars, 0.25 μm.

from each of four treatments (−NF control, K90 LPS from *R. trifolii* 0403, *R. meliloti* 102F28 LPS, and K50 LPS from *R. trifolii* 0403) are shown on a computer-generated composite map with approximate molecular weight and isoelectric point calibration in Fig. 8. Comparison of densitometric histograms plotting the relative amounts of 237 proteins matched from the composite map indicated that 13 root hair proteins were significantly affected by LPS treatment. The positions of these root hair proteins affected by LPS are indicated on the composite spot map and are arbitrarily identified by the numbers 1 to 13. Protein 1 was detected only in control root hairs (it disappeared upon all three LPS treatments). Protein 2 was detected only in root hairs treated with K50 LPS. Protein 3 was detected in root hairs treated with all LPSs but not in control root hairs, although its concentration was significantly lower in root hairs grown with K90 LPS. Proteins 4 to 8 were detected only in root hairs treated with K90 LPS. Proteins 9 and 10 were increased in root hairs treated with K90 LPS and decreased in root hairs treated with K50 LPS or LPS from *R. meliloti* 102F28. Proteins 11 to 13 were detected only in root hairs treated with LPS from *R. meliloti* 102F28. The relative levels of several other proteins changed to lesser extents in response to LPS treatment. The levels of some root hair proteins located in each quadrant of the spot map remained relatively unchanged for each of the four treatments, indicating that separation of proteins and computerized spot matching are reproducible with multiple gels in this two-dimensional PAGE analysis.

TABLE 1. Density of immunogold particles which detect *R. trifolii* 0403 K90 LPS associated with white clover root hairs

Root treatment before Au–anti-IgG	Mean no. of immunogold particles/ μm^2 ± SEM (no. of micrograph areas examined)	
	Electron dense	Electron transparent
K90 LPS, then preimmune serum	2.6 ± 0.6 (6)	0.3 ± 0.2 (15)
Anti-K90 LPS only	2.6 ± 0.5 (13)	0.3 ± 0.1 (25)
L90 LPS, then anti-K90 LPS	74.6 ± 7.1 (13)	2.6 ± 0.5 (18)

Chemical analysis of LPS from *R. trifolii* 0403. The identities and molar proportions of glycosyl components in lipid-

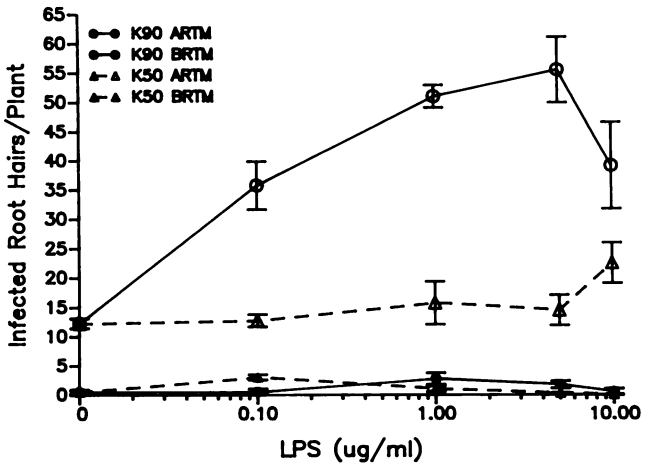

FIG. 4. Dose responses of infection-related biological activities of K50 LPS and K90 LPS from *R. trifolii* 0403. Shown are infected root hair counts above (ARTM) and below (BRTM) the root tip mark at the time of LPS treatment. Error bars are ±SEM from eight replicate plants that received the same treatment.

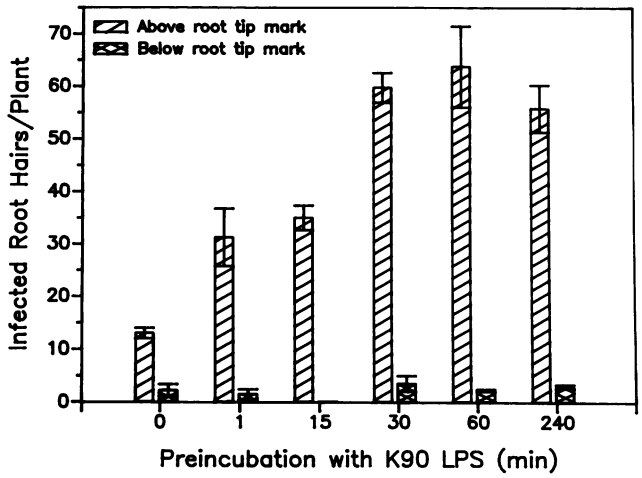

FIG. 5. Time dependence of root preincubation with 5 μg of K90 LPS from *R. trifolii* 0403 on its infection-modulating biological activity. Error bars are ±SEM from four replicate plants that received the same treatment.

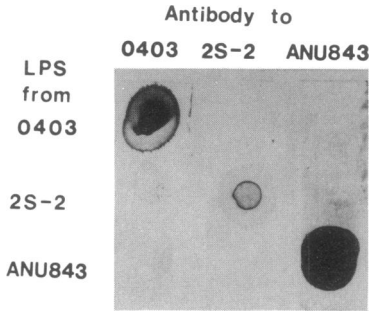

FIG. 6. Immunoblot analysis of LPSs from *R. trifolii* strains analyzed for antigenic cross-reactivity. LPSs were isolated from early-stationary-phase cultures of *R. trifolii* 0403, 2S-2, and ANU843. Rabbit antibodies were prepared against purified LPSs from *R. trifolii* 0403 and 2S-2 and against steamed and washed cells of *R. trifolii* ANU843.

free carbohydrate chains obtained after mild acid hydrolysis of K90 LPS from *R. trifolii* 0403 are presented in Table 6. The variability in the molar proportions of the components was due primarily to the wide spectrum of acid liabilities of the different glycosyl linkages and the differences in stability of the free monosaccharides in acid. Hence, the harsher conditions necessary to liberate glucosamine completely led to some decomposition of the 6-deoxysugars. The quantity of galacturonic acid present was highly variable and difficult to quantitate because of steric problems in completely methylating and reducing all of the carboxyl residues with borodeuteride prior to hydrolysis. Similar problems were encountered in quantitating the total amount of galacturonic acid in a purified R core component of LPS from another strain of *R. trifolii* (7). Despite this variability, reproducible differences were always found in the relative compositions of certain glycosyl components between different batches of K50 LPS and K90 LPS. K90 LPS always contained a lower molar proportion of fucose and higher molar proportions of 2-*O*-methylfucose, 3,6-dideoxy-3-methylamino-galactose, and quinovosamine. The molar proportions of the other glycosyl components (Table 6) remained relatively constant for K50 LPS and K90 LPS. The 1:1 Glc-Gal ratio indicates minimal detectable contamination with β-1,2-glucan, cellulose, and/or acidic heteropolysaccharide, which would increase this ratio in the LPS sample.

^{1}H NMR spectroscopy indicated the presence of *O*-methyl, *N*-methyl, and acetyl substitutions in the carbohydrate chains of *R. trifolii* 0403 LPS released by mild acid hydrolysis (Fig. 9). Resonances at approximately δ 2.1 were assigned to acetyl groups, while resonances centered at δ 2.6 and δ 3.2 were assigned to *N*-methyl and *O*-methyl groups, respectively. Each of these resonances was appreciably higher in K90 LPS than in K50 LPS (Fig. 9), indicating that the degrees of acetylation and methylation in the lipid-free carbohydrate chain(s) of *R. trifolii* 0403 LPS are different at these two culture ages. These results are consistent with the GC-MS data, which show that 2-*O*-methylfucose and 3,6-dideoxy-3-methylaminogalactose residues are more abundant in K90 LPS than in K50 LPS. Absence of proton resonances from the α-methylene group of 3-hydroxybu-

TABLE 2. Effect of *R. trifolii* 0403 K90 LPS on fixed-N inhibition of white clover root hair infection by *R. trifolii* 0403[a]

Addition to −NF plant growth medium in slide culture	Mean no. of root hair infections above root tip mark ± SE
None	13.9 ± 0.9
KNO_3 continuous	0.2 ± 0.2
NH_4Cl continuous	0.5 ± 0.3
KCl continuous	8.5 ± 0.5
KNO_3 + LPS continuous	0 ± 0
NH_4Cl + LPS continuous	0.5 ± 0.3
LPS (30 min), then KNO_3 continuous	0 ± 0
LPS (30 min), then NH_4Cl continuous	0.5 ± 0.3
LPS (30 min), then KCl continuous	48.5 ± 6.5

[a] LPS was used at 5 μg/ml. The final concentrations of KNO_3, KCl, and NH_4Cl were 15, 15, and 1 mM respectively, in −NF medium. Zero to two infections occurred below the root tip mark.

TABLE 3. Effect of LPS on infection of white clover root hairs by *R. trifolii*

LPS source	LPS concn (μg/ml)	*R. trifolii* inoculant strain	Mean no. of infected root hairs/plant ± SEM	
			Above root tip mark	Below root tip mark
−NF control	0.0	0403	15.0 ± 1.0	1.5 ± 1.5
R. trifolii 2S-2	0.1	0403	14.5 ± 0.5	2.0 ± 2.0
R. trifolii 2S-2	1.0	0403	43.5 ± 3.5	1.0 ± 0.0
R. trifolii 2S-2	5.0	0403	29.5 ± 9.5	2.5 ± 0.5
R. trifolii 2S-2	10.0	0403	2.0 ± 0.0	0.5 ± 0.5
R. meliloti 102F28	0.1	0403	14.5 ± 3.5	1.5 ± 0.5
R. meliloti 102F28	1.0	0403	17.5 ± 0.3	0 ± 0
R. meliloti 102F28	5.0	0403	13.5 ± 4.5	2.0 ± 1.0
R. meliloti 102F28	10.0	0403	14.0 ± 1.3	0.3 ± 0.3
E. coli O127:B8	5.0	0403	12.0 ± 4.0	0 ± 0
E. coli O127:B8	10.0	0403	16.5 ± 0.5	2.0 ± 1.0
S. typhimurium W	5.0	0403	4.5 ± 1.5	0 ± 0
S. typhimurium W	10.0	0403	10.5 ± 3.5	0 ± 0
−NF control	0.0	ANU843	15.9 ± 2.6	1.5 ± 0.5
R. trifolii ANU843	5.0	ANU843	57.3 ± 5.5	ND[a]
R. trifolii 0403 K90	5.0	ANU843	32.0 ± 2.0	3.0 ± 0.9
R. meliloti 102F28	5.0	ANU843	7.8 ± 1.7	1.8 ± 0.5

[a] ND, not done.

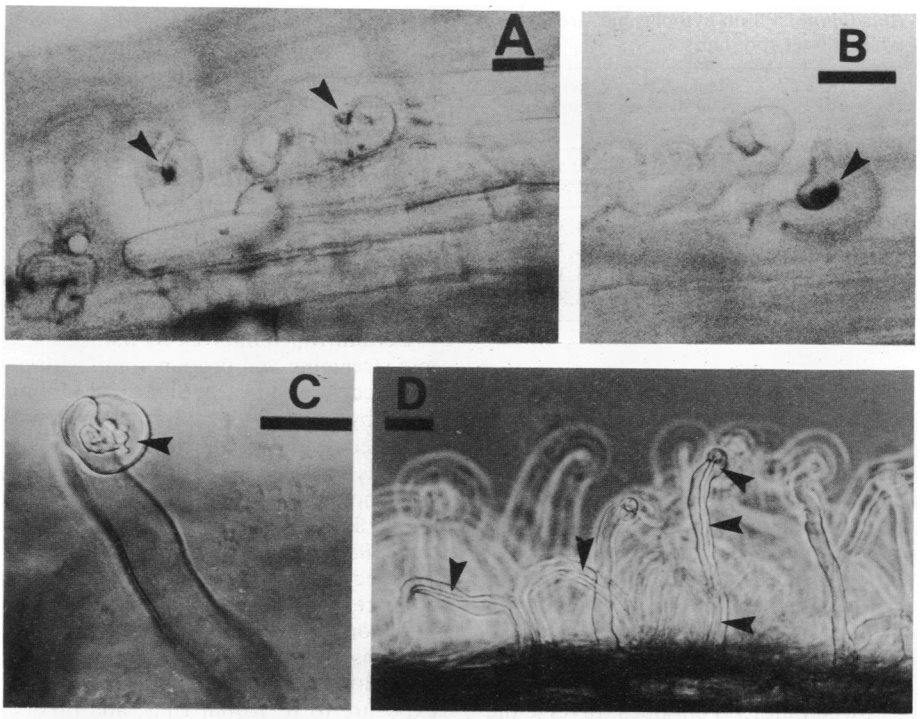

FIG. 7. Phase-contrast micrographs of white clover root hairs infected with *R. trifolii*. Roots were pretreated before inoculation with LPS from *R. meliloti* 102F28 (A and B) or with *R. trifolii* 0403 K90 LPS (C and D). Note dark (brown) deposits accumulated at the site of incipient aborted infection in panels A and B (arrowheads) but not at aborted or successful infection sites in panels C and D. Bars, 20 μm.

tyrate (δ 2.2 to 2.4) in the acidic heteropolysaccharide made by *R. trifolii* 0403 (35) also indicates a lack of detectable acidic heteropolysaccharide contamination in the LPS samples.

Flame ionization detection–gas-liquid chromatography and GC-MS analyses of the fatty acid components in *R. trifolii* 0403 LPS indicated the presence of 3-hydroxytetradecanoic acid, 3-hydroxy-12-methyltetradecanoic acid (anteiso-branched 3-hydroxypentadecanoic acid), 3-hydroxyhexadecanoic acid, 3-hydroxyoctadecanoic acid, and 27-hydroxyoctacosanoic acid. The variation in the quantities of these fatty acids between K50 LPS and K90 LPS did not exceed the variation for different batches of LPS obtained at the same culture age. The approximate molar proportion of fatty acid components in both LPSs was 2:1:1:1:1 (C_{14}–C_{15}–C_{16}–C_{18}–27-OH C_{28}). The presence of only hydroxy fatty acids indicated a lack of detectable phospholipid contamination in the LPS samples.

The Folin assay detected no protein in 40- and 50-μg samples of K90 LPS from *R. trifolii* 0403 (sensitivity, 1 μg of protein).

DISCUSSION

This study identified numerous levels of interaction between the LPS of *R. trifolii* and white clover root hairs during early stages of the infection process.

Results of the *Limulus* lysate assay suggest that some LPS is released from *R. trifolii* cultured in the external root environment and is available to interact with the root. In the well-studied enteric bacteria, release of LPS into the culture fluid occurs during normal growth as the cells divide and

TABLE 4. Relationship between LPS pretreatment and successful versus aborted infection threads in white clover root hairs above the root tip mark[a]

LPS source	LPS concn (μg/ml)	*R. trifolii* inoculant	% Successful infections	% Aborted infections	Mean no. of infected root hairs above root tip mark ± SEM
None	0	0403	70	30	15.0 ± 1.2
R. trifolii 0403 K90	5	0403	72	28	43.3 ± 1.8
R. leguminosarum 300	1	0403	28	72	16.8 ± 2.5
R. leguminosarum 300	5	0403	23	77	11.5 ± 2.5
R. leguminosarum 300	10	0403	29	71	10.5 ± 1.2
None	0	ANU843	73	27	15.5 ± 2.4
R. leguminosarum 300	1	ANU843	22	78	18.0 ± 2.0
R. leguminosarum 300	5	ANU843	17	83	16.3 ± 2.4
R. leguminosarum 300	10	ANU843	21	79	14.3 ± 1.5

[a] Successful and aborted infections are defined in the text.

TABLE 5. Effect of *Rhizobium* LPS on cytoplasmic streaming of white clover root hairs

LPS source	No. of replicates	Mean velocity (μm/s) of root hair cytoplasmic streaming ± SEM	
		Before LPS addition	20 min after LPS addition
R. trifolii 0403 K50	5	4.1 ± 0.1	4.3 ± 0.2
R. trifolii 0403 K90	7	4.0 ± 0.2	6.7 ± 0.2
R. meliloti 102F28	7	3.3 ± 0.1	3.3 ± 0.1
R. leguminosarum 300	18	4.0 ± 0.2	3.8 ± 0.1

shed excess outer membrane components, is under stringent control, and can be increased in nongrowing cells by relaxing the control of LPS synthesis (37, 62).

Immunofluorescence and immunoelectron microscopy showed that isolated LPS from *R. trifolii* binds rapidly to root hair tips. This part of the root surface is intimately involved in the infection process, since it is a site of symbiont recognition and attachment (19, 42), accumulation of host lectin (17, 42, 65), enhanced expression of pSym nodulation genes (14), marked curling and infection (24, 56), and determination of host specificity (1, 47). Immunoelectron microscopy also showed that LPS epitopes traverse the root hair wall and should be able to interact with the plasmalemma within 2 h of incubation. Further studies are necessary to determine whether the LPS is internalized within the cytoplasm of the root hair.

We devised a plant infection bioassay to test Kamberger's hypothesis (39) that *Rhizobium* LPS triggers infection-related events on host root hairs. This assay showed that pretreatment of roots with *R. trifolii* LPS significantly affects the frequency (increased or decreased) of root hair infections by this symbiont. The infection-modulating activity of *R. trifolii* LPS displays dosage and incubation time dependence. This response is restricted to the region of the root treated with LPS, indicating that it is a localized rather than a systemic response. It can be initiated by as little as 1 min of exposure of LPS to roots and is incapable of reversing inhibition of infection by combined nitrogen. We have found three infection-related characteristics which are significantly greater for *R. trifolii* 0403 in the early stationary than in the mid-exponential phase. These include the (i) efficiency of root hair infection per unit of inoculum (36), (ii) affinity for binding to trifoliin A which accumulates in particles loosely attached to root hair tips (36, 65), and (iii) infection-modulating effect of LPS on development of infection threads within host root hairs (this report). This biological activity is conserved among serologically distinct LPSs of *R. trifolii* strains.

In contrast to LPS from *R. trifolii*, preinoculation treatment of white clover roots with LPSs from certain heterologous symbionts (e.g., *R. meliloti* 102F28 and *R. leguminosarum* 300) leads to an increase in the relative proportion of undeveloped and/or aborted infection threads within root hairs. Interestingly, induction of aborted infections is also observed with rough LPS mutants of *R. phaseoli* on beans (54). The shorter infection threads could result from a delay in infection thread initiation, slower infection thread growth, or arrest of infection thread development within the root hair. The brown discoloration of aborted infection threads in white clover root hairs exposed to *R. meliloti* LPS before attempted penetration by *R. trifolii* may be analogous to plant defensive responses elicited by some incompatible microbial pathogens (30, 43) and to accumulation of osmiophilic droplets at root hair sites of attempted penetration by polysaccharide-defective mutants of broad-host-range *Rhizobium* strain NGR 234 (22). Although the heterologous rhizobia used here produced an LPS which is incompatible with successful infection of clover root hairs by *R. trifolii*, LPS from a larger number of heterologous strains and *R. trifolii* mutants altered in pSym nodulation genes must be examined to establish whether its infection-modulating biological activity is a host specificity-determining factor in symbiotic infection.

Certain *Rhizobium* LPSs induced alterations in cytoplasmic streaming and protein composition of clover root hairs. The acceleration of cytoplasmic streaming induced in root hairs by *R. trifolii* LPS is consistent with similar results obtained with an inoculum of *R. trifolii* (15, 56). This cytoskeleton response is correlated with the abilities of LPSs from homologous rhizobia to increase root hair infections (K90 LPS > K50 LPS or LPS from *R. meliloti* 102F28 or *R. leguminosarum* 300) and may influence the infection process by affecting the intracellular availability of substrates, intermediates, enzymes, cofactors, and organelles involved in infection thread development. The levels of several root hair proteins change significantly in vivo in response to incubation with different rhizobial LPSs. Some are increased upon exposure to *R. trifolii* LPS, and others are increased upon exposure to *R. meliloti* LPS. The biological activities of K50 LPS and K90 LPS from *R. trifolii* 0403 can be further differentiated by the changes they induce in the protein composition of root hairs. This macromolecular response of root hairs may be due to changes in the expression of plant genes and/or posttranslational modifications of their products. This implies active interaction between rhizobial LPS and plant receptors-signal effector systems in root hairs. Our results are consistent with preliminary reports that inoculation of pea and cowpea roots with symbiotic rhizobia leads to induction of root hair proteins (5, 27). It is important to determine whether these new root hair proteins function in the infection process.

In an earlier study, Hrabak et al. (36) found differences in quinovosamine, 2-*O*-methyl-6-deoxyhexose, and *N*-methyl-3-amino-3,6-dideoxyhexose contents between K50 and K90 LPSs from *R. trifolii* 0403. The present study advances beyond that work by identifying through chemical synthesis the exact configurations of these glycosyl components and the approximate molar stoichiometry of the various glycosyl and fatty acyl components in K90 LPS. In addition, the ^{1}H NMR study also supports the general proposal that there are chemical differences between the K50 and K90 LPSs used in the bioassays. These chemical analyses of K90 LPS detected no contaminating β-1,2-glucan, acidic heteropolysaccharide, cellulose, phospholipid, or protein. However, as is the case for natural products in general, we cannot exclude the possibility that the biological activity found in *Rhizobium* LPS is due to a trace contaminant present at levels below the limit of analytical detection. The LPSs used in this study were isolated from cells cultured without flavone inducers of pSym *nod* genes. Possible involvement of pSym *nod* genes in production or modification of biologically active LPS will be the subject of future studies.

There are several possible explanations for the chemical changes observed in LPS during various growth phases. One is that as cultures enter the stationary phase, LPS is altered by methylation of fucose or acetylation of some glycosyl component, etc. However, accurate quantitation of the

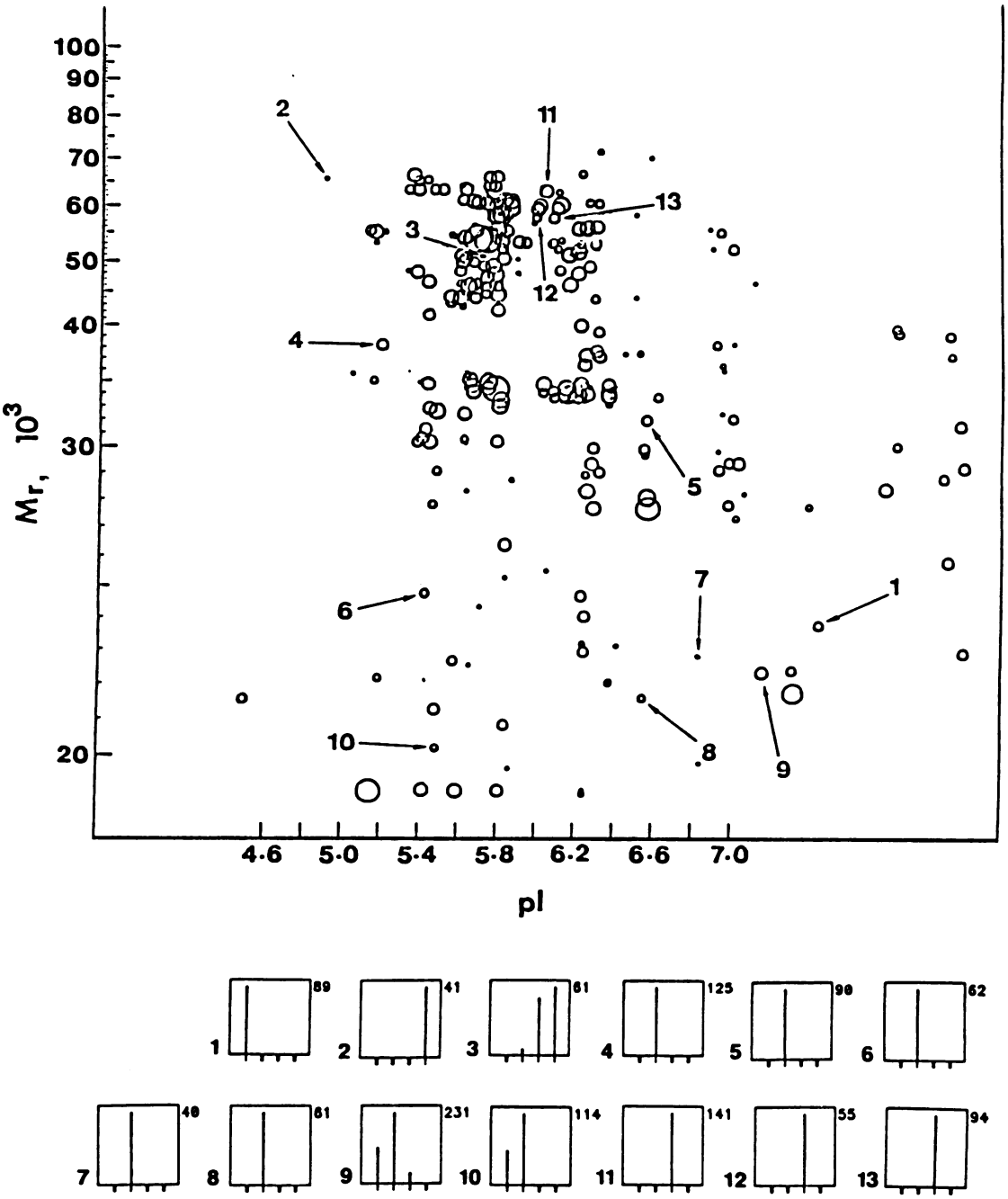

FIG. 8. Composite map of root hair proteins separated by two-dimensional gel electrophoresis. Axes are scaled for the approximate molecular masses and isoelectric points of the proteins. The 13 proteins showing the greatest change upon LPS treatment are indicated by arrows, and their relative quantities are presented below as histogram bars. The *x*-axis hash marks (left to right) correspond to protein samples from root hairs grown with −NF medium (control), K90 LPS from *R. trifolii* 0403, LPS from *R. meliloti* 102F28, and K50 LPS from *R. trifolii* 0403, respectively. The densitometry value of the highest histogram bar is at the top right corner of each graph.

resulting changes in glycosyl composition of *Rhizobium* LPS is difficult with the methods available to analyze this complex mixture produced by acid hydrolysis. An increase in methylation, acylation, and/or polymerization of the carbohydrate chains in the O antigen could decrease the rate of release of the components by acid hydrolysis. This would decrease the time of exposure of freed components to acid and thus decrease their acid-catalyzed decomposition before quantitation. The end result would be an increase in the amount of substituted sugar detected. Another possibility is that rhizobia make multiple types of LPS that differ in carbohydrate structure: one containing the immunodominant epitope and made constitutively throughout the growth cycle and one or more others made only in small quantities during imbalanced growth. Immunochemical evidence supports the latter possibility, since immunodominant LPS

TABLE 6. Relative molar proportions of glycosyl components in the lipid-free carbohydrate of K90 LPS from *R. trifolii* 0403 normalized to glucose

Glycosyl component[a]	Mean molar ratio ± SEM
2-O-Methylfucose	1.9 ± 0.1
Fucose	1.9 ± 0.1
3-Amino-3,6-dideoxy-3-*N*-methylgalactose	1.9 ± 0.1
Mannose	1.9 ± 0.1
Galactose	0.9 ± 0.1
Galacturonic acid	1.8 ± 0.2
Glucose	1.0 ± 0.1
Glucuronic acid	0.9 ± 0.2
Quinovosamine	0.9 ± 0.1
Heptose	1.1 ± 0.1
Glucosamine	0.9 ± 0.1

[a] 2-Keto-3-deoxyoctulosonic acid was detected but not quantitated.

epitopes are expressed constitutively whereas minor LPS epitopes which bind trifoliin A are transiently expressed between the lag and exponential phases and between the exponential and early stationary phases (36). Further evidence for the latter explanation has been found for a strain of *R. meliloti* having two distinct LPS chemotypes (68).

Two proposed functions for rhizobial LPS during infection of host roots are that it serves as a positive signal molecule which triggers necessary events for successful infection (39) and that it protects the invading bacterium by foiling or evading plant defensive mechanisms which would otherwise prevent infection (21). Our data showing differential responses of root hairs to LPSs from homologous or heterologous rhizobia suggest that both functions participate in formation and sustained growth of infection threads within the legume host root. We conclude that LPS-root hair interactions contribute to the complex signalling process which occurs during successful infection, leading to development of the nitrogen-fixing *Rhizobium*-legume symbiosis.

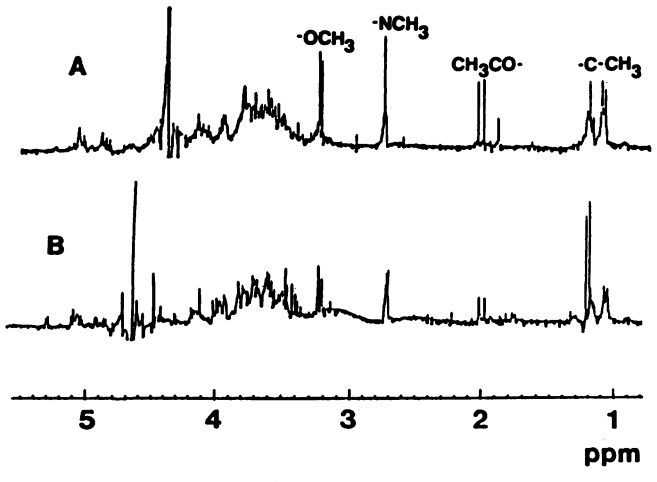

FIG. 9. Proton NMR spectra of the water-soluble glycoconjugate released by 1% acetic acid hydrolysis of LPS from *R. trifolii* 0403 in the early stationary (A) and mid-exponential (B) phases. The higher intensities of *O*-methyl, *N*-methyl, and acetyl groups are indicated in panel A. ppm, parts per million.

ACKNOWLEDGMENTS

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