CROSS REACTIVE ANTIGENS AND LECTIN AS DETERMINANTS OF HOST SPECIFICITY IN THE *RHIZOBIUM*-CLOVER SYMBIOSIS

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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To my wife, Olga

Exploration

We shall never cease from exploration,
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

Anonymous

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CROSS REACTIVE ANTIGENS AND LECTIN AS DETERMINANTS OF HOST SPECIFICITY IN THE *RHIZOBIUM*-CLOVER SYMBIOSIS

By

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The basis for host specificity in the *Rhizobium-Trifolium* (clover) symbiosis was investigated. Cross reactive antigens of clover roots and *R. trifolii* were detected on their cell surfaces by tube agglutination, immunofluorescent and radioimmunoassay techniques. Anti-clover root antiserum had a higher agglutinating titer with infective strains of *R. trifolii* than with noninfective strains. The root antiserum previously adsorbed with non-infective *R. trifolii* cells remained reactive only with infective cells, including infective revertants. When adsorbed with infective cells, the root antiserum did not react with either infective or noninfective cells. Other *Rhizobium* species incapable of infecting clover did not demonstrate surface antigens cross reactive with clover.

Radioimmunoassay indicated twice as much antigenic cross reactivity of clover roots and *R. trifolii* 403 (infective) as compared with *R. trifolii* Bart A (noninfective). Immunofluorescence using anti-*R. trifolii* (infective) antiserum was detected on the exposed surface of the root epidermal cells and diminished at the root meristem. The immunofluorescent cross reaction on clover roots was totally removed by adsorption of anti-*R. trifolii* (infective) antiserum with encapsulated infective cells but not with noninfective cells.

The cross reactive capsular antigens from *R. trifolii* strains were extracted and purified. The ability of these antigens to induce clover root hair deformation was much greater when they were obtained from infective as compared with noninfective strains. The cross reactive capsular antigen of *R. trifolii* 403 was characterized as a high molecular weight ($>4.6 \times 10^6$ daltons), amorphous, β-linked, acidic heteropolysaccharide containing 2-deoxyglucose, galactose, glucose, and glucuronic acid.

A soluble, nondialyzable substance (clover lectin) capable of binding to the cross reactive antigen and agglutinating only infective cells of *R. trifolii* was extracted from white clover seeds. This lectin was sensitive to heat, pronase, and trypsin. Inhibition studies indicated that 2-deoxyglucose was the most probable haptenic determinant of the cross reactive capsular antigen capable of binding to the root antiserum and the clover lectin. Infection

and nodulation of white clover roots by *R. trifolii* was inhibited by 2-deoxyglucose at concentrations of at least 30 mM. The adsorption of infective and noninfective *R. trifolii* cells to white clover root hairs was examined. After 12 h of incubation, four to five times more infective cells than noninfective cells were firmly adsorbed to root hairs. A model is proposed to explain host specificity based on the preferential adsorption of infective versus noninfective cells of *R. trifolii* on the surface of clover roots by a cross bridging of their common surface antigens with a multivalent clover lectin.

INTRODUCTION

The N_2 fixing *Rhizobium*-legume symbiosis is characterized by a high degree of host specificity controlled by the bacteria and the plant. Expression of host specificity is an early event which occurs prior to the formation of infection threads within the root hairs of the host (29). The purpose of the study reported here was to determine the basis for host specificity in the *Rhizobium trifolii*-*Trifolium* (clover) symbiosis. It was hypothesized that *R. trifolii* cells have specific surface antigens which are involved in the adhesion of the bacteria to the cross reactive surface antigens of the clover root hair cell wall with the aid of a clover lectin. This association was chosen for study because of the small-seeded nature of the macrosymbiont which was ideal for microscopic studies of infection and the availability of mutant strains of *R. trifolii* which have lost the ability to infect clover.

LITERATURE REVIEW

Cross Reactive Antigens

The importance of common or cross reactive antigens in the microbial invasion of a host is receiving increasing recognition. Antigenic similarities between vertebrate hosts and microbes have been implicated in the pathogenesis of rheumatic fever (55), human ulcerative colitis (41), rat glomerulonephritis (34), and rat arthritis (6). In these cases, pathogenesis may involve immunological tolerance of the cross reactive antigens by the host or deposition of cross reactive cytotoxic antibodies on the host tissue.

Common antigenic substances between invasive microbes and their plant hosts have also been found. These antigenically similar cell constituents possibly underlie host-pathogen compatibility based on their correlation with disease development. According to one theory (16, 17), a strong common antigen relationship between a plant host and a pathogen might result in the least disruption of cellular function between a pathogen and its host during infection with consequent success in disease development. Common antigen relationships have been implicated in the pathogenesis of flax rust by *Melampsora lini* (18), angular leaf spot in cotton by *Xanthomonas malvacearum* (17, 45), black rot of sweet potatoes by *Ceratocystis fimbriata* (17), common smut of corn by *Ustilago maydis* (52), crown gall tumor of

tobacco by *Agrobacterium tumefaciens* (8), and wilt of cotton by *Fusarium* and *Verticillium* (9). Host specificity is sharply defined for all of these phytopathogens except *A. tumefaciens* which has a wide host range. Cross reactive antigens have been found between 8 legumes and 3 species of *Rhizobium* (10). However, there was no correlation between the numbers of common antigens (immunoprecipitin bands in Ouchterlony plates) and the ability of the bacteria to infect their respective legume hosts.

Lectins

A lectin is a non-antibody protein or glycoprotein capable of specific interaction with carbohydrates. Lectins have been isolated from a variety of plants and non-vertebrate animals. Many lectins are phytohaemagglutinins since they can agglutinate erythrocytes by binding to their surface carbohydrate components.

Interactions between legume lectins and *Rhizobium* have been suggested (1, 48) as well as documented (4, 5, 7, 15, 22). Hamblin and Kent (22) showed that phytohaemagglutinin (*Phaseolus* lectin) could bind to *R.phaseoli* and that this lectin was present in the seeds, nodules, and on the roots below the nodules of *Phaseolus vulgaris*. Bohlool and Schmidt (4) demonstrated a high correlation between the binding capacity of soybean lectin to *Rhizobium* cells and the ability of these bacteria to nodulate soybean. They proposed that the legume lectin may serve as the basis for host specificity by interacting specifically with a polysaccharide on the surface of the *Rhizobium* cell. The

lectin binding site on the *Rhizobium* cell and the means whereby the lectin binds to the plant roots were not examined. However, some strains of *Rhizobium* do not bind to lectins obtained from the legume host that they nodulate (4, 5), and other strains of *Rhizobium* incapable of nodulating a certain legume still bind to the lectin from that host (5, 15). Thus, interactions between legume lectins and *Rhizobium* cells may not always account for the specificity expressed by the nodule bacteria for their respective legume hosts (15).

MATERIALS AND METHODS

Strains of *Rhizobium*

The sources, infectivity, and legume hosts of the *Rhizobium* strains are listed in Table 1. Infectivity is defined as the formation of root hair infection threads in small-seeded legumes using glass slide assemblies (20), or the production of root nodules on large-seeded legumes planted in cellophane pouches (51). The spontaneous infective revertants of *R. trifolii* (BA-L, BA-S, and 0435-2I) were isolated from nodules of *Trifolium repens* inoculated with the corresponding noninfective strains.

Preparation of Antigens

Bacterial cells were grown on a modified Bergersen's chemically defined medium, harvested, and sonicated as previously described (14). Root antigens of *T. repens* var. Louisiana Nolin and *T. fragiferum* var. Salina (hereafter called white and strawberry clover, respectively) were prepared. Seeds (50 g) were surface sterilized, spread on water agar plates, overlayed with sterile stainless steel wire mesh, inverted, and cold treated (38). The seeds germinated through the wire mesh into humid air at 22 C. After 3 days, seedling roots were excised along the wire mesh with razor blades and frozen in liquid N_2. Roots were macerated by grinding and thawing in 30 ml phosphate buffered saline (PBS, 0.05 M K_2HPO_4-KH_2PO_4, 0.15 M NaCl, 0.001 M $MgSO_4$,

Table 1. *Sources and Infectivity Characteristics of* Rhizobium *Strains*

Organism	Strain	Infectivity[a]	Host	Source
R. trifolii	2S	+	*Trifolium repens*	K. C. Marshall
"	2L	-	"	"
"	403	+	"	G. Fahraeus
"	Bart A	-	"	"
"	T37	+	"	Z. Lorkiewicz
"	Bio-9	-	"	"
"	0435	+	"	A. N. MacGregor
"	0435-2	-	"	"
"	0435-2I	+	"	"
"	WU290-I	+	"	R. Roughley
"	WU290-N	-	"	"
"	NA-30	+	"	W. F. Dudman
"	2S-2	+	"	F. B. Dazzo
"	BA-L	+	"	"

Table 1--*Continued*

Organism	Strain	Infectivity[a]	Host	Source
R. trifolii	BA-S	+	*Trifolium repens*	F. B. Dazzo
"	J1	+	"	J. R. Norton
"	J2	+	"	"
R. japonicum	311660	+	*Glycine max*	D. F. Weber
R. leguminosarum	3H0Q1	+	*Pisum sativum*	"
"	3H0Q51	-	"	"
R. meliloti	2001	+	*Medicago sativa*	P. S. Nutman
"	2009	-	"	"
R. phaseoli	403	+	*Phaseolus lathyroides*	M. Alexander
R. sp.	L1	-	"	"
"	229	+	"	"
"	HR1	+	*Aeschynomene americana*	H. Royals
"	CE22A1	+	*Canavalia ensiformis*	J. C. Burton
"	127E10	+	"	"

[a] *Rhizobium* strains were inoculated on their respective hosts and examined for infectivity as previously described (38).

pH 7.2) containing 0.05 M sodium ascorbate (9) and 30 g of insoluble polyvinylpyrrolidone (PVP, Bio-Rad Laboratories, Richmond, Calif.) which had been previously washed with PBS 8 times to remove 280 nm absorbing material (13). The slurry was stirred at 0 C for 20 min, then centrifuged at 27,000 x g for 30 min. The supernatant was decanted, concentrated 6-fold by dialysis at 4 C against 20% (w/v) polyethylene glycol (PEG) dissolved in PBS (14), dispensed in 2 ml aliquots, and stored at -70 C for future use. Particulate antigens were also prepared by mascerating seedling roots in PBS containing 20% soluble PVP (40,000 av. mol. wt., pharmaceutical grade, Sigma, St. Louis, Mo.) and then ultracentrifuging at 104,000 x g for 3 h. The pellet was washed twice with PBS, ultracentrifuged between washes, and then used as particulate antigens for immunization.

Preparation of Antiserum

Rhizobium and clover root antigens were emulsified with equal volumes of Freund's incomplete adjuvant and used to prepare antiserum in virgin New Zealand white rabbits according to the immunization schedule previously described (14). Sheep anti-rabbit γ globulin antiserum and fluorescein isothiocyanate (FITC) labeled γ globulin fraction of goat anti-rabbit γ globulin were purchased from Nutritional Biochemicals Co., Cleveland, Ohio, and Difco Laboratories, Detroit, Michigan, respectively.

Serological Techniques

Tube Agglutination

Tube agglutination of *Rhizobium* cells was performed

using saline (0.15 M) as the diluent (50). Eight strains of *R. trifolii* were examined, including 4 infective strains (2S-2, 403, T37, and 0435) and 4 noninfective mutants (2L, Bart A, Bio-9, and 0435-2).

Immunofluorescence

Both the direct and the indirect immunofluorescent tests were run according to standard procedures (21). Controls for detection of autofluorescence, nonspecific staining, and normal serum reactivity were included. Details of the optical system employed were described elsewhere (15).

Immunoprecipitation

A quantitative precipitin test using the purified capsular antigen of R. trifolii strain 403 and rabbit anti-white clover root antiserum was performed according to Nowotny (40). The protein contents of the washed immunoprecipitates were determined with the Folin phenol reagent (31). The supernatants were analyzed for excess antigen or antibody using the capillary precipitin test.

Radioimmunoassay

For the radioimmunoassay, 10 µg (protein) of the γ globulin fraction (21) of sheep anti-rabbit γ globulin were iodinated with 2 mCi of $Na^{125}I$ (Amersham Searle, Arlington Heights, Ill.) using the chloramine T method (25). The iodinated protein was separated from free iodine by gel filtration on a 1 x 10 cm Sephadex G-50 column (Pharmacia, Uppsala, Sweden). *R. trifolii* cells (strains 403 and Bart A) were suspended in PBS to a final density of 1.6×10^9 cells per ml as determined by direct counting

with a Petroff-Hausser chamber. Rabbit anti-white clover root antiserum (0.01 ml) was added to 0.05 ml of the cell suspensions run in duplicate, incubated at 37 C for 30 min, 0 C for 30 min, and then brought up to 1 ml volume with PBS. The suspensions were washed twice with PBS by centrifugation at 1,000 x g for 10 min each. This procedure of adding cross reactive antibody, incubation and washing was repeated twice. The washed cells adsorbed with the cross reactive antibody were incubated with the ^{125}I labeled goat anti-rabbit γ globulin added in 0.06 ml increments. The reaction mixtures were incubated for 30 min at 37 C, followed by 30 min at 0 C. Following washing of cells as before, the pellets were counted for γ emissions in a Model E116A-0 γ counter (Nuclear Equipment Co., Farmingdale, N. Y.) This procedure of adding the ^{125}I labeled anti-γ globulin was repeated until no further increase in antibody binding occurred, indicating antibody saturation. Tubes lacking anti-clover root antiserum were processed to quantitate non-specific binding of antiglobulin to cells, the inner wall of the tubes, and background γ radiation.

Adsorption of Antiserum

One ml of antiserum was incubated at 0 C with 0.05 ml packed cell volume for 15 min with constant mixing, and then centrifuged at 1,500 x g for 15 min. The supernatant was readsorbed twice with fresh cells. The adsorbed antiserum was unreactive with the adsorbing cells as determined by indirect immunofluorescence. Encapsulated cells of *R.*

trifolii strain 403 used for adsorption of cross reactive antiserum were separated from unencapsulated cells by differential centrifugation in PBS at 1,500 x g for 20 min. Clean, reliable separations were achieved under these conditions. Unencapsulated cells sedimented faster than encapsulated cells, forming a pellet under the diffuse layer of encapsulated cells. This upper layer was drawn off and centrifuged at 27,000 x g for 15 min to pellet the encapsulated cells.

Analytical Studies of *Rhizobium* Capsular Antigens

Purification

The capsular antigens of the *R. trifolii* strains were purified by procedures using cetylpyridinium chloride (40), and hexadecyltrimethyl ammonium bromide (11) as complexing agents. The material was lyophilized and stored in a desiccator for future use.

Precipitation and Immunofluorescence

The capsular antigen of *R. trifolii* 403 (hereafter called 403-AC) was tested for reactivity with 1% soluble diethylaminoethyl-dextran (DEAE-dextran) and 1% dextran sulfate (Pharmacia [19]), clover lectin (discussed later), and homologous and heterologous cross reactive antibody in capillary tubes. Some 403-AC was spread on microscope slides and tested for reactivity with rabbit anti-white clover root antiserum and anti-*R. trifolii* 403 antiserum using the indirect immunofluorescent technique.

Electrophoresis

The capsular materials (2 mg/ml) from *R. trifolii*

403, and Bart A (hereafter called BAC), were spotted on cellulose polyacetate strips (Sepraphore III, Gelman Co., Ann Arbor, Mich.) and electrophoresed for 1 h at 22 C under constant voltage (300 V) at pH 8.6 (0.05 M sodium barbital). The strips were dried, stained in 1% alcian blue in 0.01 N HCl for 10 min, and then washed in 0.01 N HCl overnight.

Gel Permeation Chromatography

A sample of 403-AC was hydrated from the lyophilized state and was passed through a 0.20 μm filter. An average molecular weight was determined by gel permeation (3) using a high pressure liquid chromatograph (Model 202/401 Waters Associates, Milford, Mass.) A sample of 403-AC was chromatographed in two stainless steel columns (3.2 x 600 mm) connected in series and packed with porous glass beads (AX through FX, Waters Assoc., and Bio-Glas 2500, Bio-Rad Laboratories) deactivated with 1% PEG, and was detected by a refractive index monitor. Degassed water was the solvent. The columns were calibrated by determining the elution volumes of soluble dextrans (5×10^3 to 2×10^6 daltons, av. mol. wt., Sigma) and purified plant viruses (Brome-grass mosaic, 4.6×10^6, and tobacco mosaic, 3×10^7 daltons, courtesy of Dr. E. Hiebert, Dept. Plant Pathology, Univ. Fla.)

Biochemical Component Analysis

Samples of 403-AC and BAC were acid hydrolyzed (11), converted to the volatile trimethylsilylated derivatives with Tri-Sil (Pierce Chem. Co., Rockford, Ill. [43]), separated and detected by gas-liquid chromatography employing

flame ionization detection as previously described (38). Sugar derivatives were identified by comparison with retention times of authentic standards (Nutritional Biochemicals Co.) The 4-0-methyl, D-glucuronic acid (24) was a gift from Dr. F. Loewus, Dept. Biology, State Univ. of New York (Buffalo, N. Y.); α-D glucuronic acid was a gift from Calbiochem, La Jolla, Calif. The neutral sugar and uronic acid content of 403-AC was quantitated colorimetrically by the anthrone and carbazole methods, repectively (28). Amino acid and amino sugar analyses of 403-AC hydrolysates were conducted on a JOEL Model JLC-6AH automated amino acid analyzer (JOEL, Inc., Cranford, N. J. [49]).

Diffraction and Spectral Analyses

X-ray diffraction and infrared spectral analyses of 403-AC and BAC were performed as previously described (38). A UV absorption spectral analysis of 403-AC dissolved in water (2 mg/ml) was performed using Suprasil cuvettes (Precision Cells Co., Hicksville, N. Y.) and a Beckman DBG grating spectrophotometer.

Antigenic Cross Reactivity of *R. trifolii* 403 After Enzymatic Digestion

Cells were fixed on microscope slides, then incubated 4 days at 22 C in the dark with the highest purity grade of pronase, trypsin, lysozyme, deoxyribonuclease, ribonuclease, pectinase, cellulase, and phospholipase dissolved in their appropriate buffers (Worthington Biochemical Corporation, Freehold, N. J. [53]). Cells were also treated with 7 M urea, 1% Triton X-100 (Sigma), 1% Tween-80 (Difco), 0.5 M

sodium periodate, aqueous HCl (pH 3, 4, 5, and 6), deionized water (pH 7), and aqueous NaOH (pH 8, 9, 10, 11, and 12). Cells plus the enzyme buffers were also examined. After incubation, cells were washed with deionized water and tested for antigenic cross reactivity using anti-white clover root antiserum by the indirect immunofluorescent technique.

Biological Assay

The effect of the capsular material (100 µg/ml, [23]) on strawberry clover root hair development was determined as previously described (46). Root hairs along the 4 optical median planes of two seedling roots per capsular preparation were examined by phase contrast microscopy. Hairs were counted, averaged, placed into straight, branched, moderately curled (<360°), or markedly curled (>360°) categories (54), and the data evaluated statistically.

Clover Lectin Studies

Extraction

White clover seeds (50 g) were ground to a fine powder and mixed with 150 ml PBS plus sodium ascorbate (0.05 M) with further grinding. Washed, insoluble PVP (20 g) was added, the slurry was stirred at 4 C for 1 h, filtered through PBS-washed cheese cloth, and centrifuged at 27,000 x g for 1 h. The upper lipid layer was removed, and the remaining supernatant was passed through 0.20 µm membrane filters (TCM-200, Gelman). This seed extract (hereafter called LAI) contained 10 mg/ml protein after dialysis against PBS to remove ascorbate. LAI was stored in 0.5 ml aliquots at -20 C for future use.

Binding to *Rhizobium* Cells

Cells washed in PBS were tested for evidence of binding with LAI by immunofluorescence, tube agglutination, and slide agglutination. Since several *Rhizobium* strains flocculate spontaneously due to cellulose microfibril production (38), uniform cell suspensions were obtained by filtering cells in PBS through glass wool loosely packed in Pasteur pipettes (5 x 10 mm). Cell suspensions were added to equal volumes of LAI, incubated at 30 C (tubes) or 22 C (slides) for 4 h, and then examined for agglutination.

In other studies, cells were mixed with LAI, incubated for 4 h, and washed twice with PBS in the centrifuge at 1,400 x g. Antigenic material in LAI capable of firmly binding to cells (not removed by washing) was detected by agglutination and indirect immunofluorescence using rabbit anti-LAI. This antiserum was prepared according to the immunization schedule for clover root antiserum and by itself was unreactive with *Rhizobium* cells using tube agglutination and immunofluorescence.

Characterization of Agglutination Factor

Dialysis. The agglutinating titer of LAI using *R. trifolii* strains 2S-2 and 403 was determined. An aliquot (3 ml) of undiluted LAI was dialyzed against PBS (3 changes in a total of 15 liters) and then titrated.

Ultracentrifugation. The agglutinating titer of LAI before and after ultracentrifugation at 104,000 x g for 1 h was determined.

Heat lability. Aliquots of LA1 were heated at 56 C or 80 C for 10 min, filtered, and titrated.

Enzymatic digestion. Aliquots of LA1 were diluted 1:8 (endpoint agglutination titer) by adding the various enzymes and other reactive materials in PBS as described above. These digestion mixtures were incubated for 24 h at 30 C, centrifuged at 3,000 x g for 10 min, and the supernatants were tested for agglutination of *R. trifolii* strain 403 cells. Strain 403 encapsulated cells were also incubated with the enzymes for 24 h at 30 C, washed twice by centrifugation at 3,000 x g for 10 min, and tested for agglutination by LA1 diluted 1:8 with PBS.

Sugar binding specificity. Inhibition of LA1-mediated cell agglutination by various carbohydrates (Nutritional Biochemicals Co.) was examined (Table 8). LA1 (0.2 ml) was diluted 1:8 with the various carbohydrates (final concentration 30 mM) dissolved in 0.15 M saline. After 1 h incubation at 30 C, *R. trifolii* cells (strains 2S-2 and 403) suspended in PBS were added. These suspensions were incubated for 2 h at 30 C, and then examined for agglutination. Filter sterilized soil extract (26) and strawberry clover root exudate (33) were also examined for inhibition of agglutination by LA1. Haemagglutination inhibition studies using LA1 and anti-white clover root antiserum preincubated with various sugars were performed on washed rabbit erythrocytes adsorbed with 403-AC according to Nowotny (40).

Inhibition of Infection of *T. repens* by 2-Deoxyglucose

Fahraeus slide assemblies (20) of *T. repens* seedlings

inoculated with 2.6 x 10^8 *R. trifolii* strain 403 cells were prepared with various increments of sterile 2-deoxyglucose (final concentrations of 0, 2.5, 5, 10, 30, 50, 100, and 200 mM) or α-D glucose (200 mM) in duplicate. Slides were incubated for 20 days in a plant growth chamber (Warren Sherer, Model CEL 255-6, Marshall, Mich.) programmed at 22 C isothermal, 12 h photoperiod, 18.6 lux light intensity, and then examined for evidence of root hair infection by phase contrast microscopy.

Adsorption of *R. trifolii* Cells to Root Hairs of *T. repens*

Slide assemblies of *T. repens* seedlings without agar were inoculated with approximately 1.9 x 10^8 infective or noninfective *R. trifolii* cells. After 12 h incubation in the growth chamber (10 h darkness followed by 2 h light), the slides were removed. The cover slips were removed, then the roots were washed on the slides by a gentle stream of saline. The slide assemblies were filled with saline, new cover slips were added, and then the roots were examined by phase contrast microscopy. Four optical median planes of two primary roots per strain were evaluated. The number of bacterial cells per strain adsorbed to root hairs approximately 200 μm in length was determined and the data were evaluated statistically.

Electron microscopic examination of *R. trifolii* associated with *T. fragiferum* root hairs was performed as previously described (38).

RESULTS AND DISCUSSION

Antigenic Cross Reactivity between *R. trifolii* and Clover Roots

Tube Agglutination

Antiserum to white clover roots cross agglutinated both infective and noninfective *R. trifolii* strains (Table 2). Endpoint cross agglutination titers were higher for the infective strains in 3 out of 4 infective-noninfective strain combinations. In the fourth combination, both the infective and noninfective strains had equal agglutination titers. Cells were not agglutinated in normal serum controls. These results indicated that cross reactive antigens between clover roots and the surface of *R. trifolii* strains exist, and that the surface antigenic composition of the infective strains may be different from the noninfective strains.

Immunofluorescence using Anti-Clover Root Antiserum

Unadsorbed anti-white clover root antiserum was reactive with the surfaces of infective and noninfective *R. trifolii* cells when examined by indirect immunfluorescence (Table 3, Fig. 1). The anti-white clover root antiserum was adsorbed with cells of each combination of infective and noninfective strains of *R. trifolii*. When adsorbed with whole cells of the noninfective strains, this antiserum remained reactive *only* with infective cells, including the three spontaneous infective revertants 0435-2I, BA-L and BA-S (Table 3). These three revertants also reacted strongly with antiserum against

Table 2. *Agglutination of Rhizobium trifolii with Rabbit Anti-Trifolium repens Root Antiserum*

Strain	Endpoint titer
2S-2[a]	128
2L[b]	128
403[a]	128
Bart A[b]	8
T37[a]	64
Bio-9[b]	4
0435[a]	64
0435-2[b]	8

[a]Infective on *T. repens*.

[b]Noninfective on *T. repens*.

Table 3. *Reactivity of Anti-Trifolium repens Root Antiserum and Rhizobium trifolii Cells Using Indirect Immunofluorescence*

Strain	Antiserum		
	Unadsorbed	Adsorbed with noninfective cells	Adsorbed with infective cells
2S-2[a]	+	+	-
2L[b]	+	-	-
403[a]	+	+	-
Bart A[b]	+	-	-
0435[a]	+	+	-
0435-2[b]	+	-	-
T37[a]	+	+	-
Bio-9[b]	+	-	-
WU290-I[a]	+	+	-
WU290-N[b]	+	-	-
0435-2I[a]	+	+	-
BA-L[a]	+	+	-
BA-S[a]	+	+	-
J1[a]	+	ND[c]	ND
J2[a]	+	ND	ND
NA-30[a,d]	+	ND	ND
NA-30[a,e]	+	ND	ND

[a]Infective on *T. repens*.

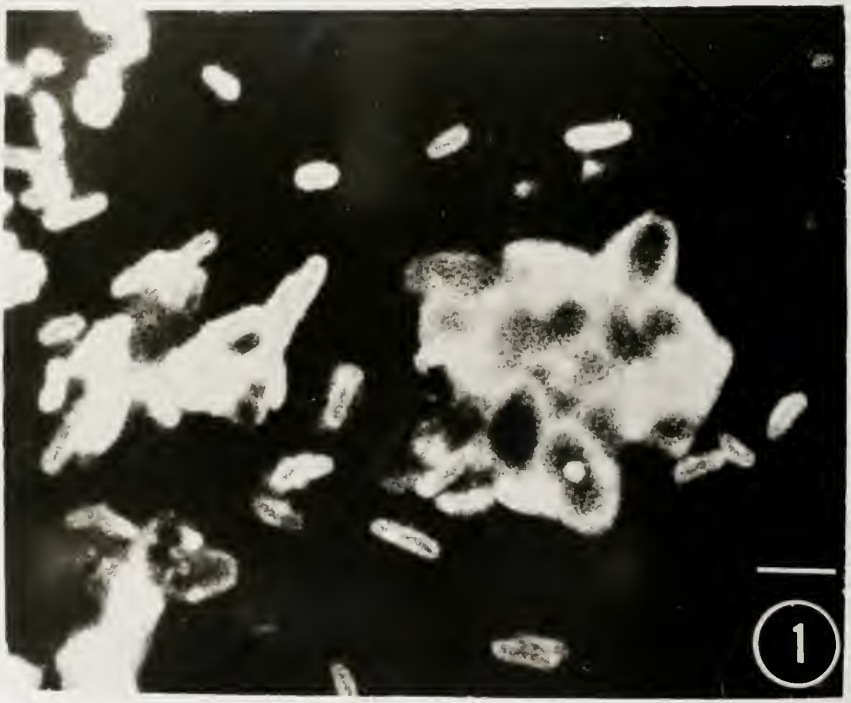
[b]Noninfective on *T. repens*.

[c]ND, not determined.

[d]Grown on a chemically defined medium.

[e]Grown in soil extract.

Fig. 1. Immunofluorescence of *Rhizobium trifolii* 403 (infective) using anti-*Trifolium repens* root antiserum. Bar scale equals 2 µm.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the parent infective strains. When adsorbed with cells of the infective strains, the root antiserum was reactive with *none* of the *R. trifolii* strains examined. These results indicated that *R. trifolii* cells have antigens on their surfaces which are cross reactive with clover roots, and there is a distinctly greater degree of antigenic cross reactivity displayed by the infective strains. A portion of this "homology" is lost when cells lose the ability to infect the root hairs of their host, but it is reacquired when the cells spontaneously revert back to their infective state. *R. trifolii* strains J1 and J2, which were recently isolated from root nodules of natural stands of white clover, were also reactive with the anti-white clover root antiserum. This indicated that the antigenic cross reactivity of *R. trifolii* and clover is not unique only to laboratory strains maintained for years as stock cultures. While growing in soil extract, *R. trifolii* NA-30 (infective) was capable of maintaining a cell surface which was antigenically cross reactive with white clover roots. Controls for autofluorescence of cells and non-specific staining of FITC-labeled anti-rabbit γ globulin were negative (no fluorescence). Preimmune sera were unreactive.

Anti-white clover root antiserum was tested for reactivity with a variety of other *Rhizobium* species, including *R. japonicum*, *R. leguminosarum*, *R. phaseoli*, *R. meliloti*, and members of the so-called "cowpea miscellany,"none of which infected clover (Table 1). None of these *Rhizobium* species were reactive with antiserum to *T. repens* (immunofluorescence) except *R.* sp. strain

HR1. In this latter case, the preimmune normal serum control was equally reactive, and therefore the rabbit had natural antibody reactive with surface antigens of this organism. These results indicate that strains of *Rhizobium* incapable of infecting clover lack specific surface components which are antigenically cross reactive with clover roots.

Antisera were prepared against 27,000 x g supernatant and 104,000 x g pellet fractions of macerated root antigens of strawberry clover. Antisera to both fractions reacted with infective strains of *R. trifolii* (2S-2, 403, T37, and 0435). This indicated that *R. trifolii* was antigenically cross reactive with at least 2 host species in the clover cross-inoculation group. It seems feasible to predict similar results with other clovers that can establish successful symbiotic relationships with *R. trifolii*. Cross reactive strawberry clover root antigen(s) remained in the supernatant at 27,000 x g for 1 h, and were sedimented at 104,000 x g for 3 h in sufficient quantity to elicit an immune response. A higher degree of antigenic similarity exists among legumes in the clover cross-inoculation groups (1).

Radioimmunoassay

Radioimmunoassay was performed to detect and quantitate the degree of antigenic cross reactivity between *R. trifolii* strains and the roots of their clover host. The amount of cross reactive antibody bound to equal numbers of strain 403 (infective) and Bart A (noninfective) cells after repeated additions is shown in Fig. 2. At saturation, approximately twice as much cross reactive antibody bound to the infective

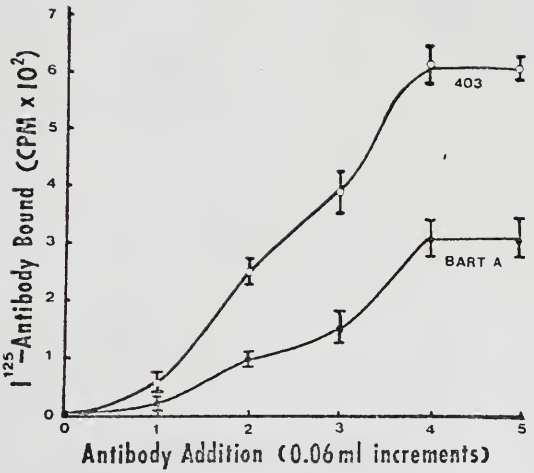


Fig. 2. Radioimmunoassay of anti-*Trifolium repens* root antiserum bound to *Rhizobium trifolii* 403 (infective) and Bart A (noninfective) cells. Cells of both strains were adjusted to equal population sizes following direct microscopic counting using a Petroff-Hausser chamber. Cells were incubated with rabbit anti-*T. repens* root antiserum, washed, and then incubated with ^{125}I-labeled goat anti-rabbit γ globulin. After each addition, cells were incubated, washed, and counted for γ emissions. Points and bars represent means and standard deviation.

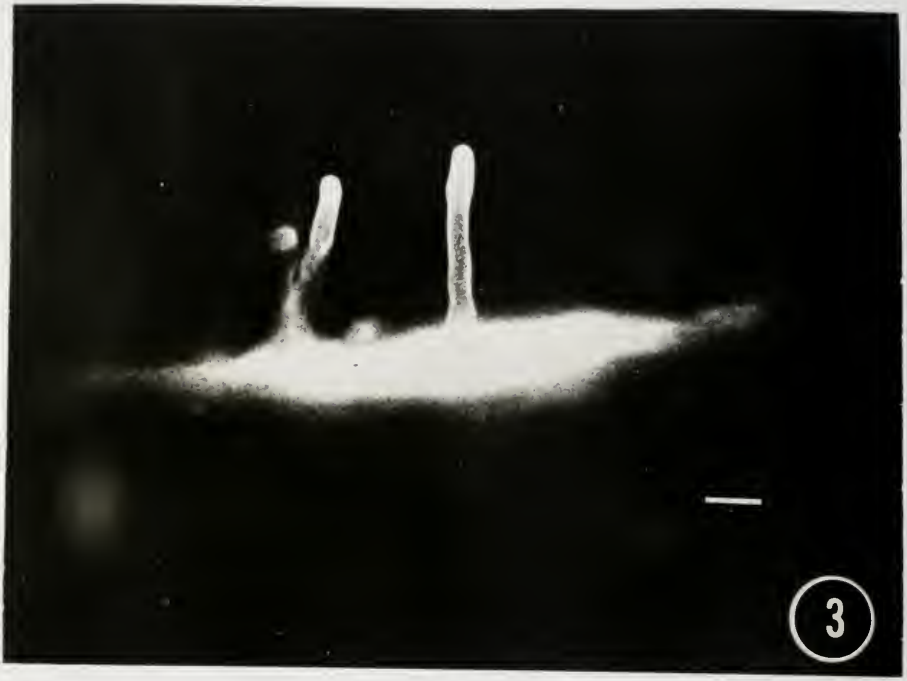
as compared with the noninfective cells. These data confirmed the earlier observations (tube agglutination and immunofluorescence) that quantitative differences in the degree of antigenic cross reactivity exist between clover roots and infective strains of *R. trifolii* as compared with noninfective strains.

Immunofluorescence using Anti-*R. trifolii* Antiserum

Axenically grown white and strawberry clover seedling roots were reacted with antisera prepared against several infective *R. trifolii* strains (2S-2, 403, T37, and 0435), and then examined for the presence of bound cross reactive antibody using the indirect immunofluorescent technique. Antibody to each of the infective strains bound to the surface of sterile clover roots. Immunofluorescence by anti-0435 antibody appeared brightest at the growing root hair tip of white clover as compared with other external root parts. In all cases, immunofluorescence was present on the exposed surface of the root epidermal cells including root hairs (Fig. 3) and diminished at the root meristem. These results indicated that clover has antigens on its exposed root surface which are cross reactive with antigens from infective *R. trifolii* cells.

The blue autofluorescence of fresh root tissue (27) was removed by a K490 barrier filter. Jones and Russell (27) used immunofluorescence to identify *R. trifolii* strains in *T. repens* nodules. Interestingly, their photographs provided clear evidence of antibody to *R. trifolii* binding to surface antigens of clover roots without the authors actually reporting the observation.

Fig. 3. Immunofluorescence of a *Trifolium repens* root using anti-*Rhizobium trifolii* 2S-2 antiserum. Bar scale equals 45 µm.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Antisera to the infective strains of *R. trifolii* previously adsorbed with whole cells of the corresponding noninfective strains bound to the antigens of the white clover root surface and the homologous infective *R. trifolii* strains. Antibody to *R. trifolii* 403 (infective) which was cross reactive with the surface of clover roots could be removed by adsorption with encapsulated 403 cells. These results indicated important differences in the degree of antigenic cross reactivity between infective vs. noninfective *R. trifolii* strains and the roots of a clover host.

Analytical Studies of *Rhizobium* Capsular Antigens

Since the cross reactive antigen of *R. trifolii* 403 was localized on a morphologically distinct capsule as viewed by immunofluorescence, the capsular material was isolated and characterized.

Precipitation and Immunofluorescence

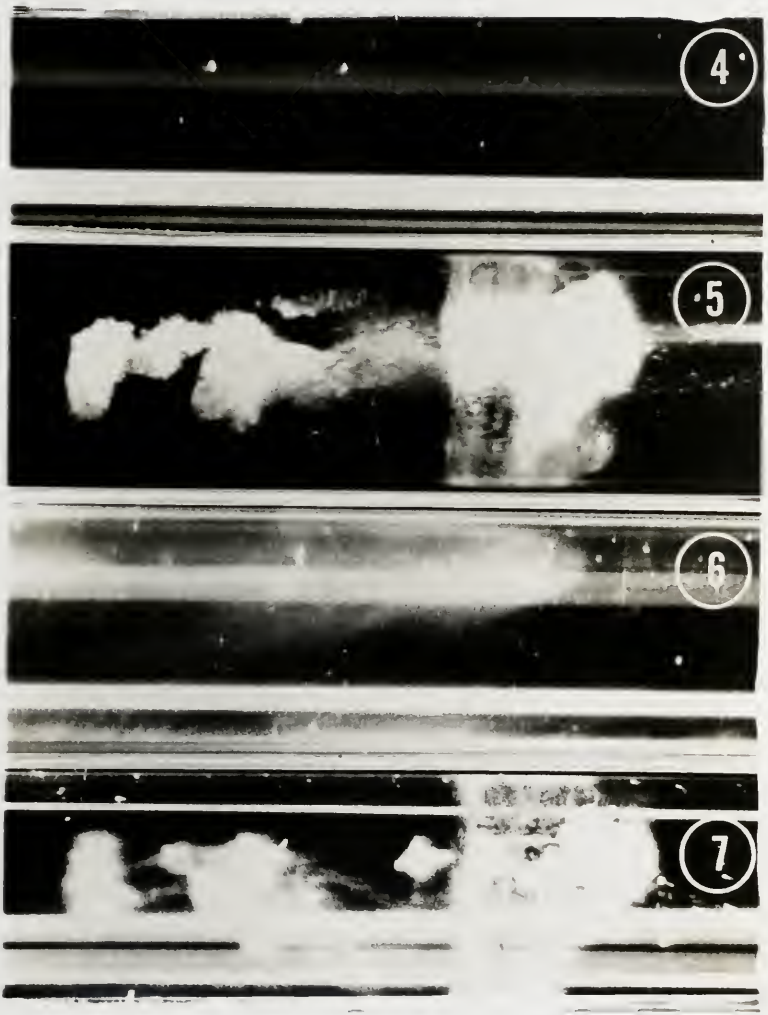
When dissolved in water, 403-AC was optically clear (Fig. 4). The 403-AC and the capsular material from Bart A (BAC) complexed with soluble DEAE-dextran (Fig. 5), cetylpyridinium chloride, hexadecyltrimethyl ammonium bromide, and alcian blue. These compounds form specific precipitates with acidic polysaccharides (11, 19, 35, 40). 403-AC and BAC did not react with dextran sulfate which precipitates basic polysaccharides (19). The 403-AC also formed a precipitate when reacted with anti-white clover root antiserum (Fig. 6) and a clover seed extract (Fig. 7, discussed later). The quantitative precipitin curve (Fig. 8) indicated that 403-AC was cross reactive with white clover roots. A maximum of

Fig. 4. *Rhizobium trifolii* 403 capsular antigen dissolved in water (2 mg/ml).

Fig. 5. Reaction of *Rhizobium trifolii* 403 capsular antigen with DEAE-dextran.

Fig. 6. Reaction of *Rhizobium trifolii* 403 capsular antigen with anti-*Trifolium repens* root antiserum.

Fig. 7. Reaction of *Rhizobium trifolii* 403 capsular antigen with *Trifolium repens* seed extract.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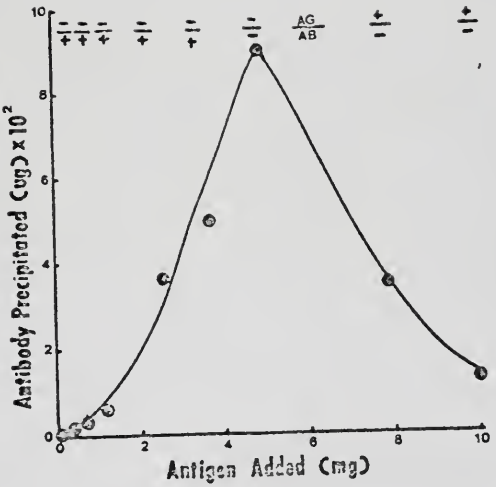

Fig. 8. Quantitative precipitin curve of *Rhizobium trifolii* 403 capsular antigen with anti-*Trifolium repens* root antiserum. Symbols at the top represent excess antigen or antibody in the supernatants after removal of the immunoprecipitates by centrifugation.

930 µg cross reactive anti-white clover root antibody protein was present per ml of undiluted antiserum. Antibody against white clover roots and *R. trifolii* 403 reacted with 403-AC using immunofluorescence.

Electrophoresis

At pH 8.6, 403-AC and BAC were electrophoretically homogeneous. They both had a net negative charge, and migrated as single bands of equal electrophoretic mobilities (2.08×10^{-5} cm^2/V sec).

Gel Permeation Chromatography

The capsular antigen 403-AC eluted as a sharp peak just after the void volume of the glass bead columns of AX through FX (Waters Assoc.) and Bio-Glas 2500 (Bio-Rad, Fig. 9). No additional peaks of lower molecular weight in 403-AC were eluted within the selective permeation range of the beads. When compared with the elution volumes of various markers, these results indicated that 403-AC had an average molecular weight in excess of 4.6×10^6 daltons. This material was not contaminated with detectable amounts of any smaller compounds that would have changed the refractive index of water.

Biochemical Component Analysis

The capsular antigens 403-AC and BAC consisted primarily of carbohydrate. Total neutral sugar (anthrone) and uronic acid (carbazole) contents of 403-AC were 68.0 $\pm$ 0.5% and 32.0 $\pm$ 0.5%, respectively. Gas-liquid chromatography of the trimethylsilylated (TMS) derivatives of the sugars released by acid hydrolysis of 403-AC indicated 2-deoxyglucose,

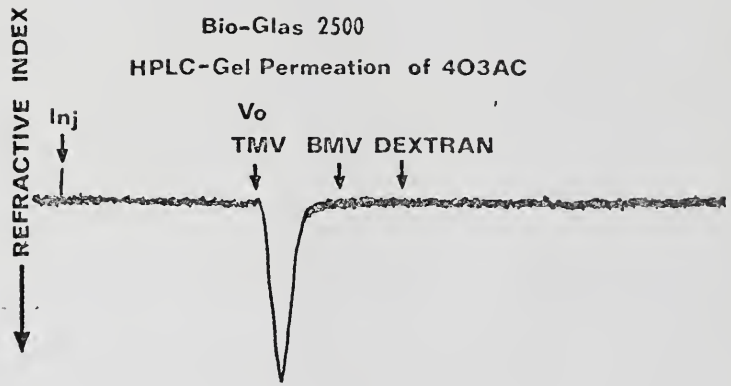

Fig. 9. High pressure liquid chromatography of the *Rhizobium trifolii* 403 capsular antigen on Bio-Glas 2500. Tobacco mosaic virus (TMV), Bromegrass mosaic virus (BMV), and dextran markers are 3×10^7, 4.6×10^6, and 2×10^6 daltons, respectively.

α-D galactose, α and β-D glucose, α-D glucuronic acid, and one unidentified compound (Fig. 10). The unidentified compound (peak 2) did not correspond to the trimethylsilylated derivative of L-sorbose, L-fucose, 2-deoxyribose, 2-deoxy-galactose, 3-O-methyl D-glucose, D-ribose, 6-deoxy-l-galactose, D-mannose, D-fructose, L-arabinose, D-xylose, 4-O-methyl D-glucuronic acid, D-melibiose, or L-rhamnose. Hexa-methyldisiloxane was also present in the mixture as a normal by-product of the silylation reaction (43). BAC gave an identical chromatogram (not shown). The absence of deoxyribose and ribose indicated a lack of nucleic acid contamination. The analytical system employed could detect 1 nanogram of α-D glucose TMS as a lower limit of sensitivity.

Trace amounts (1-10 nanomoles per mg dry wt) of lysine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, and isoleucine were identified in acid hydrolysates of 403-AC by comparison with $\frac{440nm}{570nm}$ ratios and identical retention times of authentic standards. The total amino acid content could account for approximately 0.26% of the dry weight of 403-AC and therefore was not considered a major structural component of the capsule. Neither glucosamine nor galactosamine was detected within the sensitivity of the amino acid analyzer employed (10 nanomoles).

Diffraction and Spectral Analyses

X-ray diffractograms of 403-AC and BAC revealed no reinforcement peaks within the range 2° 2θ to 60° 2θ. This

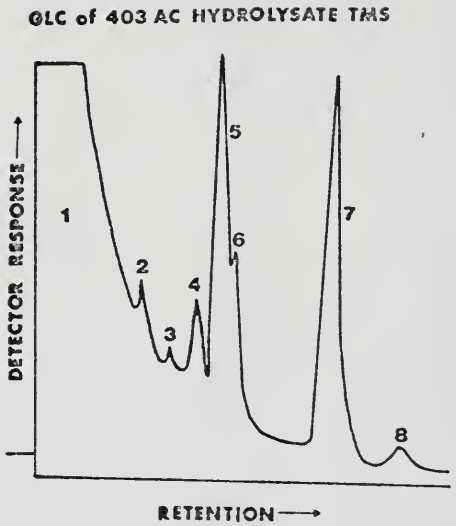

Fig. 10. Gas-liquid chromatography of trimethylsilylated sugars in an acid hydrolysate of *Rhizobium trifolii* 403 capsular antigen. Peak 1= pyridine solvent, peak 2= unidentified, peak 3= 2-deoxyglucose, peak 4= α-D galactose, peak 5= α-D glucose, peak 6= hexadisiloxane, peak 7= β-D glucose, peak 8= α-D glucuronic acid.

indicated an amorphous, non-crystalline structure, and the lack of detectable contamination with cellulose microfibrils which were produced by both strains (38). The infrared spectra of both materials were essentially identical (Fig. 11). The spectra were consistent with a carbohydrate structure rich in hydroxyl and carboxyl groups. The absorption band at 890-900 cm^{-1} was characteristic of a β glucosidic linkage and absence of a band at 870 cm^{-1} indicated absence of an α glucosidic linkage (2). The UV spectrum of 403-AC indicated absorption in the short UV range only (210-230 nm). Lack of absorption down to 230 nm indicated no detectable nucleic acid and/or protein which would absorb in this range.

It is concluded from these analytical studies that 403-AC is a water soluble, amorphous, high molecular weight, β-linked, acidic heteropolysaccharide.

Antigenic Cross Reactivity of *R. trifolii* Strain 403 After Enzymatic Digestion

Encapsulated *R. trifolii* 403 cells were treated with various enzymes and other reactive materials and then tested for cross reactivity with anti-white clover root antiserum using immunofluorescence. The antigenic cross reactivity of strain 403 capsules was eliminated by lysozyme, periodate, acid (HCl, pH 3) and alkaline (NaOH, pH 12) treatments. Lysozyme treatment lysed the cells. Apparently the capsular antigen was washed away from the cell walls solubilized by lysozyme. The capsules of the cells were removed by washing after periodate treatment. However, the underlying cell

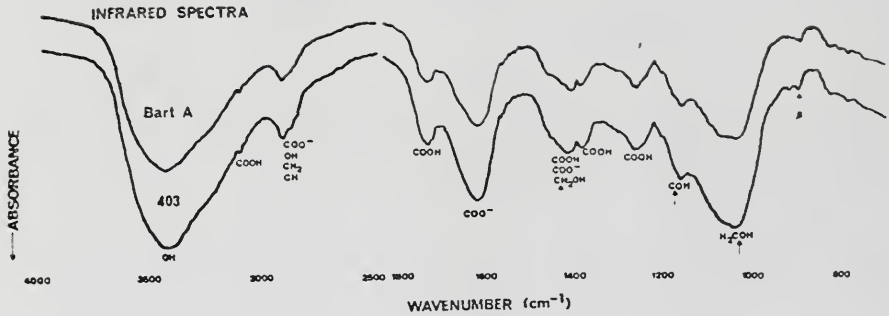

Fig. 11. Infrared spectra of *Rhizobium trifolii* 403 and Bart A capsular antigens.

surface remained antigenically cross reactive with clover roots. This indicated that these cells maintained an antigenically cross reactive surface even if they lost their capsules. Maintaining an underlying cross reactive layer could possibly be important for the cell in the rhizosphere if the C/N ratio of root exudates restricted abundant capsule formation.

Effect of pH on Antigenic Cross Reactivity

The antigenic cross reactivity of *R. trifolii* 403 cells following acid and alkaline treatment was examined in more detail. The degree of antigenic cross reactivity remained high in the pH range 6-8, but diminished outside of this range (Fig. 12). The acid sensitivity of the cross reactive surface antigen (at pH 5 and below) corresponded to the acid-sensitive step where root hair curling, infection, and nodulation abort (36). If possession of the cross reactive antigen is essential to the infection process as proposed here, then its loss at high H^+ concentration may provide a biochemical explanation of nodulation failure in acid soils despite proper inoculation.

Biological Activity of *R. trifolii* Capsular Antigens

Rhizobium extracellular nondialyzable material has been known to induce deformation of legume root hairs (23, 30, 33, 54). A markedly curled deformation of root hairs has been reported to be restricted to inoculation of a legume with live, virulent, homologous *Rhizobium* cells (54). Other have shown that root hair deforming substances produced by

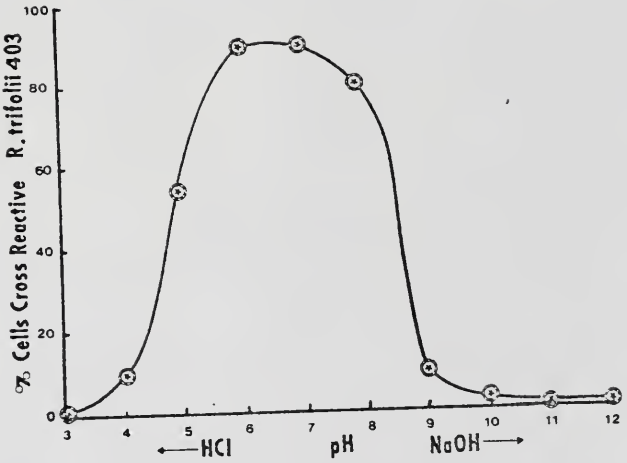

Fig. 12. The effect of pH on the immunofluorescent cross reactivity of encapsulated cells of *Rhizobium trifolii* 403 with anti-*Trifolium repens* root antiserum. The K470 barrier filter was used to differentiate reactive and unreactive cells.

R. trifolii could be adsorbed to white clover roots and eluted again by acetic acid or urea (46).

The antigenically cross reactive material, which was isolated by the method of Nowotny (40) from infective and noninfective cells grown on a chemically defined medium, was tested for its ability to deform root hairs of strawberry clover grown axenically. The results are reported in Table 4. A χ^2 test indicated a highly significant dependence (at 99.9% level) of root hair deformation on the addition of this capsular material. Another χ^2 test indicated (at 99% level) that the capsular material from all the strains examined did not induce root hair deformation to the same degree. Several χ^2 tests were performed to determine if the degree of deformation was independent of whether or not the curling factor came from the infective or the corresponding noninfective strain. These tests were all rejected at the 99% level, indicating that the degree of root hair deformation induced by the curling factor was significantly dependent on the strain from which it was isolated. One-tailed Z tests were performed to test if the frequency of observations within a deformation category (straight, branched, moderately or markedly curled) was greater for one strain than for another (e.g., infective vs. noninfective). The frequency of straight root hairs was significantly less (at 99% level) when incubated with the capsular material of the infective strains as compared with the corresponding noninfective strains. In all cases except the branching

Table 4. *Deformation of Trifolium fragiferum Root Hairs Induced by Capsular Material from Rhizobium trifolii Strains*

Strain	Deformation categories[a,b]				Total root hairs examined
	Straight	Branched	Moderately curled	Markedly curled	
403[c]	9.2	13.7	39.9	37.2	336
Bart A[d]	96.1[e]	0.6[e]	3.3[e]	0.0[e]	362
0435[c]	9.8	13.4	51.8	25.0	224
0435-2[d]	90.7[e]	3.3[e]	6.0[e]	0.0[e]	246
T37[c]	53.4	20.9	22.3	3.4	296
Bio-9[d]	89.2[e]	3.6[e]	7.2[e]	0.0[f]	195
2S-2[c]	18.1	2.5	42.9	36.5	408
2L[d]	93.3[e]	2.8	3.9[e]	0.0[e]	281
Control	98.8	0.8	0.4	0.0	513

[a]Final concentration of capsular material was 100 µg/ml.

[b]Percentage of total root hairs examined.

[c]Infective on *T. fragiferum*.

[d]Noninfective on *T. fragiferum*.

[e]Statistically significant difference from corresponding infective strain (at 99% level).

[f]Significantly less than corresponding infective strain (at 95% level).

frequency of 2S-2 and 2L, the degree of root hair deformation was significantly greater for the *infective* strains. Thus, the antigenically cross reactive capsular material of these R. *trifolii* strains possessed biological activity characterized by the ability to deform root hairs of the clover host. Photomicrographs of control root hairs and root hairs deformed by 403-AC and BAC are presented in Figs.13, 14, and 15, respectively.

Clover Lectin Studies

A clover lectin capable of differentiating infective and noninfective R. *trifolii* strains was sought. A heat sensitive, nondialyzable, soluble protein or glycoprotein agglutinating factor with definable sugar specificity has been found in LAI. The agglutinating factor reacted with the cross reactive surface antigens which these symbionts share.

Binding to *Rhizobium* Cells

The spectrum of cell agglutination by LAI (Table 5) included *all* R. *trifolii* strains capable of infecting white clover (including the infective revertants) but *none* of the noninfective R. *trifolii* strains or other *Rhizobium* species incapable of infecting this legume. R. *leguminosarum* 3HOQ1 and R. sp. HR1 could not be examined due to autoagglutination. Control and LAI-agglutinated cell suspensions of R. *trifolii* 2S-2 are illustrated in Figs.16 and 17, respectively. The ability to agglutinate indicated that the proposed clover lectin was at least divalent, i.e., had at least two reactive binding sites.

Fig. 13. *Trifolium fragiferum* root hairs. Bar scale equals 45 µm.

Fig. 14. *Trifolium fragiferum* root hair deformation in the presence of the capsular material from *Rhizobium trifolii* 403 (100 µg/ml). Bar scale equals 45 µm.

Fig. 15. *Trifolium fragiferum* root hair deformation in the presence of the capsular material from *Rhizobium trifolii* Bart A (100 µg/ml). Bar scale equals 45 µm.


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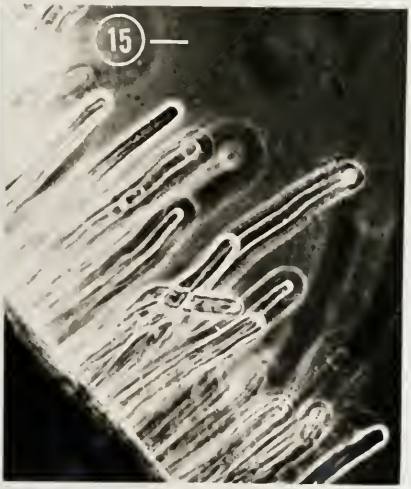
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Table 5. *Agglutination of Rhizobium Cells by Trifolium repens Seed Extract*

Species	Strain	Agglutination	Species	Strain	Agglutination
R. trifolii	2S-2[a]	+	*R. japonicum*	311660[b]	-
"	2L[b]	-	*R. leguminosarum*	3H0Q1[b]	AAG[c]
"	403[a]	+	"	3H0Q51[b]	-
"	Bart A[b]	-	*R. meliloti*	2001[b]	-
"	0435[a]	+	"	2009[b]	-
"	0435-2[b]	-	*R. phaseoli*	403[b]	-
"	T37[a]	+	*R. sp.*	229[b]	-
"	Bio-9[b]	-	"	HR1[b]	AAG[c]
"	J1[a]	+	"	CE22A1[b]	-
"	J2[a]	+	"	127E10[b]	-
"	BA-L[a]	+	"	L1[b]	-
"	BA-S[a]	+			
"	0435-2I[a]	+			

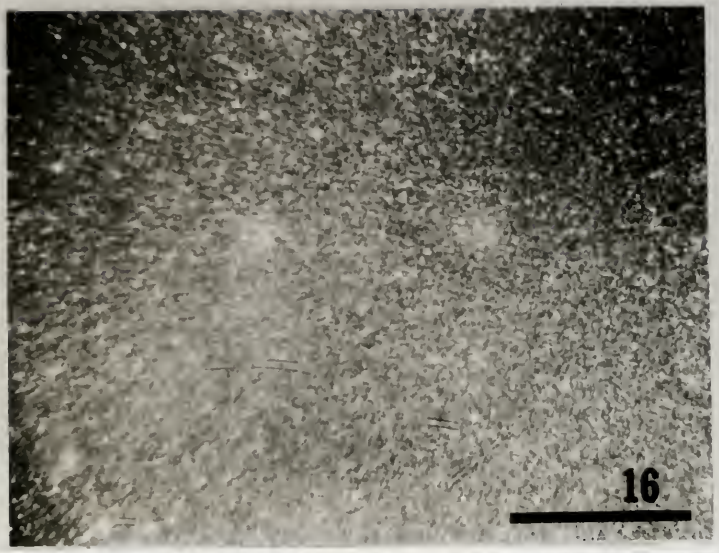
[a]Infective on *T. repens*.

[b]Noninfective on *T. repens*.

[c]AAG, autoagglutinated.

Fig. 16. *Rhizobium trifolii* 2S-2 cells suspended in saline. Bar scale equals 0.5 mm.

Fig. 17. Agglutination of *Rhizobium trifolii* 2S-2 cells by the *Trifolium repens* seed extract. Bar scale equals 0.5 mm.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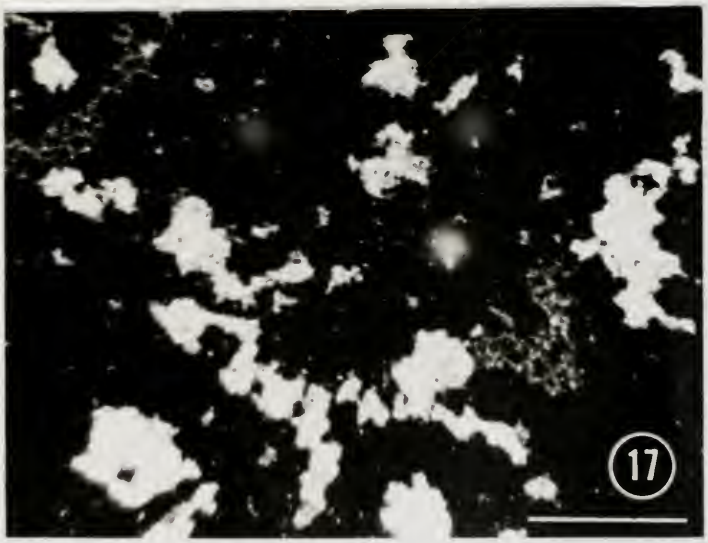
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Whether the agglutinating factor could bind to cells at levels below the sensitivity of the agglutination test was then tested. Cells were incubated with LAI, washed, and then reacted with anti-LAI using tube agglutination and indirect immunofluorescence. Antigenic material in LAI capable of firmly binding to the cells was detected on both infective and noninfective *R. trifolii* strains but not on other *Rhizobium* species incapable of infecting white clover (Table 6). Indirect immunofluorescence showed that the proposed clover lectin bound to the distinct capsules of *R. trifolii* strain 403.

Characterization of the Agglutination Factor

The endpoint agglutinating titer of LAI did not change following extensive dialysis (3,500 molecular weight cut-off) and ultracentrifugation at 104,000 x g for 1 h. Treatment of LAI at 56 C and 80 C for 10 min completely destroyed its ability to agglutinate infective strains of *R. trifolii*. These results indicated that the agglutinating factor was nondialyzable, nonparticulate (soluble), and heat labile.

The effect of various enzymes and other reactive materials on the agglutination of *R. trifolii* 403 cells by LAI was examined. Pronase, trypsin, periodate, acid (HCl, pH 3), alkaline (NaOH, pH 12), and 7 M urea treatments of LAI destroyed its ability to agglutinate the cells (Table 7). This indicated that the agglutination factor (lectin) in LAI was an acid and alkali sensitive protein (or

Table 6. *Binding of Clover Lectin to* Rhizobium *Cells*

Species	Strain	Anti-LA1[a] agglutination	Anti-LA1 immunofluorescence
R. trifolii	2S-2[b]	+	+
"	2L[c]	+	+
"	403[b]	+	+
"	Bart A[c]	+	+
"	T37[b]	+	+
"	Bio-9[c]	+	+
"	0435[b]	+	+
"	0435-2[c]	+	+
R. japonicum	311660[c]	-	-
R. leguminosarum	3H0Q1[c]	-	-
R. meliloti	2001[c]	-	-
"	2009[c]	-	-
R. sp.	229[c]	-	-
"	CE22A1[c]	-	-
"	127E10[c]	-	-
"	L1[c]	-	-
R. phaseoli	403[c]	-	-

[a]LA1, *T. repens* seed extract.

[b]Infective on *T. repens*.

[c]Noninfective on *T. repens*.

Table 7. *Effect of Various Treatments on Agglutination of Rhizobium trifolii 403 by Clover Seed Extract*

Treatment	LAl[a] treated	Cells treated
Pronase	-	+
Trypsin	-	+
Deoxyribonuclease	+	+
Ribonuclease	+	+
Phospholipase	+	+
Cellulase	+	+
Pectinase	+	+
Periodate	-	-
HCl (pH 3)	-	-
NaOH (pH 12)	-	-
7 M Urea	-	+
1% Triton X-100	+	+
Soil extract	+	+
Clover root exudate[b]	+	+
Untreated[c]	+	+

[a] LAl, *T. repens* seed extract.

[b] *T. fragiferum*.

[c] Enzyme buffers alone.

possibly glycoprotein) which may require the participation of H-bonding for agglutinability. These experiments did not distinguish between the possible need of H-bonding for intramolecular stability of the lectin and for intermolecular binding of the lectin to the surface of the *Rhizobium* cells. Periodate, acid, and alkaline treatments rendered the 403 cells non-agglutinable by LAI as with the anti-white clover root antiserum as described earlier. Clover root exudate and soil extract did not prevent the agglutination of *R. trifolii* 403 cells by the clover lectin. *R. trifolii* NA-30 was also agglutinated by clover lectin after growth for one generation in soil extract.

Sugar Binding Specificity

Inhibition of LAI-mediated agglutination of *R. trifolii* 403 and 2S-2 cells by various carbohydrates was examined. The sugar inhibition patterns were identical for both infective strains. Preincubation of LAI with 30 mM 2-deoxyglucose or N-acetylgalactosamine inhibited its ability to agglutinate the cells (Table 8). None of the other carbohydrates at this concentration were inhibitory.

LAI agglutinated rabbit erythrocytes coated with 403-AC only if followed by anti-LAI. This antiserum did not agglutinate cells coated only with 403-AC. LAI-mediated passive haemagglutination was inhibited by 30 mM 2-deoxyglucose and N-acetylgalactosamine (Table 8). Identical results were obtained with anti-white clover root antiserum. Since 2-deoxyglucose was a component of this cross reactive

Table 8. *Inhibition of Clover Lectin-Mediated Cell Agglutination by Various Carbohydrates*

Carbohydrate	Cell agglutination[a]	
	R. trifolii strains 403 and 2S-2[b]	Rabbit erythrocytes coated with 403-AC[c]
α-D Glucose	+	+
β-D Glucose	+	+
α-D Galactose	+	+
α-D Glucuronic acid	+	+
α-D Galacturonic acid	+	+
Cellobiose	+	+
D-Mannose	+	+
D-Fructose	+	+
D-Xylose	+	+
α-Methyl D-mannoside	+	+
L-Sorbose	+	+
2-Deoxyglucose	-	-
N-Acetylglucosamine	+	+
N-Acetylgalactosamine	-	-
Untreated[d]	+	+

[a] Agglutination scored as +'ve, no agglutination as -'ve.

[b] Tube agglutination.

[c] Passive haemagglutination of purified cross reactive capsular antigen of *R. trifolii* 403 following addition of anti-white clover seed extract.

[d] No carbohydrate added.

capsular antigen, the sugar binding site of both the clover lectin and the anti-white clover root antibody was probably directed toward this sugar.

Inhibition of Infection of *T. repens* by 2-Deoxyglucose

Since 2-deoxyglucose inhibited the binding of the clover lectin to the cross reactive antigen of *R. trifolii*, it was hypothesized that this sugar may interfere with the bacterial infection of clover by occupying all the lectin binding sites. Infection of white clover root hairs by *R. trifolii* 403 was completely inhibited by 2-deoxyglucose at concentrations of 30 mM and above (Table 9). Partial inhibition was evident at lower concentrations (2.5-10 mM). The inhibition was specific for 2-deoxyglucose and was not due to hypertonic osmotic effects since infection and nodulation occurred in the presence of 200 mM α-D glucose. However, 2-deoxyglucose is known to interfere with glucan wall synthesis, microfibril production, and cell metabolism in *Saccharomyces cerevisiae* (32), and therefore the inhibitory effect should be regarded at the present time as a result of multiple effects.

Adsorption of *R. trifolii* to Root Hairs of *T. repens* Comparison of Infective and Noninfective *R. trifolii* Strains

The mean number of *R. trifolii* cells adsorbed to root hairs of approximately 200 μm in length after 12 h incubation are presented in Table 10. When pooled together, the mean number of adsorbed infective and noninfective cells were 25.8 ± 5.9 and 5.6 ± 1.9, respectively. The populations were compared statistically using the *t* tests computed after

Table 9. *Inhibition of Infection and Nodulation of Trifolium repens with Rhizobium trifolii 403 by 2-deoxyglucose*

Concentration of 2-Deoxyglucose (mM)	Infection threads on two roots	Nodules on two roots
0.0	25	12
0.0[a]	23	9
2.5	6	0
5.0	3	0
10.0	1	0
30.0	0	0
50.0	0	0
100.0	0	0
200.0	0	0

[a]Containing 200 mM α-D glucose.

Table 10. *Adsorption of Rhizobium trifolii Cells on Trifolium repens Root Hairs*

Strain	Adsorbed cells[a] (means ± std. dev.)
2S-2[b]	21.00[d] ± 1.00
2L[c]	3.00 ± 1.73
0435[b]	22.50[d] ± 2.81
0435-2[c]	6.67 ± 1.63
T37[b]	22.75[d] ± 2.22
Bio-9[c]	4.80 ± 1.64
WU-290-I[b]	25.50[d] ± 4.12
WU-290-N[c]	8.00 ± 2.45
403[b]	25.67[d] ± 0.58
Bart A[c]	5.33 ± 2.31
BA-L[b]	37.33[d] ± 9.48

[a]On root hairs of approximately 200 µm in length.

[b]Infective on *T. repens*.

[c]Noninfective on *T. repens*.

[d]Significantly greater than corresponding noninfective mutant (at 99.5% level).

the square root transformation (47) of the means. For every strain combination examined, the mean number of infective cells adsorbed was significantly greater (at 99.5% level) than the noninfective cells. Of particular importance was the finding that the infective revertant BA-L had a significantly greater frequency of adsorbed cells than the corresponding noninfective strain Bart A. Thus, it can be concluded that the phenotypic trait of infectivity for *R. trifolii* is directly correlated with the extent of cell adsorption to root hairs of the clover host. Another study has indicated no correlation between the extent of root adsorption with the infective capabilities of the microorganisms (42). This study was based on indirect measurements involving viable colony counts of cells following their removal from roots by shaking. Unfortunately this latter approach did not distinguish root hairs from other adsorptive root surfaces, nondispersable flocs from single cells, and the variability of root surface areas among individual plants.

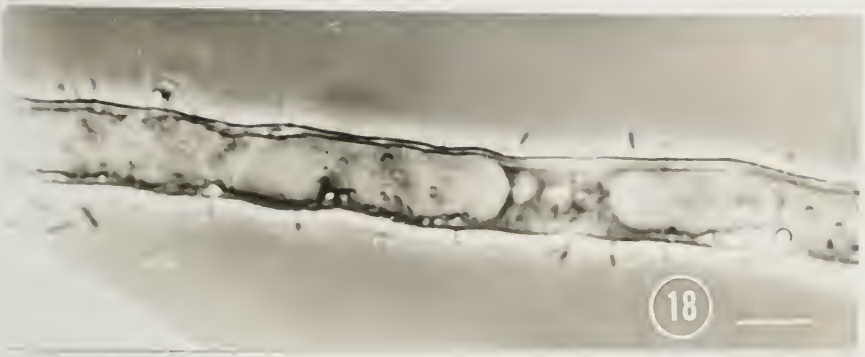
Adsorption of the rod-shaped bacteria occurred primarily through attachment of one of their poles to the root hair surfaces (Fig. 18). Polar orientation has been reported as the position of attachment of single cells of *R. trifolii* (38), *R. meliloti* (12), *R. japonicum* (44), and *R.* sp. (*Aeschynomene* nodulating strain, [37]) on their appropriate legume host root surface.

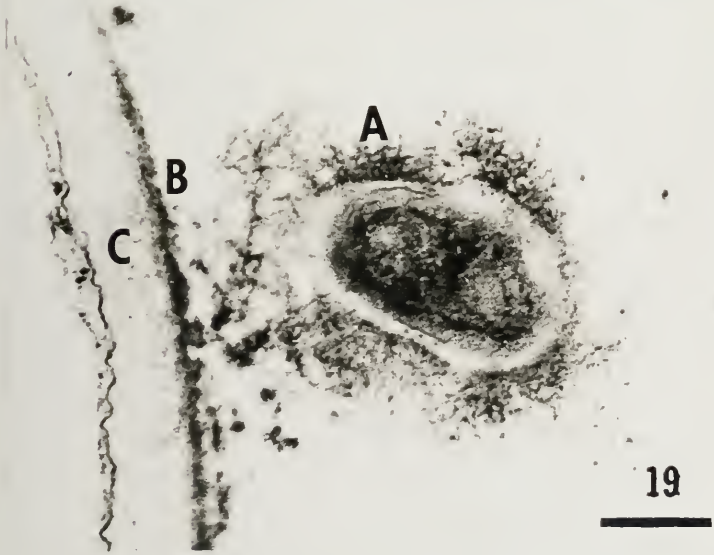
Ultrastructure of Adsorption

The ultrastructural details of the association of

Fig. 18. Adsorption of *Rhizobium trifolii* 403 cells on a *Trifolium repens* root hair. Cells are polarly attached. Bar scale equals 20 µm.

Fig. 19. Electron photomicrograph of an ultrathin section of *Rhizobium trifolii* NA-30 in association with a *Trifolium fragiferum* root hair. The fibrillar capsule (A) of the bacterium is in contact with electron dense particles (B) on the outer periphery of the root hair cell wall (C). Bar scale equals 1 µm.


18


A
B
C
19

R. trifolii NA-30 with the clover root hair wall are shown in Fig. 19. This photograph was typical of the polar orientation that was routinely observed. The fibrillar capsule of the bacterium was in physical contact with electron dense, globular aggregates lying on the exterior periphery of the fibrillar root hair cell wall.

Based on the data of these investigations, the following model was proposed (Fig. 20) to explain this preinfective adsorption event which contributes to host specificity in the *Rhizobium*-clover association. Infective strains of *R. trifolii* have on their surfaces a polysaccharide which is antigenically cross reactive with an antigen on the root surface of the clover host, and also is capable of deforming root hairs. It is proposed that the clover lectin which recognizes these surface antigens cross-bridges them to form a correct molecular interfacial structure which allows for specific adhesion of the bacteria to the root surface. Following this specific cell adhesion, the invagination process of the root hair wall begins, resulting in infection thread formation (39). Noninfective *R. trifolii* cells have cross reactive surface antigens but either in reduced quantity or sterically blocked as neither antibody nor lectin binds to them as efficiently as to infective cells. Other *Rhizobium* species lack this surface cross reactive antigen, and therefore do not bind to the clover lectin. The model is consistent with the findings of Hamblin and Kent (22), and Bohlool and Schmidt (4) who introduced the role of lectin in the adsorption of the *Rhizobium*. But the

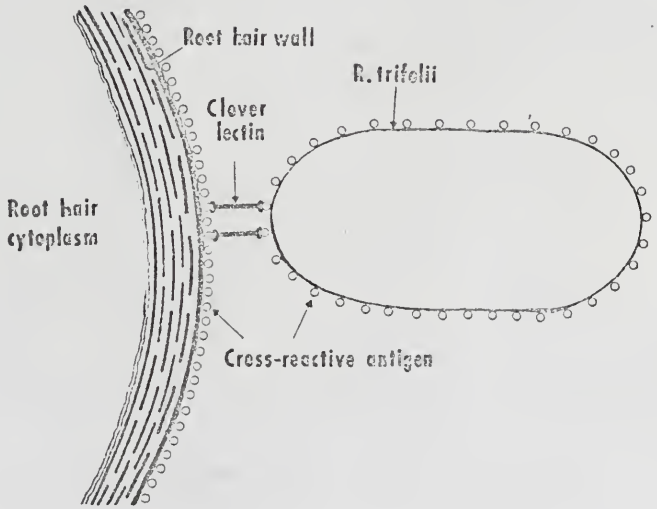


Fig. 20. Schematic diagram of the proposed cross-bridging of the cross reactive antigens of *Rhizobium trifolii* and *Trifolium* root hair with a clover lectin.

lectin alone does not confer specificity. It is one component of a multimembered specificity-determining complex. Two other components of this complex are the cross reactive surface antigens.

LITERATURE CITED

1. Baldwin, I. L., E. B. Fred, and E. G. Hastings. 1927. Grouping of legumes according to the biological reactions of their seed proteins. Bot. Gaz. 83:217-243.

2. Barker, S., E. Bourne, and D. Whiffen. 1956. Use of infrared analysis in the determination of carbohydrate structure. Meth. Biochem. Anal. 3:213-245.

3. Belue, G. P., and G. D. McGinnis. 1974. High pressure liquid chromatography of carbohydrates. J. Chromatogr. 97:25-31.

4. Bohlool, B. B., and E. L. Schmidt. 1974. Lectins: a possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science 185:269-271.

5. Brethauer, T. S., and J. D. Paxton. 1975. Interaction of soybean lectin with extracellular material from *Rhizobium* bacteria. Abstr. North Central Division Meeting, Amer. Phytopathol. Soc., June 23-24, 1975, West Lafayette, Ind.

6. Cahill, J. F., B. C. Cole, B. B. Wiley, and J. R. Ward. 1971. Role of biological mimicry in the pathogenesis of rat arthritis induced by *Mycoplasma arthritidis*. Inf. Immun. 3:24-35.

7. Cappelletti, C. 1926. The bacteroid-like form and immunity in leguminous plants. Internatl. Congr. Plant Sci., Ithaca, N. Y. Proc. 1:59-60.

8. Chada, K. C., and B. I. Strivastava. 1971. Evidence for the presence of bacteria-specific proteins in sterile crown gall tumor tissue. Plant Physiol. 48: 125-129.

9. Charudattan, R., and J. E. DeVay. 1972. Common antigens among varieties of *Gossypium hirsutum* and isolation of *Fusarium* and *Verticillium* species. Phytopathology 62:230-234.

10. Charudattan, R., and D. H. Hubbell. 1973. The presence and possible significance of cross reactive antigens in *Rhizobium*-legume associations. Antonie van Leeuwenhoek J. Microbiol. Serol. 39:619-627.

11. Clapp, C. E., and R. J. Davis. 1970. Properties of extracellular polysaccharides from *Rhizobium*. Soil Biol. Biochem. 2:109-117.

12. Dart, P. J., and F. V. Mercer. 1964. The legume rhizosphere. Arch. Mikrobiol. 47:344-378.

13. Dazzo, F. B., and D. H. Hubbell. 1974. A quantitative assay of insoluble polyvinylpyrrolidone. Plant Soil 40:435-440.

14. Dazzo, F. B., and D. H. Hubbell. 1975. Antigenic differences between infective and noninfective strains of *Rhizobium trifolii*. Appl. Microbiol. 30:172-177.

15. Dazzo, F. B., and D. H. Hubbell. 1975. Concanavalin A: lack of correlation between binding to *Rhizobium* and specificity in the *Rhizobium*-legume symbiosis. Plant Soil 43:717-722.

16. DeVay, J. E. 1975. Protein specificity in plant disease development: protein sharing between host and parasite. NATO Conference on Specificity in Plant Diseases, May 4-16, 1975, Sardinia Advanced Study Institute, Sardinia.

17. DeVay, J. E., W. C. Schnathorst, and M. S. Foda. 1966. Common antigens and host-parasite interactions. p. 313-328. *In* C. Mirocha and I. Uritani (eds.), The dynamic role of molecular constituents in plant-parasitic interactions. Bruce Pub. Co., St. Paul, Minn.

18. Doubly, J. A., H. H. Flor, and C. O. Clagett. 1960. Relation of antigens of *Melampsora lini* and *Linium usitatissimum* to resistance and susceptibility. Science 131:229.

19. Dudman, W. F. 1972. Detection of acidic polysaccharides in gels by DEAE-dextran. Anal. Biochem. 46:668-673.

20. Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. 16:374-381.

21. Goldman, M. 1968. Fluorescent antibody methods. Academic Press, N. Y. 303p.

22. Hamblin, J., and S. P. Kent. 1974. Possible role of phytohaemagglutinin in *Phaseolus vulgaris* L. Nature New Biology 245:28-30.

23. Hubbell, D. H. 1970. Studies on the root hair "curling factor" of *Rhizobium*. Bot. Gaz. 131:337-342.

24. Humphrey, B. A. 1959. Occurrence of 4-O-methyl glucuronic acid in *Rhizobium* gums. Nature (London) 184:1802.

25. Hunter, W. 1973. Radioimmunoassay. *In* D. Weir (ed.), Handbook of experimental immunology. p. 17.1-17.33. Blackwell Scientific Pub., Oxford, England.

26. Johnson, L. F., and E. A. Curl. 1972. Methods for research on the ecology of soil-borne plant pathogens. p. 92-96. Burgess Pub. Co., Minneapolis, Minn.

27. Jones, D. G., and P. E. Russell. 1972. The application of immunofluorescence techniques to host plant/nodule bacteria selectivity experiments using *Trifolium repens*. Soil Biol. Biochem. 4:277-282.

28. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry, 2nd ed. Charles Thomas Pub., Springfield, Ill. 905p.

29. Li, D., and D. H. Hubbell. 1969. Infection thread formation as a basis of nodulation specificity in *Rhizobium*-strawberry clover associations. Can. J. Microbiol. 15:1133-1136.

30. Ljunggren, H. 1969. Mechanism and pattern of *Rhizobium* invasion into leguminous root hairs. Physiol. Plant. Suppl. V. 82p.

31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-269.

32. Lratky, A., P. Biely, and S. Bauer. 1975. Mechanism of 2-deoxy-D-glucose inhibition of cell wall polysaccharide and glycoprotein biosynthesis. Eur. J. Biochem. 54:459-467.

33. MacGregor, A. N., and M. Alexander. 1972. Comparison of nodulating and non-nodulating strains of *Rhizobium trifolii*. Plant Soil 36:129-139.

34. Markowitz, A. S., S. H. Armstrong, and D. S. Kushner. 1960. Immunological relationships between the rat glomerulus and nephritogenic streptococci. Nature (London) 187:1095-1097.

35. Mowry, R. 1956. Alcian blue techniques for the histochemical study of acidic carbohydrates. J. Histochem. Cytochem. 4:470.

36. Munns, D. N. 1968. Nodulation of *Medicago sativa* in solution culture. I. Acid-sensitive steps. Plant Soil 28:129-146.

37. Napoli, C., F. Dazzo, and D. Hubbell. 1975. Ultrastructure of infection and "common antigen" relationships in *Aeschynomene*. 5th Australian Legume

Conference, March 18-21, 1975, Brisbane, Australia.

38. Napoli, C. A., F. B. Dazzo, and D. H. Hubbell. 1975. Production of cellulose microfibrils by *Rhizobium*. Appl. Microbiol. 30:123-131.

39. Napoli, C. N., and D. H. Hubbell. 1975. Ultrastructure of *Rhizobium* induced infection threads in clover root hairs. Abstr. Ann. Meeting, Amer. Soc. Microbiol. April 27 - May 2, 1975, New York.

40. Nowotny, A. 1969. Basic exercises in immunochemistry. Springer-Verlag, N.Y. 197p.

41. Pearlman, P., S. Hammarstrom, R. Lagercrantz, and B. E. Gustafson. 1965. Antigen from colon of germfree rats and antibodies in human ulcerative colitis. Annu. N. Y. Acad. Sci. 124:337-394.

42. Peters, R. J., and M. Alexander. 1966. Effect of legume exudates on the root nodule bacteria. Soil Science 102:380-387.

43. Pierce Chemical Company. 1972. Handbook of silylation. Pierce Chemical Company, Rockford, Ill. 48p.

44. Reporter, M., D. Raveed, and G. Norris. 1975. Binding of *Rhizobium japonicum* to cultured soybean root cells: morphological evidence. Plant Science Letters 5:73-76.

45. Schnathorst, W. C. 1973. Comparative antigenic structures of virulent and avirulent isolates of *Xanthomonas malvacearum*. p. 1330. Proc. 2nd International Congr. Plant Pathology, Amer. Phytopathol. Soc., Sept. 5-12, 1973, Minneapolis, Minn.

46. Solheim, B., and J. Raa. 1973. Characterization of substances causing deformation of root hairs of *Trifolium repens* when inoculated with *Rhizobium trifolii*. J. Gen. Microbiol. 77:241-247.

47. Steel, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York. 481p.

48. Stevens, I. 1967. The ecology and etiology of human disease. p. 106-107. Charles Thomas Pub. Co., Springfield, Ill.

49. Vaught, R., and A. Bleiweis. 1974. Antigens of *Streptococcus mutans*. II. Characterization of an antigen resembling a glycerol teichoic acid in walls of strain BHT. Inf. Immun. 9:60-67.

50. Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria. IBP Handbook No. 15. Blackwell Scientific Pub., Oxford, England. 164p.

51. Weaver, R., and L. Frederick. 1972. A new technique for most-probable-number counts of *Rhizobium*. Plant Soil 36:219-222.

52. Wimalajeewa, D. L. S., and J. E. DeVay. 1971. The occurrence and characterization of a common antigen relationship betwen *Ustilago maydis* and *Zea mays*. Physiol. Pl. Path. 1:523-535.

53. Worthington Biochemical Corporation. 1972. Worthington enzyme manual. Worthington Biochemical Corporation, Freehold, N. J. 216p.

54. Yao, P. Y., and J. M. Vincent. 1969. Host specificity in the root hair "curling factor" of *Rhizobium* spp. Aust. J. Biol. Sci. 22:413-423.

55. Zabriskie, J. B. 1967. Mimetic relationships between group A streptococci and mammalian tissue. Adv. Immunol. 7:147-188.

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Frank Bryan Dazzo was born April 8, 1948, in Miami, Florida. He was graduated from Coral Gables High School in Coral Gables, Florida, in June, 1966. In June, 1968, he received an Associate of Science degree from Young Harris College, Young Harris, Georgia, where he was elected to Who's Who in American Junior Colleges. In August, 1970, he received his Bachelor of Science degree with a major in bacteriology from The Florida State University, Tallahassee, Florida. He began his graduate studies at the University of Florida in September, 1970, and received his Master of Science degree in microbiology there in June, 1972.

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He is married to Olgalina G. Dazzo.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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