



Sainfoin: rhizobium, lectin and host specificity
by James Patrick Beltzer

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
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Abstract:

Experiments were conducted to determine if the lectin from sainfoin (*Onobrychis viciifolia*, Scop.) is a determinant of host specificity in the Rhizobium-legume symbiosis. For the purpose of these experiments, three pure strains of Rhizobium were isolated from root nodules of sainfoin. They were isolated under controlled conditions by means of a trap host technique. These strains were examined and their purity assured by using light and electron microscopy with standard microbiological techniques for the identification of the genus Rhizobium. Growth of two of the three strains was measured by optical density on a yeast-mannitol medium and a defined medium. Classical growth kinetics were demonstrated in three out of four cases.

The interaction of lectin and Rhizobium was examined using a lectin bacteria agglutination assay, an F.I.T.C.-lectin binding study, a time course ¹⁴C-lectin binding assay and a ferritin conjugated concanavalin A binding study. Bacterial suspensions were not agglutinated when incubated with increasing concentrations of sainfoin lectin. F.I.T.C.-lectin did not bind rhizobia cultured in liquid or solid media. Low levels of lectin-Rhizobium binding were observed and binding varied with culture age and nutrition using a ¹⁴C-lectin. Ferritin-labeled concanavalin A was used, with appropriate parallel controls, to examine the cell surface architecture of lectin receptors on the transmission electron microscope. To examine possible cell surface polysaccharide differences, stationary growth phase organisms were cultured on two separate media before staining with ferritin-Con A. Binding was observed and quantitated in ferritin-Con A stained cat erythrocyte positive controls. However, with ferritin-Con A stained Rhizobium bacteria and bacteria pretreated with trypsin and glutaraldehyde no significant binding could be demonstrated. These data are interpreted as suggesting that simple Rhizobium-lectin binding is not the basis of, nor does it significantly contribute to, host-symbiont specificity in the sainfoin system.

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by

JAMES PATRICK BELTZER

A thesis submitted in partial fulfillment
of the requirements for the degree

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ABSTRACT

Experiments were conducted to determine if the lectin from sainfoin (Onobrychis viciifolia, Scop.) is a determinant of host specificity in the Rhizobium-legume symbiosis. For the purpose of these experiments, three pure strains of Rhizobium were isolated from root nodules of sainfoin. They were isolated under controlled conditions by means of a trap host technique. These strains were examined and their purity assured by using light and electron microscopy with standard microbiological techniques for the identification of the genus Rhizobium. Growth of two of the three strains was measured by optical density on a yeast-mannitol medium and a defined medium. Classical growth kinetics were demonstrated in three out of four cases.

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INTRODUCTION

Symbiosis is the intimate living together of two kinds of organisms such that there is a biological advantage to this relationship. As man looks more closely at the world that surrounds him, the number and variety of symbioses steadily increase. One of the most remarkable examples of symbiosis that has been brought to light with the advent of the electron microscope is the protozoan Myxotricha paradoxa, which inhabits the digestive tract of Australian termites. This motile protozoan supplies the enzymes that digest the cellulose to usable carbohydrate and leaves behind the undigestible lignin. The flagella which propel the Myxotricha under closer scrutiny with an electron microscope turn out to be regularly spaced spirochetes which have attached themselves to the surface of the protozoan. Embedded in the surface close to this point of attachment are oval organelle-like structures which are also found in the cytoplasm. These oval structures are, in fact, bacteria living in symbiosis with the spirochetes and the protozoan and probably contributing enzymes necessary for the breakdown of cellulose. The whole animal seems to be a model for the development of more advanced cells as well as a fantastic example of symbiosis. There seems to be an underlying force that drives together several creatures as in the Myxotricha, and then produces a union [1]. Valuable insights could be gained by understanding the forces causing such a union.

Nitrogen is an essential element in all forms of life. Next to water, it is often the limiting factor in crop production. Biological nitrogen fixation is the process by which atmospheric nitrogen is converted to ammonia and eventually to protein. Biological nitrogen fixation by leguminous plants is an example of a symbiosis with great agronomic importance. In contrast to fixation of nitrogen by industrial means, biological nitrogen fixation supplies approximately three and one-half times as much fixed nitrogen and takes place in the fields, forests and oceans where nitrogen is utilized [2]. To utilize biological nitrogen fixation for the greatest benefit to mankind, it is essential to understand the biology, including the ecological relationships of those organisms that have evolved this unique capability. Intimate and specific symbiotic associations between leguminous plants and bacteria of the genus *Rhizobium* provide most of the biologically fixed nitrogen available for agriculture as well as an excellent opportunity for management and the harnessing of this symbiosis for increased production [3]. With the increasing scarcity of fossil fuels for industrial nitrogen fixation, leguminous plants are in the spotlight.

Characterization of the Rhizobium

- Legume Symbiosis

The N_2 -fixing *Rhizobium*-legume symbiosis is characterized by a high degree of host specificity that is expressed by both the bacteria

and the plant [4]. The population of bacteria in the soil is huge and varied. Presently, a biochemical explanation of the selection process is lacking. The specificities expressed by the host legume and Rhizobium species culminate in root nodule formation. The root nodule is the product of the symbiosis and the actual site of nitrogen fixation. Certain morphological changes are evidenced during nodulation [5]. First, bacteria accumulate on the legume root surface and induce root hair curling. Bacteria enter the root with infection thread initiation. The infection thread invaginates, directed by unknown forces, toward a tetraploid cell in the root cortex. Release of the bacteria within the tetraploid cell is followed by a morphological change of the bacteria to bacteroids. Bacteroids are branched or enlarged cells unable to grow on media that support unmodified cells [6]. Within the nodule the bacteroid's nitrogen fixing machinery is protected from oxygen poisoning by leghaemoglobin, an oxygen scavenging compound provided by the host legume [6]. The host legume also furnishes the bacteroids with nutrients resulting from photosynthesis and, in turn, receives fixed atmospheric nitrogen from the bacteroids. However, a complete discussion of the biochemistry of fixation is not within the scope of this work and is best left to the current reviewers [7,8,9].

The bacteria which nodulate legumes, Rhizobium spp., are specific in that a single strain of the bacterium can only infect certain species of legume. This specificity is the basis of species

differentiation in the genus Rhizobium [5]. Legumes are also said to display cross-inoculation group specificity, that is, groups of legume species are nodulated by a single species of Rhizobium [6]. Bergey's manual gives six major cross-inoculation groups of host plants and associated bacteria [6].

Characterization of Rhizobium spp.

Rhizobium is a genus of aerobic, heterotrophic, non-spore forming soil bacteria [6]. They possess the ability to invade leguminous roots and form nodules as described above. This ability to form root nodules is the most important characteristic for distinguishing Rhizobium from other bacteria genera. Rhizobium cells are small to medium sized ($0.5 - 0.9 \times 1.2 - 3.0 \mu\text{m}$) gram negative rods, often motile when young by polar, subpolar or peritrichous flagella [6]. Within the organisms' cytoplasm can be found characteristic granules of β -hydroxy-butyrate which stain with Sudan Black [10]. The surface of most strains is covered with an extrapolsaccharide slime layer of highly variable composition. This slime layer is believed to be involved in the legume host specificity and will be discussed in more detail [10]. In short, staining techniques and cultural tests help to distinguish Rhizobium from other genera but are not diagnostic. The principle test remains to be the ability to form root nodules in the host legume.

Brief Characterization of Sainfoin
(Onobrychis viciifolia, Scop.)

The legume of interest in this study is Sainfoin. The 'Eski' variety was brought to the United States from Turkey and released as a variety in 1964 by the Montana Agricultural Experiment Station [11]. Sainfoin is a long lived, deep rooted, perennial legume crop which has gained popularity in Montana. As a forage for cattle it has the advantage of being non bloating and is resistant to infection by the alfalfa weevil. Root and crown rot have limited Sainfoin production in recent years. Sainfoin is also a poor nitrogen fixer and has a short stand life.

Host Specificity in the Rhizobium
- Legume Symbiosis

Plant proteins called phytohemagglutinins or lectins have been isolated from a large number of leguminous plants [12]. Many reviewers have discussed the interesting characteristics of these proteins [12,13,14,15]. Because of their ability to agglutinate erythrocytes and to bind specific saccharides, they have been used as probes of cell surface architecture. However, not much is known concerning the biological role of these proteins within the plant. The ability to bind specific saccharides, which originally brought attention to lectins, is now postulated to function in recognition processes between plants and bacteria [16]. Several investigators have recently examined the possibility that the lectins of legumes may serve as

determinants of host specificity [3]. One hypothesis states that root lectins may bind a distinctive polysaccharide on the Rhizobium cell surface thereby initiating nodulation [17]. Because lectins are multivalent they could cross-bridge the similar surface polysaccharides of the Rhizobium bacteria and legume root hairs [18]. A number of approaches have been taken to investigate the potential cross-bridging role of lectins in the Rhizobium-legume symbiosis. Hamblin and Kent showed that a Phaseolus lectin could bind to host specific rhizobia and localized the lectin in the seeds, nodules and on the roots below the nodules of Phaseolus vulgaris [19]. Bohlool and Schmidt, using a fluorescent tagged soybean lectin, found that 22 of 25 strains of the symbiotic rhizobia tested bound the lectin whereas all 23 nonsymbiotic strains of rhizobia did not [20,21]. Dazzo and Hubbell, using the white clover-Rhizobium trifolii system, have found a common antigen present on the clover roots and on the surfaces of infective strains of rhizobia [4]. This "cross reactive antigen" was isolated from the capsular polysaccharide of infective strains of rhizobia. Dazzo and Hubbell also showed that a lectin isolated from the clover seed could bind the capsular polysaccharide antigen [4], as well as infective strains of rhizobia. Wolpert and Albersheim used a somewhat different approach; they isolated lectins from the seeds of four legumes and obtained lipopolysaccharide (LPS) preparations from the four respective symbiotic nodulating Rhizobium strains [22]. In all cases the

bacterial LPS bound only to the lectin of the host legume and not to the lectins of legumes the bacterium could not nodulate. Schmidt and Tsien (in 16) located the lectin-binding sites at one end of the bacterium and associated binding with the exopolysaccharide or polysaccharide excreted from the cell. And to add to the confusion, Planque and Kinje found a pea lectin bound to a species of polysaccharide which is neither lipopolysaccharide nor exopolysaccharide [23]. These results, although different, all imply the involvement of lectins in the host specificity of the Rhizobium-legume symbiosis. One important fact to bear in mind is that each symbiosis is potentially unique. In summary, the nature of the lectin binding site on bacteria may be: (1) exopolysaccharide, (2) capsular polysaccharide, (3) lipopolysaccharide, or (4) another saccharide containing fraction that is part of the cell surface architecture.

Lectin Involvement in Host Specificity in the Sainfoin System

A lectin has been isolated from Sainfoin seed using ammonium sulfate fractionation and affinity chromatography [38]. This lectin specifically agglutinates cat erythrocytes and has a saccharide specificity for α -D-mannose and α -methylmannoside which are similar to the specificities exhibited by concanavalin A [24,38]. A lectin has also been isolated from the root tissue of sainfoin and has been shown to be similar to the seed lectin by amino acid analysis and

immunoprecipitation. Sainfoin root lectin was localized on the root surface by an indirect rhodamine immunohistofluorescence technique [25].

Research Objectives

The possible role of sainfoin lectin as a determinant or one of the determinants of host specificity was examined. Since the sainfoin lectin protein had been localized on the outer root surfaces, a number of techniques were used to examine sainfoin lectin binding to the surface of the host specific rhizobia. The specific objectives of this work were:

1. Isolation and characterization of pure strains of sainfoin Rhizobium bacteria.
2. Development of techniques to study the interaction of lectin with Rhizobium bacteria cultures.
3. Cell surface architecture studies of lectin receptor sites on Rhizobium bacteria using a ferritin labeled lectin.

MATERIALS AND METHODS

I. Isolation and Characterization of Pure Cultures of Rhizobium

A. Growth medium. One of the most commonly used complex mediums used for growing Rhizobium bacteria is yeast mannitol. This medium consists of: NaCl 1.7×10^{-6} M; CaCl_2 7.7×10^{-6} M; MgSO_4 7.4×10^{-6} M; K_2HPO_4 4.6×10^{-6} M; KH_2PO_4 1.4×10^{-6} M; mannitol 10 g/l and yeast extract (Difco) 1 g/l. The solid medium contains noble agar 15 g/l.

Defined media replaces the yeast extract with inorganic combined nitrogen, several amino acids and vitamins. The defined medium used was modified from Jensen's chemically defined medium [26] by Currier et al. [5]. This medium is prepared as follows: 5.5×10^{-2} M glucose, 1.07×10^{-3} M l-asparagine, 7.14×10^{-4} M l-methionine, 8.72×10^{-4} M KCl, 7.99×10^{-4} M $\text{Ca}(\text{NO}_3)_2$, 7.91×10^{-4} M KNO_3 , 1.46×10^{-3} M MgSO_4 , 1.38×10^{-4} M NaH_2PO_4 , 1.41×10^{-3} M Na_2SO_4 , 2.98×10^{-5} M MnSO_4 , 1.54×10^{-4} M ZnSO_4 , 2.5×10^{-5} M H_3BO_4 , 4.52×10^{-6} M KI, and 7.40×10^{-6} M FeCl_3 . After autoclaving, 1 ml of a filter sterilized solution of biotin, nicotinic acid and calcium pantothenate was added to the cooled medium. Fifty ml of the vitamin solution contained 2.5 mgs biotin, 25 mgs nicotinic acid and 25 mgs calcium pantothenate.

B. Staining techniques for characterization of Rhizobium. One of the most useful staining techniques in bacteriology is Gram's Differential Stain. Burke's modification of this technique was used

[27] for staining organisms grown in liquid cultures of defined medium. The solutions used were: Solution A 1% crystal violet, Solution B 5% NaHCO_3 with Merthiolate diluted to 1:20,000, Iodine solution 1% KI in 0.5% iodine, Decolorizing solution consisting of ether and acetone in a 1:3 v/v ratio and a Counterstaining solution of Safranin 0 in 0.5% concentration. All solutions are aqueous unless otherwise specified. The staining procedure for organisms fixed to clean glass slides involves flooding the slide with Solution A. Three to five drops of Solution B are then added and allowed to stand for one minute. Wash the slide well with water. Cover the organisms with the iodine solution and let stand for 1 minute. Rinse well with water. Decolorize with ether-acetone mixture by adding mixture to the slide until no more color washes off the slide. Wash with water. Counterstain 10-15 seconds with Safranin 0. Wash with water, dry and examine. Gram-positive organisms stain blue; gram negative, red.

The Fat Staining Procedure for Rhizobium for the light microscope was done according to the technique of Burdon [28]. Organisms grown in defined media were heat fixed to a clean glass slide. Flood slide with sudan black for 15 minutes (0.3 g/100 ml 70% ETOH). Drain excess stain and blot dry. The slide is then cleared using xylene and blotted dry. Counterstain with 0.5% aqueous safranin 0 for 5-10 seconds. The slide is then washed with distilled water, blotted dry, and examined.

The silver impregnation stain for Leptospira and flagella was done according to the method of Blenden and Goldberg [29]. Rhizobium strains 116A8, 116A12, 116A14, 116A15, 116A17, and 95C13 (Nitragen) were used for the staining procedure after 24 hours growth in defined medium. A positive control organism Pseudomonas aeruginosa was also stained. First, a loopful of sterile distilled water was placed on a very clean slide. Next to it was placed a loopful of the culture. This forms a gradient. The slide was then allowed to air dry. Reagent A was applied to the slide for 2-4 minutes. Reagent A consists of 100 ml of sterile distilled water containing 5 grams of tannic acid, 1.5 grams of ferric chloride, 2.0 ml of 15% formalin and 1.0 ml of 1% sodium hydroxide. After the allotted time the slide was rinsed with water. Reagent B was then added for 30 seconds before washing with water. Reagent B; an ammoniated silver nitrate solution, was prepared by use of 10 ml of 2% silver nitrate. To the silver nitrate solution ammonium hydroxide was added dropwise until the heavy precipitate that formed was dissolved. Reagent B was then backtitrated with 2% silver nitrate until a slight clouding appeared and persisted. After staining, slides were allowed to air dry before viewing under the microscope.

Congo red yeast mannitol petri plates were prepared according to the method of Gibbs and Shapton [6]. The congo red dye was added 1 ml per 100 ml of yeast mannitol medium with the congro red previously

diluted 1:400 and autoclaved separately. The plates were streaked with a loopful of each stock culture of Rhizobium and incubated for 5 days at 21°C before scoring. They were scored (+) for congo red uptake and (-) for a pure culture, usually a milky white color.

Phase contrast microscopy was performed routinely on Rhizobium grown in defined medium. A Zeiss Standard microscope with phase contrast and integral illumination and photometer was used to check purity as well as initial morphological examinations. This instrument was also used for all photomicrography.

For shadowing Rhizobium were grown on yeast mannitol medium on petri plates [37]. One small loopful of each strain was diluted in a separate drop of sterile distilled water on a clean glass slide. After mixing, a small amount of each dilution mixture was drawn off in a 1 cc tuberculin syringe. Then a small drop of each mixture was placed on formvar coated grid (300 mesh) and the bacteria were allowed to settle for 30 seconds. All excess water was then removed with a piece of filter paper. All strains of Rhizobium were shadowed at once with platinum at a shadow length:object height ratio of 3:1 using a Varian VE-10 vacuum evaporator. The grids were then viewed immediately in a Zeiss EM9S2 transmission electron microscope. The objective aperture, 50 μ m in diameter, was left in place to allow more precise study of the cell surface architecture. A phosphotungstic acid negative stain was also performed for the transmission electron.

microscope. The phosphotungstic acid (P.T.A.) stain solution consisted of 1.0 gram of PTA, 0.2 grams sucrose and 50 ml of distilled water. The pH was adjusted to 6.5 with sodium hydroxide. The droplet staining technique was used [30]. First, formvar coated grids were placed on a glass slide. Then 4 drops of the bacterial culture in defined medium (1 mg/ml) were mixed with 4 drops of the PTA solution. The liquid was drawn up in a tuberculin syringe (1 cc). One drop of the mixture was then added to each grid. The excess liquid was removed from the grid after 30 seconds with a piece of filter paper. All grids were then viewed immediately in the electron microscope.

C. Isolation of pure cultures. Nodulation of sainfoin was achieved under controlled conditions in 10 inch test tubes. Five inches of vermiculite were placed in the bottom of each tube. To each tube was added 10 ml of Thornton's nitrogen-free medium [31]. This medium contained: 1.71×10^{-3} M NaCl, 7.3×10^{-4} M MgSO_4 , 5.7×10^{-4} M K_2HPO_4 , 7.3×10^{-4} M KH_2PO_4 , 4.63×10^{-5} M H_3BO_3 , 9.10×10^{-6} M MnCl_2 , 7.34×10^{-7} M ZnCl_2 , 3.20×10^{-7} M CuSO_4 , 5.21×10^{-7} M NaMoO_4 , 6.17×10^{-5} M FeCl_3 , and 5×10^{-3} M CaCO_3 . The tubes were then capped and autoclaved. Sainfoin seeds were surface sterilized in 5.25% sodium hypochlorite for 15 minutes and rinsed twice for 15 minutes in sterile distilled water. The seeds were then planted aseptically (2 per tube) just below the surface of the vermiculite. Seeds were germinated for

two days in the dark before placing in a growth chamber. The roots remained in the dark. The growth chamber was operated with an 18:6 day, night cycle and 26°C day:18° night temperature cycle. The plants were then grown for 5 days before inoculation. To prepare the inoculum, non congo red absorbing Rhizobium colonies were picked from petri plates of yeast mannitol medium and transferred to defined liquid medium. The inoculum was incubated at 27° on a shaker bath 150 RPM for 4 days in the dark. An appropriate volume of inoculum was removed from each flask and the bacteria were pelleted with a clinical centrifuge. The bacteria were resuspended in sterile distilled water. This washing procedure was repeated twice with the final concentration of approximately 10^7 cells/per ml of inoculum. Inoculation of the plants with the bacteria was done by transfer of the bacteria to the tubes with a sterile pipette. Four weeks after inoculation, the plants were carefully removed from the test tubes. The roots were washed with sterile distilled water to remove pieces of vermiculite. The nodules were removed from the roots with a razor blade. The nodules were surface sterilized for 5 minutes in 5.25% sodium hypochlorite, 5 minutes in 70% ethanol, and 5 minutes in sterile distilled water. The nodule was placed on a clean glass slide in a drop of sterile distilled water and was crushed with a pair of forceps. One loopful of the mixture was streaked out on a yeast mannitol petri plate with congo red indicator dye in the agar. The bacteria were

allowed to incubate for 5 days at 21° in the dark. Colony morphology and dye absorbing characteristics were recorded.

Once pure cultures had been obtained, growth curves were performed on each strain of bacteria. Ten ml aliquots of each medium were inoculated with an equal number of cells. All culture tubes were then placed on a shaker bath (140 RPM) in the dark at 27°. Growth was measured as a function of optical density at 600 nm and the results were plotted. Two media were used for growth curve studies yeast mannitol medium and defined medium.

Once strains of bacteria had been characterized by the aforementioned techniques, pure cultures were maintained on slants of yeast mannitol medium stored at 4°. These stock cultures were transferred on the average of once every two months to fresh medium.

II. Interaction of Lectin with Rhizobium Cultures

A. Agglutination experiments. Four day cultures of strains 116A14 and 116A15 grown on liquid defined medium were washed and resuspended in sterile phosphate buffered saline pH = 7 (PBS). Approximately 10^{12} organisms per 0.5 ml was used in this assay. In large depression slides, 0.5 ml of the bacteria solution was added to 1 ml of a sainfoin lectin solution in PBS. Lectin concentrations tested were 298 µg/ml, 149 µg/ml, 29.8 µg/ml and 0 µg/ml. The bacteria and lectin mixtures were allowed to incubate for 5 hours at room

temperature. At 1 hour, 3 hours, and 5 hours, each was examined macro and microscopically for signs of agglutination.

B. F.I.T.C.-lectin binding assay. Fluorescein isothiocyanate was coupled to sainfoin lectin using the technique of Goldman [32]. The fluorescent lectin was dialyzed against PBS and had an $OD_{280} = 1.26$ and $OD_{490} = 0.34$ and a protein concentration of 0.8 mg/ml. The fluorescein to protein ratio was ~ 2 . This conjugate was stored at 4°C in PBS with no glucose present and no azide present.

Stationary growth phase bacteria of strains 116A12, 116A14, and 116A15 grown in yeast mannitol liquid medium and yeast mannitol agar grown organisms of the same age were tested for the ability to bind FITC lectin. Cat erythrocytes which had been trypsinized and cross-linked with glutaraldehyde [33] as well as sepharose-mannose beads were used as positive controls. All organisms and controls were washed twice and resuspended in 0.5 ml sterile distilled water. FITC lectin was added to the bacteria and controls to a final concentration of 200 $\mu\text{g/ml}$. The mixtures were incubated for one hour at room temperature. PBS was used for the autofluorescence controls. Bacteria and control beads and erythrocytes were then washed, pelleted, and diluted 10 fold in sterile distilled water before placing 20×10^{-6} ml of the stained mixtures on a glass slide. The organisms and controls were all coverslipped immediately and the edges sealed to prevent

evaporation. The bacteria and controls were viewed using a 495 nm interference excitation filter and K510 barrier filter in a Leitz fluorescence microscope with transmitted darkfield illumination.

C. ^{14}C - lectin binding study. Ten ml aliquots of yeast-mannitol medium in capped culture tubes were inoculated with 0.1 ml of 5 day cultures of bacterial strains 116A12 and 116A14. All culture tubes were incubated at 27° on a shaker bath (80 RPM) in the dark. Before examining the binding characteristics of the bacteria, the optical density was measured at 600 nm and flocculation of the cells noted if present. In a clinical centrifuge tube, the bacteria were then pelleted and washed with sterile distilled water. After a second washing, the bacteria were resuspended in only 1 ml of sterile distilled water. ^{14}C -labeled lectin was prepared by reaction with ^{14}C -acetic anhydride according to the method of Riordan and Vallee [38,39]. ^{14}C -lectin (specific activity 289,000 cpm/mg) was added to a final concentration of ~ 100 $\mu\text{g/ml}$ and the mixture was allowed to incubate for 2 hours at room temperature. After incubation, the bacteria were pelleted and 0.1 ml of the supernatant was removed and counted in a Beckmann LS100 scintillation counter. With each sampling, a PBS + ^{14}C -lectin negative control was also counted to allow for pipetting errors. The same procedure was repeated for organisms grown in defined medium. Counts per minute per 0.1 ml of supernatant were recorded

until the organisms reached stationary growth phase as monitored by OD_{600} nm. Binding was expressed as the percent of the total counts which disappeared from the supernatant.

III. Rhizobial Cell Surface Architecture Studies

A. Characterization of ferritin stain. A 100 fold dilution of the Ferritin-Concanavalin A stain (Cappel Laboratories, Inc.) displayed an $OD_{280} = 0.84$ and an $OD_{350} = 0.72$. Using cat erythrocytes, a serial hemagglutination titer of 1/512 was also determined for a 100 fold dilution of the stain. The ferritin conjugate in phosphate buffered saline pH 6.8 was determined to be pure by immunoelectrophoresis (Cappel Laboratories, Inc.). The conjugate was stored at 4° without preservative until used.

B. Staining with ferritin-Concanavalin A. Four samples of each of five different organisms were used. The organisms include: 1) cat erythrocyte positive controls, 2) strain 116A12 in stationary phase in liquid defined medium, 3) strain 116A12 in stationary phase in liquid yeast-mannitol medium, 4) strain 116A14 in stationary phase in liquid defined medium, and 5) strain 116A14 in stationary phase in liquid yeast-mannitol medium. The four samples of each organism were stained differently as follows: A) the labeling reaction was stained with undiluted Fer-Con A, B) the blocking reaction, sainfoin lectin was applied before staining with undiluted Fer-Con A, C) the nonspecific

binding control was stained with an inactivated stain consisting of a mixture of α -methyl mannoside and undiluted Fer-Con A, and D) the non-ferritin staining control, received no ferritin stain but only those stains necessary for visualization under the electron microscope. Staining protocol: cells were washed in sterile PBS pH 7.0 and suspended in 0.4 ml PBS. To sample B) 0.2 ml of 1.5 mg/ml sainfoin lectin in sterile PBS was added and incubated for one hour at room temperature. During this incubation a 1:1 mixture of Fer-Con A and 0.1 M α -methyl mannoside (α mm) in PBS was made and allowed to incubate for one hour at room temperature. All samples were then washed and resuspended in 0.4 ml PBS. One tenth ml of undiluted Fer-Con A was added to samples A and B. At the same time 0.2 ml of the Fer-Con A and α mm mixture was added to the sample C. Samples A, B, and C were allowed to incubate for two hours at room temperature. During this procedure, sample D was left unstained. After incubation all samples were washed three times with sterile PBS pH 7.0 and twice with sterile distilled water 0.5 ml per wash. Samples A, B, C, and D were pre-embedded in 0.5 ml of sterile noble agar in water (Difco). Fixation, dehydration, and infiltration then proceeded according to the schedule of Mullens which was slightly modified [34]. Fixation of preembedded cells was for two hours in 6% glutaraldehyde diluted with Kellenberger's buffer containing tryptone. All samples were washed for twelve hours with Kellenberger's buffer containing tryptone. Post

fixation was for 20 hours in 1% osmium tetroxide in Kellenberger's buffer. A brief 15 minute washing in Kellenberger's buffer preceded post staining for one hour in 1% uranyl acetate in Kellenberger's buffer. This was followed by a stepwise dehydration with the ethanol concentration increasing from 25% to 100%. The samples were transferred to 100% propylene oxide. On a shaker table (60 rpm), Spurr's epoxy was added gradually over a three hour period to a final concentration of nearly 100%. Finally, samples were placed in pure Spurr's and allowed to shake for 2 hours. All samples were then transferred to beam capsules filled with pure Spurr's. Embedded samples were polymerized for 8-16 hours at 70° [35]. Samples were thin sectioned on a Reichert OmU2 ultramicrotome with glass knives made with a LKB 7800 A Knifemaker. All sections were post-section stained for 3-5 minutes with Reynold's lead citrate stain [36]. Sections were viewed in a Zeiss EM9S2 electron microscope and photographed using Kodak electron microcope film no. 4489 at a magnification of 9,000x. Finally, strain 116A14 grown to stationary growth phase in defined medium, was pretreated with trypsin and glutaraldehyde [33] before staining with ferritin-Con A as per the schedule discussed above.

C. Quantification of ferritin labeling. Ten fields were photographed of each sample and printed using a Durst Laborator S-45 EM enlarger and a Kodak Ektamatic Processor to a total magnification of

32,400 x magnification. From these prints, ferritin particles associated with the membranes were counted and the total length of membrane calculated using a K+E map measure. Ferritin binding was expressed as the average number of particles bound per centimeter of membrane surface in the final print.

RESULTS AND DISCUSSION

I. Isolation and Characterization of Rhizobium Bacteria

A. Characterization. Sainfoin Rhizobium strains 116A8, 116A12, 116A14, 116A15, and 116A17, as well as a trefoil strain 95C13, were examined for classical cultural characteristics as indicated in Table 1. All strains proved to be gram negative rods using Gram's differential stain. The fat staining procedure revealed inclusions of β -hydroxy-butyrate in all strains. These fat body inclusions varied in size and staining density within each culture tested. The flagella staining procedure revealed peritrichous flagella. Many of the flagella had been broken during manipulation. Although every culture stained exhibited flagellated organisms, a few nonflagellated organisms were noted in some of the fields viewed with the light microscope. Congo red dye testing for culture purity of initial strains of Rhizobium revealed mixed cultures. Both congo red absorbing and non-absorbing organisms were found on yeast-mannitol plates containing this dye. Examination of initial Rhizobium cultures with shadowing and negative staining for the electron microscope revealed rods of the characteristic size and morphology. Electron microscope studies were performed only on stationary growth phase organisms and no flagellated organisms were observed. Strains of Rhizobium are classified according to their ability to nodulate legumes, therefore, as expected, none

Table 1. Initial Characterization of Rhizobium

	Strain #					
	116A8	116A12	116A14	116A15	116A17	95C13
gram negative	+	+	+	+	+	+
fat stain	+	+	+	+	+	+
flagella stain	+	+	+	++	+	NT
congo-red uptake	++	++	+	+	++	NT

+ = pos. for test

NT = not tested

of the techniques discussed above evidenced any strain specific staining or morphological characteristics.

The establishment of pure cultures of sainfoin Rhizobium must precede further experimentation for the sake of reproducibility and interpretation. Rhizobium bacteria are gram-negative rods with characteristic inclusions of β -hydroxy-butyrate. Kleczkowska et al. [6] state that as a Rhizobium bacterium grows older, the amount of β -hydroxy-butyrate found in the cytoplasm increases. The size variability of the fat inclusions seen in stained cultures could be a reflection of this age differential. Flagella are believed to be present only on young organisms [6]. They are also fragile. Manipulation or culture age could account for the broken or missing flagella seen in light and electron microscope examinations.

The most common contaminant of Rhizobium cultures is a very common soil bacterium Agrobacterium. Congo red dye uptake by Agrobacterium is one of the few means to distinguish it from Rhizobium. Since all initial cultures of sainfoin Rhizobium were believed to be contaminated with Agrobacterium, efforts were made to establish pure cultures.

B. Isolation of pure cultures of Rhizobium. To obtain pure cultures of sainfoin Rhizobium, it was convenient to utilize the natural specificity exhibited by both the bacterium and its host legume

through the 'trap host' technique. Surface sterilized sainfoin seeds germinated under sterile conditions using Thorton's nitrogen free medium appeared healthy before inoculation. After inoculation with single strain cultures of contaminated Rhizobium bacteria, nodulation occurred in three of five strains tested. Rhizobium bacteria were successfully reisolated in all three cases. Qualitatively, strain 116A12 produced fewer nodules than did strains 116A14 and 116A15. Congo red dye testing of all three reisolated strains produced milky white colonies indicating pure cultures of Rhizobium free of Agrobacterium contamination.

Strains 116A12 and 116A14 were selected for further study. Growth curves of strains 116A12 and 116A14 were plotted as optical density at 600 nm versus time in hours in Figures 1 and 2. Growth was recorded on yeast-mannitol medium and defined medium. In Figure 1, strain 116A12 grew to a greater optical density than did strain 116A14 in stationary growth phase on yeast-mannitol. No flocculation of organisms was observed on yeast-mannitol medium until the organisms were many days older than represented on the graph. Strains 116A12 and 116A14 cultured in defined medium grew more slowly than in the yeast-mannitol medium as shown in Figure 2. Strain 116A12 flocculated after 72 hours of growth. Strain 116A14 reached stationary growth phase after about 300 hours in defined medium.

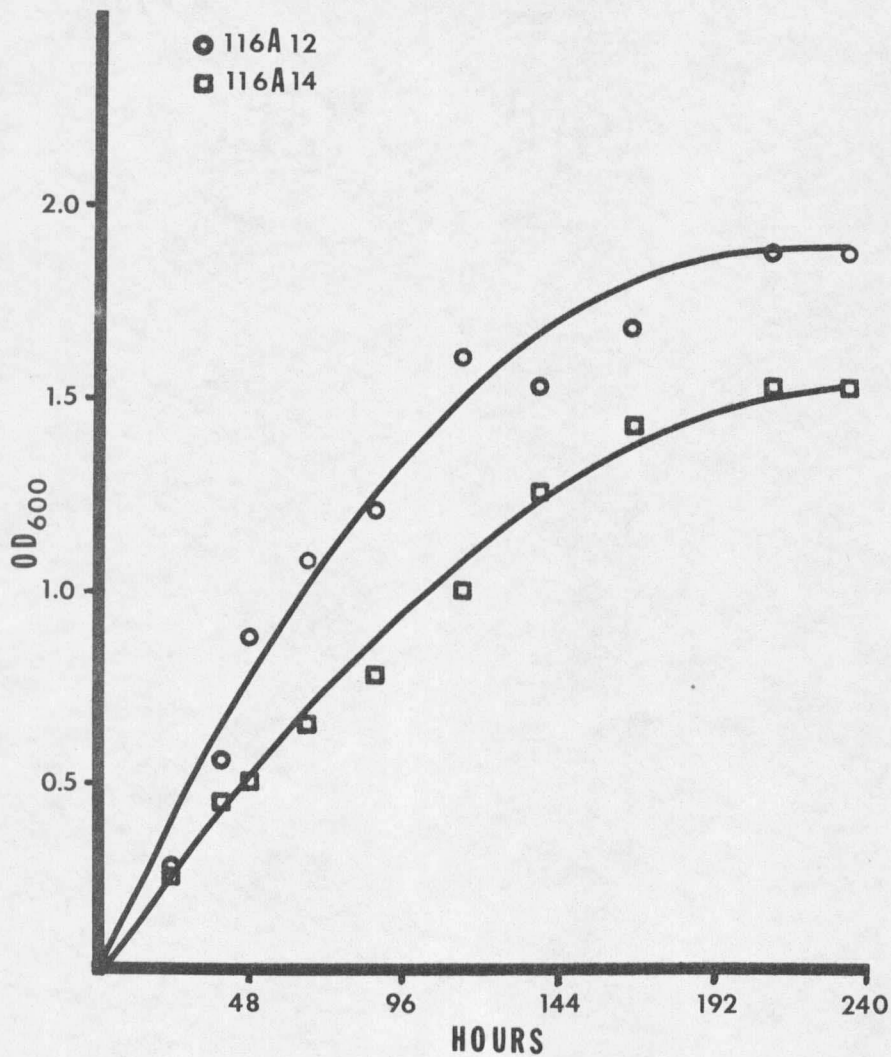


Figure 1. Growth Curves of Two Strains of Sainfoin *Rhizobium* Cultured on Yeast-Mannitol Medium at 27°.

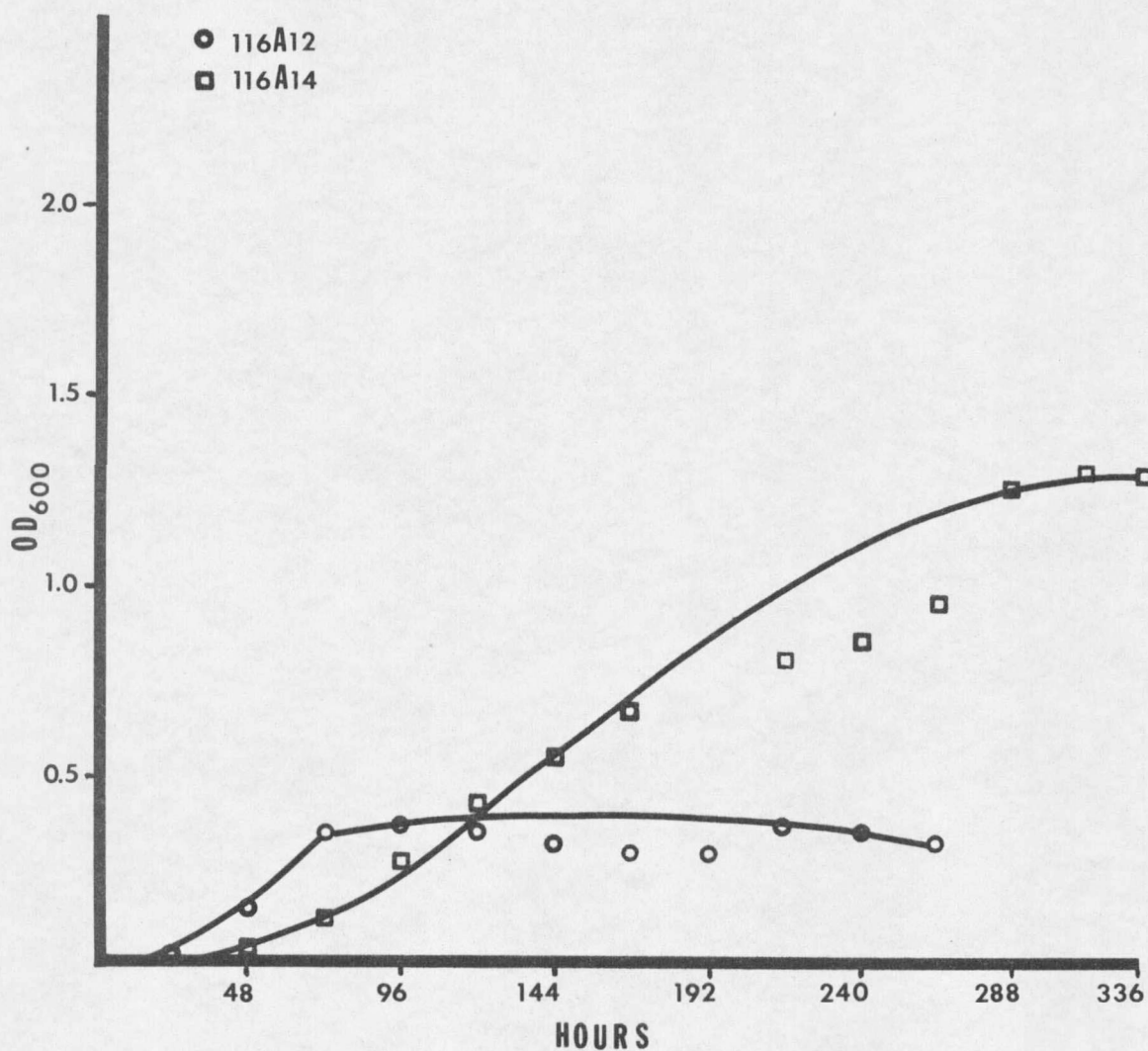


Figure 2. Growth Curves of Two Strains of Sainfoin *Rhizobium* Cultured on Defined Medium at 27° (compare to Figure 1).

The ability to form nodules in the host legume is the only diagnostic test for the identification of the genus Rhizobium [6]. Nodulation under controlled conditions or a 'trap host' technique utilizes the natural host specificity of the bacteria and the host legume. Bacteria reisolated from those nodules proved to be free of Agrobacterium contamination after testing on yeast-mannitol petri plates containing congo red dye. During the reisolation of pure cultures of Rhizobium bacteria, it was noted that strain 116A12 produced fewer root nodules per plant than did strains 116A14 and 116A15. Measurements of the nitrogen fixing capacities by acetylene reduction and quantitation of nodulation with these strains were performed in another laboratory [40]. These quantitative data verified that strain 116A12 was a poor nodulator-nitrogen fixer when compared with the nodulating and nitrogen fixing capabilities of strains 116A14 and 116A15 [40].

Growth of the reisolated pure strains on yeast-mannitol medium and defined medium produced almost classical growth kinetics in three out of four cases. The exception was strain 116A12. When cultured in defined medium, it flocculated after 72 hours of growth and made accurate measurement of even the relative phases of growth impossible. Flocculation of bacteria during manipulation also prevented dilution plate techniques for the actual quantitation of viable organisms during growth curves as well as other studies. This flocculation is believed to be caused by the sticky cell surface polysaccharides

recently termed the "glycocalyx" by Costerton [45]. Unlike many other genera of bacteria, Rhizobium bacteria do not shed this glycocalyx when cultured in the laboratory [46]. Although the actual number of viable organisms remained speculative it was possible to delineate the lag, log, and stationary growth phases as shown in Figures 1 and 2.

II. Interaction of Sainfoin Lectin with Rhizobium Cultures

A. Agglutination of Rhizobium with sainfoin lectin. Stationary growth phase organisms cultured in defined medium and resuspended in PBS were examined for agglutinability in the presence of unlabeled sainfoin lectin. Results are shown in Table 2. Neither increasing lectin concentration or increasing incubation time produced agglutination of the bacteria. Bacterial aggregation in the absence of lectin did, however, increase with time. This background aggregation was indistinguishable from any possible lectin induced agglutination.

Dazzo and Hubbell demonstrated a common antigenic determinant on the surface of white clover roots and their symbiont rhizobia [4]. Dazzo and Brill then isolated a specific agglutinating protein capable of agglutinating only symbiotic strains of Rhizobium from a saline extract of white clover root tissue [18]. This specific agglutinating protein was hypothesized to be a multivalent lectin. It was concluded that symbiotic bacteria would be agglutinated by lectins isolated from the host legume. Agglutination of symbiotic bacteria would result

Table 2. Agglutination of Sainfoin Rhizobium with Sainfoin Lectin

Lectin Conc.	Strain 116A14			Strain 116A15		
	1 hr	3 hr	5 hr	1 hr	3 hr	5 hr
0 µg/ml	*	*	*	*	*	*
28.9 µg/ml	--	--	--	--	--	--
149 µg/ml	--	--	--	--	--	--
298 µg/ml	--	--	--	--	--	--

* = background spontaneous aggregation (Results and Discussion).

- = no change above background.

from the cross linking of cell surface polysaccharides by a multivalent lectin isolated from the host legume.

Sainfoin Rhizobium bacteria were examined for agglutinability by incubation with varied concentrations of sainfoin lectin. However, spontaneous aggregation of the bacterial suspensions was observed even in the lectin free controls. This spontaneous aggregation of rhizobia was also observed by Dazzo and Hubbell [4]. The spontaneous aggregation background increased with incubation time and may have masked any lectin induced agglutination. Spontaneous aggregation could have been flocculation resulting from the intrinsic properties of the glycocalyx as described above, or it could have been due to cellulose microfibrillar extensions of the cell's polysaccharide coat as hypothesized by Dazzo and Hubbell [4].

B. F.I.T.C. - Lectin binding assay. Fluorescein labeled sainfoin lectin was used in a qualitative microscopic technique for the examination of Rhizobium-lectin interaction. Unstained control bacteria displayed no autofluorescence. Unstained sepharose-mannose bead controls and unstained cat erythrocyte controls, likewise displayed no autofluorescence when examined under the microscope. When stained with F.I.T.C.-lectin sepharose-mannose beads and cat erythrocytes displayed a strong fluorescence. F.I.T.C.-lectin stained purified strains of Rhizobium bacteria failed to display any visible fluorescence. Results are shown in Table 3. No difference in labeled-lectin

Table 3. F.I.T.C.-Lectin Binding Assay

Strain	Fluorescence
116A12	
Agar	--
Liquid	--
116A14	
Agar	--
Liquid	--
116A15	
Agar	--
Liquid	--
Cat RBC's	+
Seph-Mann	+

+ = fluorescence

-- = no fluorescence

binding capacity was observed between liquid and agar cultured bacteria. Both liquid and agar cultured organisms of each strain were examined for lectin binding in an attempt to explore possible cell surface polysaccharide differences resulting from culture conditions. A number of fluorescent globs were noticed. These globs did not appear to be flocs of organisms and were not directly associated with any bacteria. The fluorescent globs were randomly observed on stained slides of both liquid and agar cultured organisms. No fluorescent globs were observed in the labeled-lectin stained erythrocytes or sepharose-mannose bead positive controls.

F.I.T.C.-lectin was used for a qualitative microscopic examination of Rhizobium-lectin interactions as well as the examination of possible differences in cell binding due to culture conditions. Although no binding was observed in any of the bacterial preparations, positive controls displayed a high affinity for the F.I.T.C.-lectin stain. This lack of binding could have been the result of a number of variables within the sainfoin system. First, lectin receptors may not have been present on the surface of the bacteria. Second, if lectin receptors were present, they may have been inaccessible to the lectin. And finally, the binding constant of sainfoin lectin may have been too low to produce any visible staining.

Bohloul and Schmidt demonstrated a high correlation between the binding capacity of a F.I.T.C.-lectin to Rhizobium cells and the

ability of these cells to nodulate the host legume--soybean [20]. Dazzo and Hubbell using F.I.T.C.-labeled concanavalin A could not demonstrate preferential binding to Rhizobium bacteria capable of nodulating jackbean [17]. In the white clover system, however, Dazzo and Hubbell could demonstrate preferential binding [4]. Chen and Phillips using a quantitative fluorochrome-labeled lectin technique revealed no relationship between lectin-Rhizobium interactions and the ability to nodulate host legumes [41].

Fluorescent globs not directly associated with the bacterial cells as reported above have been observed by other investigators. Bhuvaneswari et al. noticed particles of F.I.T.C.-labeled material not attached to bacterial cells [3]. They hypothesized that these globs may be aggregates of receptor material which have become detached from the cells and precipitated by the fluorochrome-labeled lectin.

C. ^{14}C -labeled lectin binding assay. The possibility that Rhizobium-lectin binding could be transient in nature was examined using ^{14}C -labeled lectin. This technique offered increased sensitivity as well as a quantitative estimation of binding. Two strains of bacteria were examined for lectin binding activity as a function of culture age: strain 116A12 and strain 116A14. ^{14}C -lectin binding was examined using the above strains cultured on both yeast-mannitol and defined liquid media. The organisms were washed and resuspended in sterile distilled water prior to incubation with ^{14}C -lectin.

^{14}C -labeled lectin was not present in the growing cultures because of the resultant counting inaccuracy due to the quenching effects of the growth medium. Organisms cultured in yeast-mannitol medium displayed the greatest degree of binding in early log and late log growth phases, as is shown in Figure 3. Organisms cultured in defined medium displayed different binding characteristics as shown in Figure 4. Strain 116A12 cultured in defined medium bound 30% of the labeled lectin in the early log phase of growth and then lost all binding capacity. This loss of binding capacity in strain 116A12 corresponded with the flocculation of the strain when cultured in defined medium, as was shown in Figure 2. Sainfoin Rhizobium strain 116A14, when cultured in defined medium, produced less clear results. Binding of between 15% and 20% of the labeled lectin was generally observed throughout the maturation of the organism. However, during the early and late logarithmic phases of growth, sharp decreases in the binding capacity of strain 116A14 were noted. Sepharose-mannose bead controls were utilized to assure that changes in Rhizobium-lectin binding was not a function of changes in the labeled lectin molecule. Periodically during the course of the ^{14}C -lectin binding assay, a few sepharose-mannose beads were incubated with the labeled lectin. These control beads consistently bound 50% of the labeled lectin. If larger numbers of sepharose-mannose beads were incubated with the labeled lectin, 90% bind could easily be demonstrated. Counting accuracy was maintained

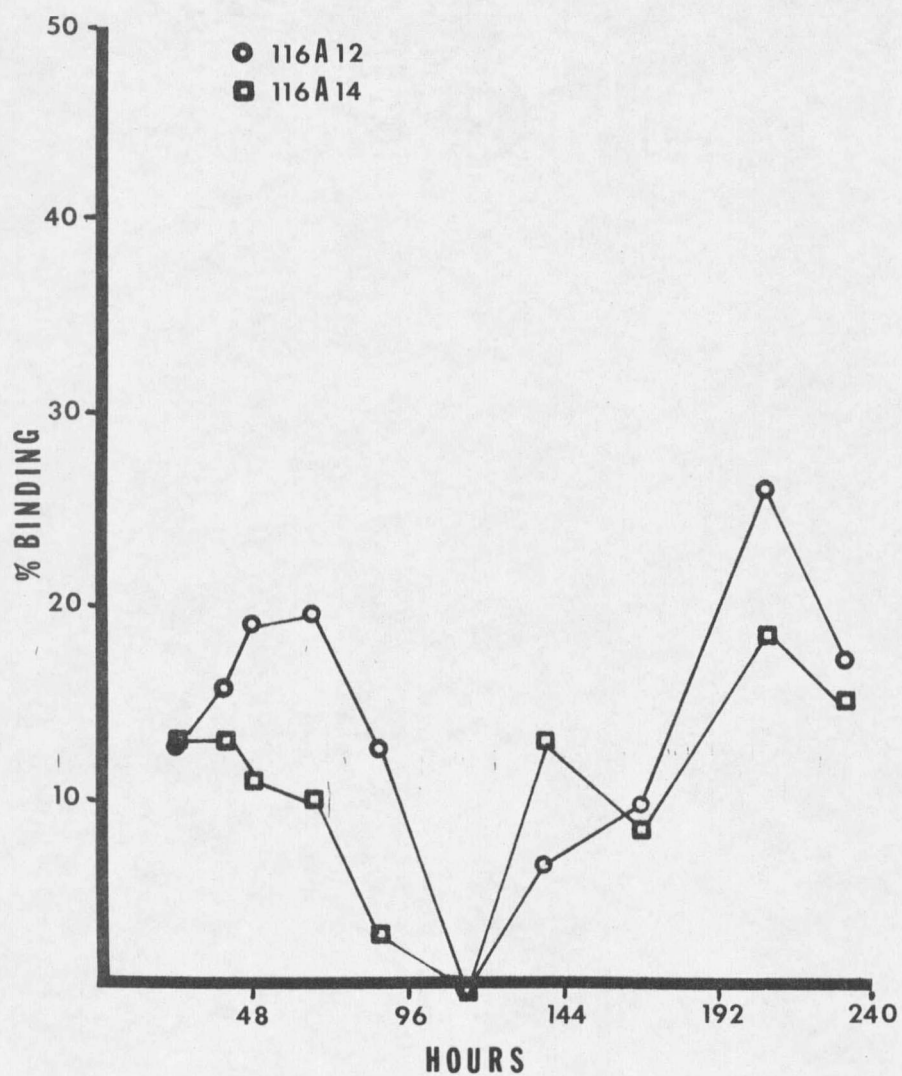


Figure 3. ^{14}C -labeled Lectin Binding Assay of Two Strains of Sainfoin Rhizobium Cultured on Yeast-Mannitol Medium.

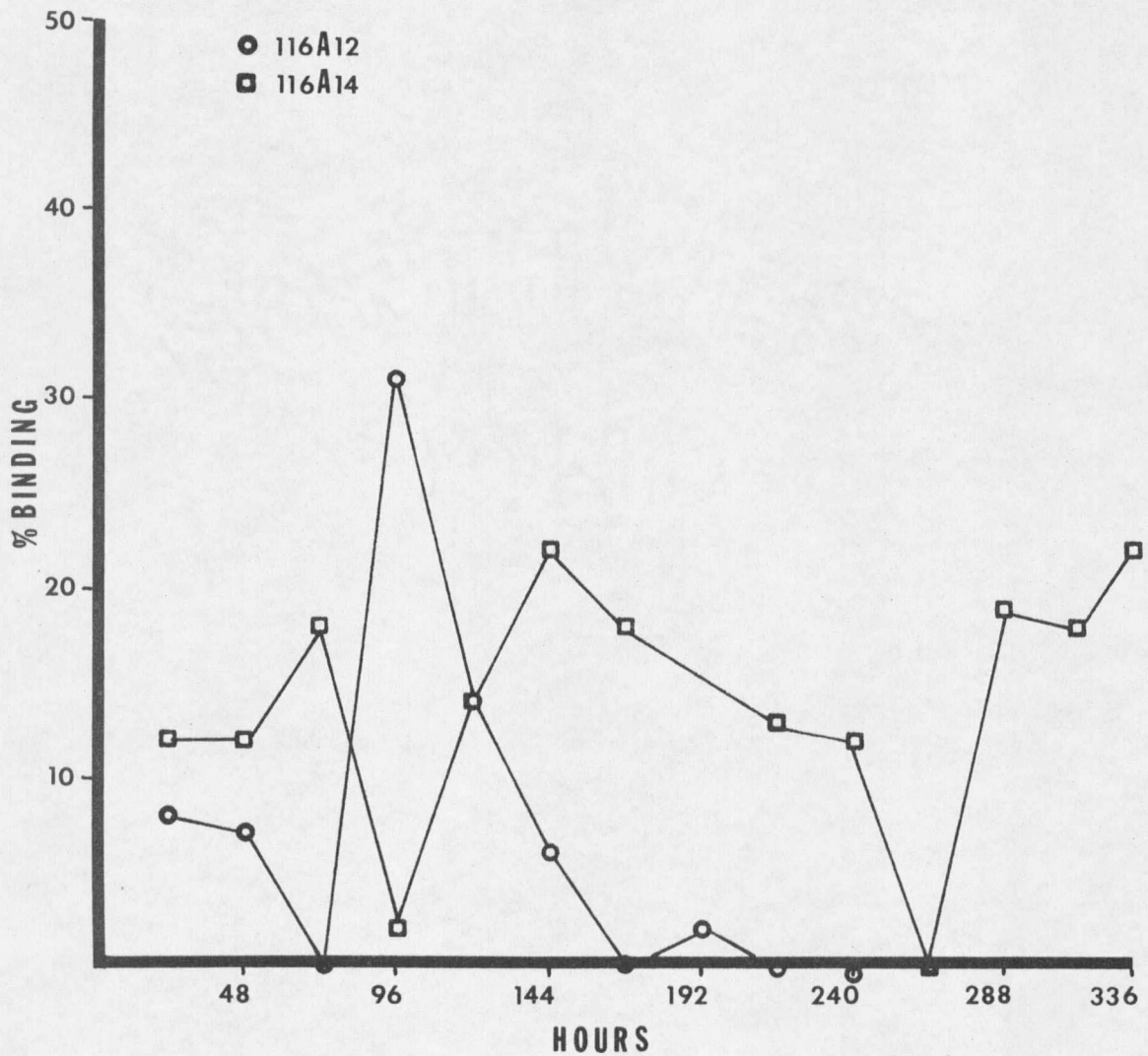


Figure 4. ^{14}C -labeled Lectin Binding Assay of Two Strains of *Sainfoin Rhizobium* Cultured on Defined Medium.

at 5%. If each bacterium had bound 10 molecules of labeled lectin, 100% binding would have been observed, based on the concentration of ^{14}C -lectin and a minimum of 10^6 organisms per sampling.

The modulation of Rhizobium-lectin interactions by nutritional factors and growth phase, as observed with the sainfoin system, was first hypothesized by Bohlool and Schmidt [21]. Bhuvaneswari et al. reported the greatest degree of Rhizobium-lectin binding to be at mid-log phase and reach zero binding by the stationary phase using a qualitative system employing a fluorochrome-labeled lectin. Sainfoin Rhizobium bacteria exhibit transient low levels of lectin binding. Nutritional factors as well as culture age elicit changes in binding capacity. These changes in binding capacity were considered to be reflections of corresponding changes in the cell surface polysaccharide coat or glycocalyx. Mimicry of the rhizosphere environment would be helpful in eliminating these media induced binding difference which remain unexplained in strict biochemical terms. Until rhizosphere conditions can be duplicated, the degree of importance of these Rhizobium-lectin interactions will remain complicated by too many variables.

III. Rhizobial Cell Surface Architecture Studies

A. Rationale behind the use of ferritin conjugated concanavalin

A. Initial attempts in the examination of the Rhizobium cell surface architecture by lectin binding were focused on a direct

ferritin-labeled lectin staining technique. Ferritin conjugated sainfoin lectin was to be used to examine the ultrastructure of bacterial cell surfaces. Preparation and purification of the conjugate was unsuccessful. The coupling of the proteins was attempted using the techniques of Shick and Singer [47] and the coupling procedure of Avrameas [48]. Initial purification involved the removal of the uncoupled ferritin on a sepharose-mannose affinity column and removal of uncoupled lectin by gel filtration on Ultrogel AcA 34. Because no ferritin conjugated sainfoin lectin was recovered using these techniques, the initial affinity chromatography step was eliminated and gel filtration column fractions were assayed for activity according to ability to agglutinate cat erythrocytes. These final attempts followed the techniques of Nicolson and Singer [43]. However, after numerous trials, no active ferritin conjugated sainfoin lectin was obtained. Because no active conjugate could be obtained, an alternative ferritin conjugated lectin was chosen: ferritin-concanavalin A. Concanavalin A and sainfoin lectin have overlapping monosaccharide specificities [24,38]. With the use of appropriate controls, it was felt that a demonstration of limited sainfoin lectin binding to Rhizobium bacteria cell surfaces could be achieved.

B. Staining with ferritin-concanavalin A. Ferritin-concanavalin A binding was observed with thin sections of the cat erythrocyte positive controls under the electron microscope, as shown

in Figure 5. Quantification of the ferritin bound per unit of erythrocyte membrane showed a significant difference between ferritin-Con A staining (sample A) and both the nonspecific staining control (sample C) and the nonferritin staining control (sample D), as shown in Figure 6. The figure is a diagrammatic representation of the ferritin-Con A staining procedure as well as parallel control reactions performed on cat erythrocytes and Rhizobium bacteria. Sample A refers to the labeling reaction, as shown in Figure 6, B refers to the blocking reaction and so on through the nonferritin staining control, sample D. The sainfoin lectin blocking reaction (sample B) did not decrease the number of ferritin-Con A particles bound per unit of erythrocyte membrane, as illustrated in Figure 7. Figure 7 is the quantitative estimation of particles of ferritin bound per unit length of cell membrane for all samples of the cat erythrocyte position control binding study. Examination of sample A, the undiluted ferritin-Con A labeling reaction and sample D, the nonferritin staining control, of non pretreated Rhizobium strains 116A12 and 116A14 cultured on both yeast-mannitol medium and defined medium displayed few bound particles of ferritin-Con A, as can clearly be seen by comparing Figures 8 and 5. Pretreatment of strain 116A14, cultured in defined medium at stationary phase, with trypsin and glutaraldehyde [33] before ferritin-Con A staining did not affect binding. Pretreatment of cells, as described above,

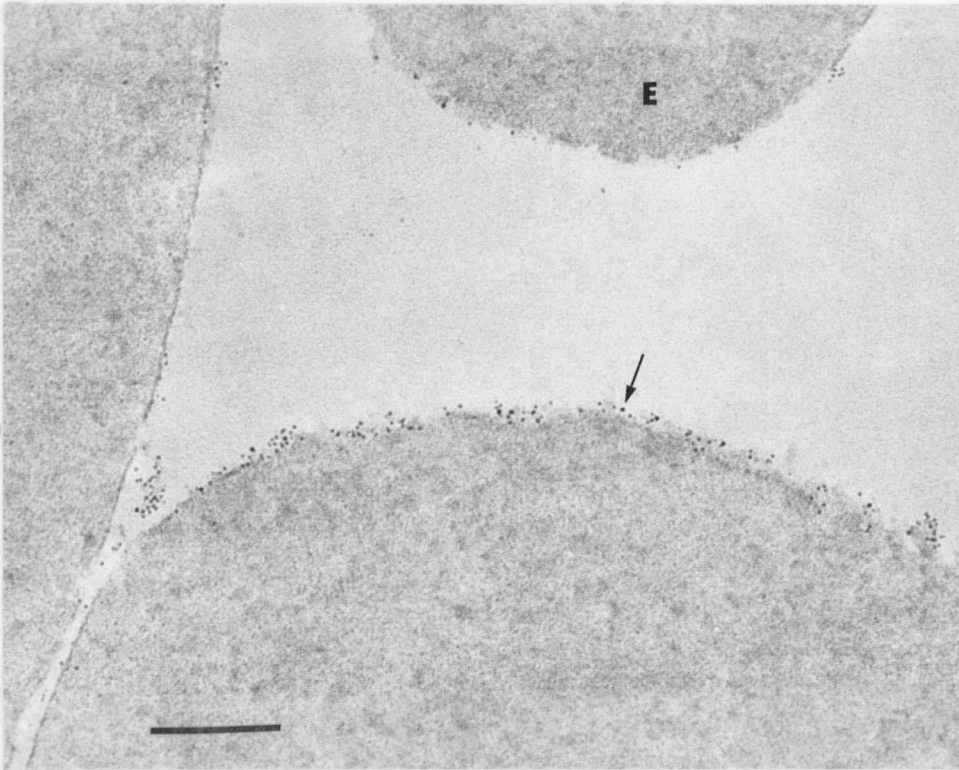
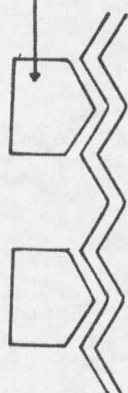


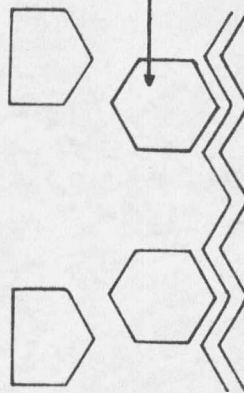
Figure 5. Ferritin-concanavalin A bound to the Cell Membranes of Portions of Three Cat Erythrocytes that were Thin Sectioned. The Erythrocytes served as Positive Controls (Sample A in Figure 6). A Bound Ferritin Particle is indicated by the Arrow. The Cytoplasm of an Erythrocyte (E) is also shown. Magnification is 69,300x. Scale Bar represents 0.25 microns.

Fer-ConA

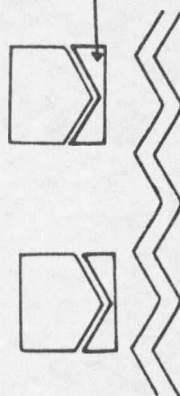


A. LABELING RXN

Sainfoin Lectin



B. BLOCKING RXN

 α -methyl-mannosideC. NONSPECIFIC BINDING
CONTROL

Cell membrane

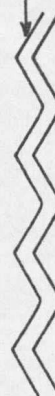
D. NONFERRITIN
STAINING CONTROL

Figure 6. Diagrammatic Representation of Ferritin-Concanavalin A Staining and Parallel Control Reactions.

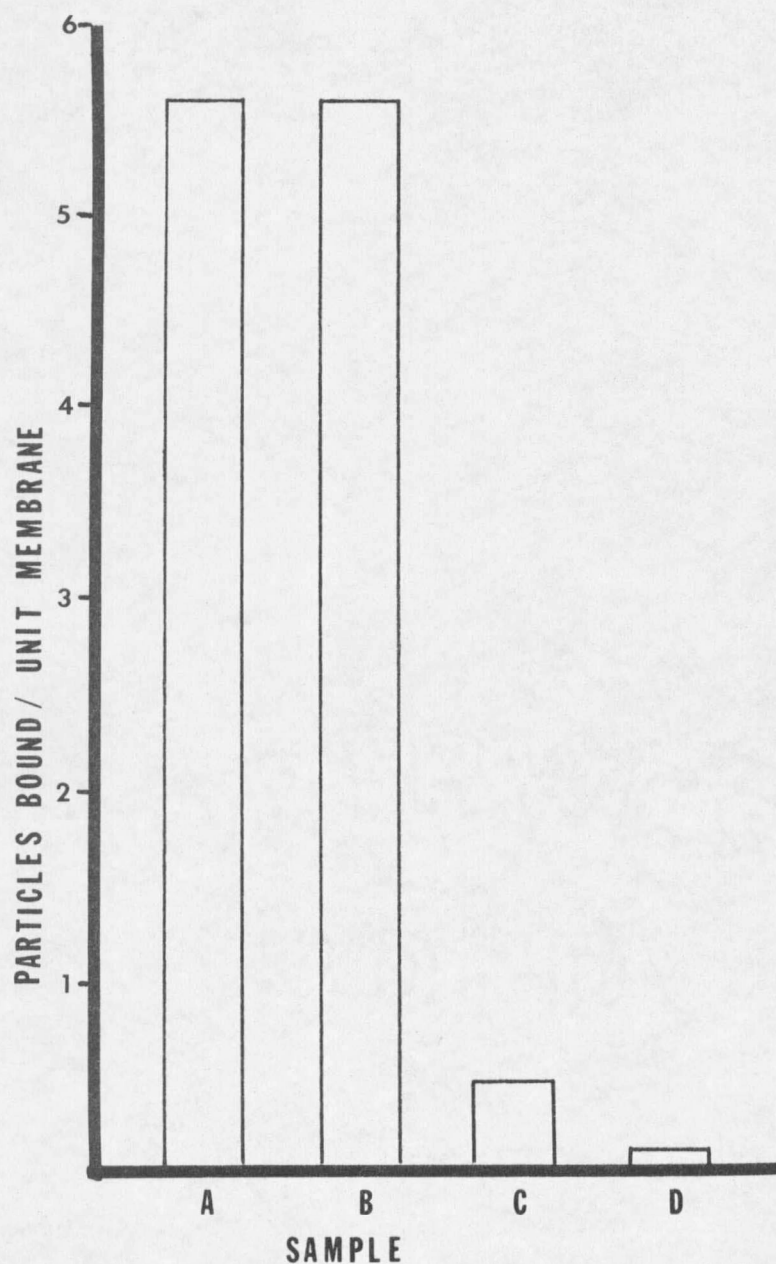


Figure 7. Quantification of Ferritin-Concanavalin A Staining of Cat Erythrocytes Positive Controls (refer to Figure 6).
Sample Designation: A Labeling rxn, B Blocking rxn, C Nonspecific Binding Control, D Nonferritin Staining Control.

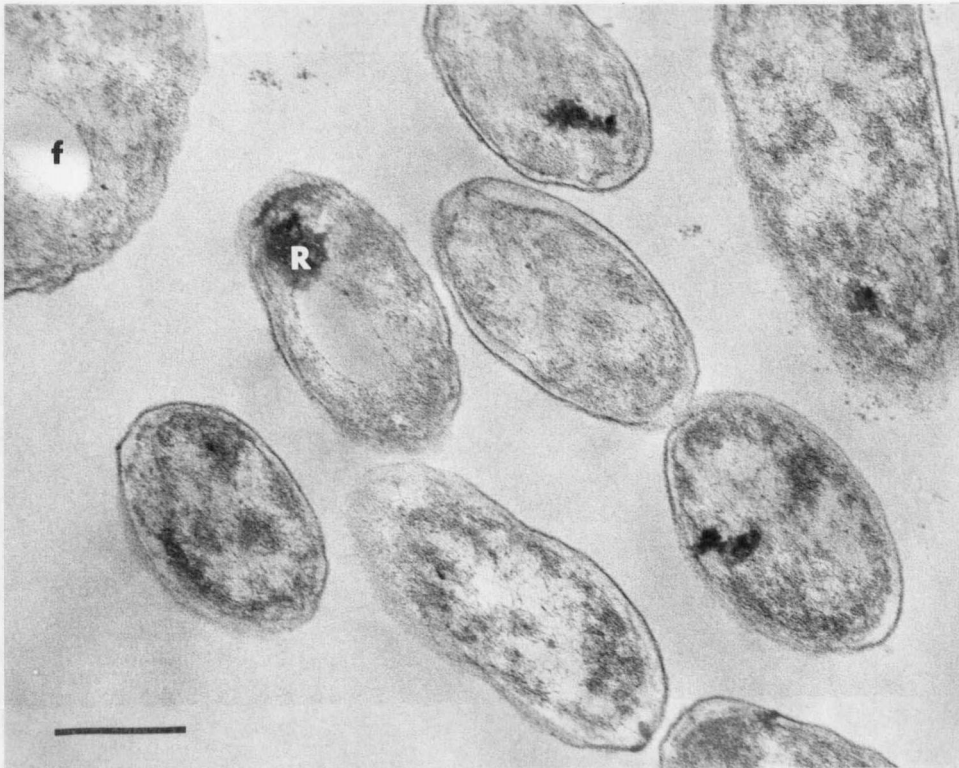


Figure 8. Cross-sectional Views of Bacterial Cells of Sainfoin Rhizobium (R), Strain 116A12, which have been grown to Stationary Phase on Defined Medium before Staining with Ferritin-Concanavalin A (Sample A). Very few Bound Ferritin Particles are Visible on the Bacterial Cell Walls. A Fat Inclusion (f) occurs inside one of the Bacteria. Magnification is 69,300x. Scale Bar represents 0.25 Microns.

did strengthen the cell surfaces and they displayed a more uniform cell surface, however, no ferritin-Con A bound these cell surfaces.

Ferritin-Con A has been previously demonstrated to bind erythrocytes by Marikovsky et al. [42]. Parallel controls using hapten inactivated stain (sample C) and ferritin free preparations (sample D) made quantification of nonspecific staining straightforward, as illustrated in Figure 6. A ten fold difference in bound ferritin-Con A between samples A and C in erythrocyte positive controls was considered significant (refer to Figure 7). The sainfoin lectin blocking reaction (sample B) produced no difference in the average number of ferritin-Con A particles bound to the erythrocyte cell surface. The specificity of ferritin conjugated lectins is the specificity of the lectin itself [43]. Concanavalin A and sainfoin lectin have overlapping monosaccharide specificities [24]. Both lectins agglutinate cat erythrocytes and are mitogenic for lymphocytes [24,38]. The failure of the sainfoin lectin blocking reaction (sample B) to produce a significant reduction in the amount of ferritin-Con A bound to the erythrocyte cell surface could have been the result of a number of unknowns. First, sainfoin lectin may not have been an effective competitor for the saccharide receptor sites. Concanavalin A is tetravalent and sainfoin lectin is divalent with respect to saccharide hydrophobic binding sites as well as the specific saccharide binding sites. For equilibrium reasons alone, concanavalin A could have displaced sainfoin lectin on

erythrocyte membranes. Or second, the receptor sites for concanavalin A and sainfoin lectin may be distinctly different in spite of monosaccharide specificities [44]. Monosaccharide binding specificities have been determined through the use of simple sugar hapten inhibition studies for most lectin characterization studies [24]. This method in itself could present an oversimplification of the actual lectin-cell surface interaction. Current research points to the actual cell surface lectin receptors as being complex oligosaccharides [24]. A dichotomy was found between different lectins: some interact strongly with terminal sugar residues while others interact most strongly with oligosaccharide complexes. Concanavalin A is believed to fall into the latter group [24]. No oligosaccharide binding studies have been performed using sainfoin lectin.

The failure of ferritin-Con A to bind sainfoin Rhizobium bacteria could have been a reflection of the possible dichotomy in saccharide specificities discussed above. Host specificities as expressed by both the bacteria and host legume could account for the lack of bound ferritin-Con A. An electron microscopic examination of the transient nature of cell surface lectin binding sites, as seen earlier with ^{14}C -labeled lectin, was beyond the limits of this work. The lack of concanavalin A binding to sainfoin Rhizobium could have also been a reflection of the importance of lectin-Rhizobium interactions within the sainfoin system's method of host specificity determination. Once

again, however, lectin receptor sites may not have been present, may have been unavailable even after trypsinization and prefixation with glutaraldehyde, or the lectin may have a very low binding constant in this system. The possible dichotomy of specificities between sainfoin lectin and concanavalin A as well as the lack of demonstrable lectin-Rhizobium binding present a number of stumbling blocks in the illustration of the biological role of lectins as determinants of host specificity within the sainfoin system.

SUMMARY AND CONCLUSIONS

Original Rhizobium strains used in these studies were received from Nitragen and were tested for their ability to form root nodules in their appropriate host plant. Three pure strains of Rhizobium (116A12, 116A14, and 116A15) were isolated under controlled conditions using a 'trap host' technique from root nodules of sainfoin (Onobrychis viciifolia, Scop.). These bacterial strains displayed the appropriate staining characteristics when examined with gram's stain, a fat inclusion body stain, and a flagella stain under the light microscope. Characteristic morphology was also observed using a phosphotungstic acid negative stain and a shadow technique for electron microscopic examination. In culture, the bacteria failed to absorb congo red dye showing them to be free of the common contaminant Agrobacterium. When sainfoin Rhizobium strains were cultured on yeast-mannitol medium they displayed classical growth kinetics reaching stationary growth phase in about 250 hours. However, when cultured on defined medium strain 116A12 flocculated after 72 hours, whereas, strain 116A14 grew more slowly than on yeast-mannitol medium but did not flocculate and reached stationary growth phase in about 330 hours. The flocculation of strain 116A12 was believed to be a glycocalyx induced phenomenon which varied with culture conditions. Therefore, by use of a trap host technique and appropriate characterization methodology, it is possible to isolate and characterize pure strains of sainfoin rhizobia.

The interaction of sainfoin lectin and Rhizobium was explored to test the specific hypothesis that lectin-Rhizobium binding is a determinant of host specificity among legumes. Control preparations of cat erythrocytes were readily agglutinated when incubated with low concentrations of lectin. However, bacterial suspensions were not agglutinated when incubated with increasing concentrations of lectin up to a final concentration of about 300 micrograms per ml. Spontaneous aggregation of the bacteria suspension was observed in lectin free controls and this aggregation increased with incubation time and may have masked any lectin induced agglutination. F.I.T.C.-labeled sainfoin lectin bound cat erythrocytes and they displayed a strong fluorescence, whereas F.I.T.C.-lectin did not bind to Rhizobium cultured on liquid or solid media when examined with a fluorescence microscope. Possible nutritional as well as culture age effects on Rhizobium-lectin binding were examined using ¹⁴C-lectin. Transient low levels of binding were observed and binding capacity varied with growth medium. When cultured on yeast-mannitol medium, strains 116A12 and 116A14 displayed a maximum of 20% binding in the early and late log phases of growth. When cultured on defined medium strain 116A14 consistently bound between 15% and 20% of the lectin, whereas strain 116A12 bound 30% of the labeled lectin in early log phase and then lost all binding capacity. Therefore, it was possible to monitor

these low levels of Rhizobium-lectin binding through the use of both qualitative and semiquantitative techniques.

The cell surface architecture of lectin binding was examined via the transmission electron microscope using ferritin-labeled concanavalin A and appropriate parallel controls. Cat erythrocyte positive controls, when stained with ferritin-Con A, displayed a significant amount of bound ferritin. A ten-fold difference between bound ferritin-Con A and nonspecifically adherent ferritin-Con A was quantitatively estimated for the cat erythrocyte positive controls. A sainfoin lectin blocking reaction failed to reduce the amount of ferritin-Con A which bound erythrocyte membranes. The failure of the sainfoin lectin blocking reaction to reduce the amount of bound ferritin-Con A cannot be explained in strict biochemical terms but probably resulted from subtle differences in the nature of the complex oligosaccharide lectin receptor sites. Rhizobium strains 116A12 and 116A14 were grown to stationary phase on both yeast-mannitol medium and defined medium before staining with ferritin-Con A. However, Rhizobium cells when viewed under the electron microscope after ferritin-Con A staining displayed little bound ferritin-Con A. Pretreatment of Rhizobium strain 116A14, grown to stationary phase in defined medium, with trypsin and glutaraldehyde before ferritin-Con A staining did not effect lectin binding. Although no Rhizobium-lectin binding could be

demonstrated, suitable techniques were developed for the study of lectin receptor sites on cell surfaces.

The literature concerning Rhizobium-lectin binding as the determinant of host specificity in legumes is divergent from system to system. Although these data do not support the popular role of lectins in host specificity, neither do they disprove the basic concept that host-symbiont specificity may result from some type of recognition and attachment phenomenon. Sainfoin lectin does not appear to function as the determinant of host specificity within this system.

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