AN ABSTRACT OF THE THESIS OF

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Peter J. Bottomley

Although much has been learned about the comparative nodulating behavior of simple mixtures of rhizobial strains under non-soil situations, it is unclear how these findings relate to the factors influencing nodulation success by the complex mixtures of strains found within soil-borne rhizobial populations. Information on the structure and physiological behavior of soil populations is almost non-existent. To achieve a better understanding of the situation in soil, studies were carried out with the following objectives. (i) To delineate by serological analysis the population composition of nodule occupants of *Rhizobium leguminosarum* bv.*trifolii* recovered from a variety of annual and perennial clover (*Trifolium*) species planted into Abiqua soil. (ii) To further the development of an assay to evaluate the substrate responsiveness of specific indigenous

serotypes of *R. leguminosarum* bv. *trifolii* while they reside within the soil microbial community. Immunodiffusional analysis of isolates recovered from nodules of five annual (*T. subterraneum, T. incarnatum, T. vesiculosum, T. parviflorum, T. patens*) and three perennial (*T. pratense, T. repens, T. hybridum*) species of clover revealed that the serotypic composition of the natural population of *R. leguminosarum* bv. *trifolii* in Abiqua soil is almost completely known. With antisera to 14 antigenically distinct serotypes at our disposal, only 19 of 272 isolates recovered from these eight clover species were antigenically unknown. While the perennial species showed no pronounced preference for particular serotypes, a substantial proportion (37-75%) of nodule occupants from each of the annual clovers (with the exception of *T. vesiculosum*) reacted with antiserum AS6. These isolates could be subdivided by their serological reactions of non-identity with either antisera AS6, AS27, or both antisera AS21 and AS27. Using multi-locus allozyme electrophoresis (MLAE) to analyze population structure within serotypes, isolates representing serocluster AS6 were found to be rooted at a similarity of 0.82 and clustered with the other three serotypes (AG4, AS21, and AS27) only at a similarity of 0.37. In contrast to AS6, MLAE analysis revealed that "genotypic distances" between the 7 ETs representing AG4 could be large. The chapter on the nalidixic acid cell-elongation assay only represents the second report of its use on soil microbial populations.

Nalidixic acid was found to be the most suitable DNA gyrase inhibitor for rhizobial studies since norfloxacin and ciprofloxacin at extremely low concentrations (2.0 and 0.5 mg/l, respectively) reduced the proportion of elongating cells significantly. In contrast to other indigenous serotypes, the majority of members of serotype AR23 did not elongate in response to yeast extract (YE). Regardless of nutrient type, or concentration, the percentage of elongated cells of AR23 remained low (<16%) even after 24 h of incubation. While the cell elongation response of serotype AS6 occured more rapidly to YE than did AR23, a less vigorous response by AS6 was observed when other nutrient sources were used. The appearance of elongated cells was delayed and the final percentage of elongated cells was reduced.

Symbiotic and Saprophytic Characteristics of a Soil Population of *Rhizobium leguminosarum* bv. *trifolii*

by

Kathryn H.M. Yap

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Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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Symbiotic and Saprophytic Characteristics of a Soil Population of *Rhizobium leguminosarum* bv. *trifolii*

CHAPTER 1

Literature Review

Chapter 1

LITERATURE REVIEW

Biological nitrogen fixation is the process by which dinitrogen gas (N_2) is reduced to ammonia by prokaryotic microorganisms. Bacteria of the genera *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* are capable of forming symbiotic dinitrogen-fixing associations with leguminous plants. Since nitrogen is the mineral nutrient which limits crop productivity in the majority of soils, leguminous plants are able to circumvent this problem through their dinitrogen fixing ability. Consequently, leguminous crops can be produced independently of fertilizer N inputs, and non-dinitrogen fixing crops grown subsequently in the same soil may need less fertilizer N to achieve profitable yields (Postgate, 1982).

Inoculant and indigenous strains

During the first half of the twentieth century, failure of leguminous crops to achieve profitable yields was often due to the absence of symbiotically effective rhizobia in the soil (Miller and May, 1991). In many parts of the world, legume seeds are inoculated routinely before sowing with selected strains of the appropriate rhizobia in order to guarantee that an effective dinitrogen-fixing symbiosis will

be formed. When soils are known to contain a native rhizobial population capable of effectively nodulating the legume in question, attempts to enhance the rate of dinitrogen fixation and improve legume productivity by inoculating seed with highly effective strains of rhizobia invariably fail (Lynch and Sears, 1952; Ham et al., 1971; Bohlool and Schmidt, 1973). Representatives of the native rhizobial populations occupy the majority of root nodules and are an effective barrier against inoculant strains forming many nodules (Johnson et al., 1965; Ireland and Vincent, 1968; Holland, 1970; Weaver and Frederick, 1974). Ironically, the successful nodulating members of the indigenous population are often sub-optimally effective on the host legume (Bottomley, 1991; Cregan et al., 1991). There are several possible explanations for this complex phenomenon.

(i) Despite their superior N_2-fixing ability, inoculant strains may be inherently less competitive for nodulating sites than some members of the soil populations. *Bradyrhizobium japonicum* USDA serogroup 123 is a good example of an indigenous competitive subpopulation (Cregan et al., 1991). Many reports have shown the prevalence of strains of serogroup 123 in the nodules of field-grown soybean throughout the north-central states of the USA, regardless of the inoculant strains and technologies used in an attempt to displace them (Ham et al., 1971; Kapusta and Rouwenhorst, 1973 and Ellis et al., 1984). Moawad et al.

(1984) noted that even when other strains of *B. japonicum* were present in soil and soybean rhizospheres in numbers similar to serogroup 123, most nodules were still formed by strains of serogroup 123. The authors concluded that the prevalence of strains of serogroup 123 in nodules is not a function of numerical superiority but results from their inherent competitiveness for nodule formation.

(ii) In other situations the possibility exists that the most prevalent nodule-occupying subpopulations are not more competitive per se, but are simply more numerous in the soil due to being better adapted (saprophytically competent) than inoculant strains to the soil conditions prevailing both at planting time and during the absence of the host (Bushby, 1982; Bottomley, 1991). Over the years many studies (mostly non-soil) have shown a relationship between nodulation success and the relative numbers of strains either in the inoculum or in the rhizosphere (Amarger and Lobreau, 1982; Fuhrmann and Wollum, 1989).

(iii) Indigenous strains may have better access to nodulation sites while inoculant strains placed with the seed have limited access to the developing root system (Danso and Bowen, 1989; McDermott and Graham, 1989).

(iv) Reports about the performance of inoculant strains being inconsistent within and between locations may be due to the variable composition of indigenous populations at different sites and their subpopulation dynamics on a specific site (Chatel and Greenwood, 1973; Gibson et

al.,1976; Roughley et al., 1976; Brockwell et al.,1982; McLoughlin et al.,1984).

Threads woven throughout the above issues are the roles that the composition and physiological ecology of soil rhizobial populations play in relation to nodulation success by either inoculant strains or by subpopulations within the soil populations. If we are to develop an understanding of the factors which limit nodulation of soil-grown legumes by superior N_2-fixing rhizobia, we must elucidate the composition of soil rhizobial populations at different locations, identify and enumerate dominant nodule occupying types, and determine the mechanism behind their competitive ability.

Influence of host preference on delineating the composition of a soil population

The outcome of competitive nodulation among mixtures of different strains of a rhizobial species has long been known to be influenced by the particular legume species and even by the cultivar (Vincent and Waters, 1953; Jones and Handarson, 1979; Dowling and Broughton, 1986; Triplett, 1990; Bottomley, 1991). Red clover (*Trifolium pratense, L.*) was shown to nodulate predominantly with different serotypes from a soil population than had previously been recovered on the annual species, subclover (Valdivia et al., 1988). Compatible with this finding, Robinson (1969) and Masterson

and Sherwood (1974) showed that annual and perennial clovers planted in the same soil nodulated with different members of the soil rhizobial population. Weaver et al. (1989) showed that two annual species of clover (*T. vesiculosum* and *T. incarnatum*), when planted in the same soil, nodulated with different members of the same soil population. Recently, evidence for host preference has been obtained even at the genus level (*Vicia faba* and *Pisum sativum*). In this case, plasmid profile patterns formed the basis of subpopulation breakdown of *R. leguminosarum* bv.*viceae* (Hynes and O'Connell, 1990).

Obviously, the composition and diversity of a soil rhizobial population could be grossly underestimated if a host with a restricted nodulating habit was used as a trap host. Since the studies described above involved no more than two host species it is not clear if the host preference phenomenon occurred because one host selected a restricted subset of the population while the other was promiscuous, or if both hosts were restrictive and selected different subgroups from the populations. One objective of this thesis was focused upon this issue.

Saprophytic competence within soil rhizobial populations

Parker et al.,(1977) raised the issue of our ignorance of the sources of nutrients utilized by rhizobia in soil and their influence on saprophytic competence. Despite numerous

studies having revealed phenotypic diversity among rhizobial strains, little evidence has accumulated from studies about indigenous soil rhizobia for the existence of sufficient interstrain variation to account for nodulation success or failure (Bottomley, 1991). Immunofluorescence studies from our laboratory indicate that statistically significant differences exist between the densities of serotype subpopulations within the *R. leguminosarum* bv.*trifolii* population in Abiqua soil (Almendras and Bottomley, 1987; Valdivia et al., 1988 and Bottomley and Dughri, 1989). Intuitively, one can speculate that subpopulational density differences are showing that differences in "fitness" do exist for survival in the soil niche. By combining immunofluorescence with a substrate responsiveness assay data were generated about the physiological status of the serotype subpopulations in soil (Bottomley and Maggard, 1990). The rationale behind this method lies principally in the action of the DNA gyrase inhibitor, nalidixic acid. This antibiotic specifically inhibits DNA replication (Goss et al., 1964) while allowing transcription and translation to operate. Due to impairment in cell wall formation, these cells elongate thereby revealing their viability and also their ability to grow and respond to the added substrates. Bottomley and Maggard (1990) observed that the proportion of substrate-responsive cells as well as the rate of appearance of elongated cells differed between rhizobial serotypes indicating that "physiological readiness" varied among

subpopulations. It was of interest, therefore, to determine if these effects were reproducible in soil populations sampled two years later, and to extend these observations by evaluating the response of rhizobial subpopulations to other nutrient sources including seedling exudates. Ultimately, I wished to evaluate if a subpopulation which was a dominant nodule occupant on a specific host responded more rapidly to exudates from that host than did a subpopulation representing a minor nodule occupant. In addition, Bottomley and Maggard (1990) reported that nalidixic acid had occasionally been inadequate in controlling cell divisions within the soil bacterial population. As a result other DNA gyrase inhibitors, norfloxacin and ciprofloxacin were evaluated as possible substitutes for nalidixic acid.

OBJECTIVES

The research described in this thesis was conducted with the following objectives in mind: 1) to delineate by serological analysis the population composition of nodule occupants recovered from a variety of annual and perennial clover species planted into Abiqua soil. 2) To further the development of an assay to evaluate the substrate responsiveness of specific indigenous serotypes of *R. leguminosarum* bv. *trifolii* while they reside within the soil microbial community. As a result of the findings from objectives 1) and 2), I examined the relatedness within subpopulations of serotypes AS6, AG4, and AR23 of *R. leguminosarum* bv. *trifolii* using multi-locus allozyme electrophoretic analysis.

CHAPTER 2

Nodule Occupancy by Indigenous Serotypes on a Variety of Annual and Perennial Clover Species Planted into Abiqua Soil

INTRODUCTION

When leguminous plants are grown in agricultural systems, they are nodulated predominantly by bacteria from poorly-defined soil populations of the genera *Bradyrhizobium* or *Rhizobium*. While bacteria from these genera confer N_2-fixing ability on legumes, a great deal of variation exists among isolates in their effectiveness at fixing nitrogen (Bottomley, 1991). From an agricultural perspective it is probably significant that mediocre N_2-fixers can occupy the majority of nodules on field-grown plants, while superior fixers are often relegated to minor nodule-occupying roles (Leung, Wanjage, and Bottomley, unpublished observations). Despite many scientific man-hours having been consumed on this problem, there has been no technological success at reversing the situation described above (Johnson et al., 1965; Weaver and Frederick, 1974; Wadisiruk et al., 1989; Cregan et al., 1991).

Although we have learned much about the comparative nodulating behavior of simple mixtures of rhizobial strains under non-soil situations (Date and Brockwell, 1978; Amarger, 1984; Dowling and Broughton, 1987; and Triplett, 1990), it is unclear how these findings relate to the factors influencing nodulation success by different members of soil-borne rhizobial populations. Information on the

structure and physiological behavior of sub-populations in soil is almost non-existent (Bottomley, 1991).

As a step toward achieving a better understanding of the situation in soil, one of our aims has been to delineate the composition of a soil population of *Rhizobium leguminosarum bv. trifolii,* the microsymbiont of clover (*Trifolium*) species. Unfortunately, any attempt to carry out such a task is hindered by the phenomenon known as 'host preference'. This term describes the observation that only a fraction of the total diversity within a soil population of a particular rhizobial type is recovered by a specific host. Other hosts susceptible to nodulating with the same rhizobial type, but belonging to different genera (Hynes and O'Connell, 1990) or to different species (Robinson, 1969; Masterson and Sherwood, 1974; Valdivia et al., 1988; Weaver et al., 1989), may recover different types from the same soil population.

In this regard studies from our laboratory which were completed in 1988-89 showed that only four of 146 isolates of *Rhizobium leguminosarum bv. trifolii* obtained from soil-grown clover plants could not be serotyped with antisera at our disposal (Dashti and Bottomley, unpublished observations). These isolates were recovered from four different clover species and were placed into fourteen different serotypes. A similar number of serotypes has been observed previously in soil rhizobial populations (Holland, 1966; Gaur and Lowther, 1980; and Fuhrmann, 1989). In these

studies, however, a significant number of isolates were untypable indicating that the populations were even more serotypically complex.

To confirm whether or not the rhizobial population structure in Abiqua soil is reasonably well defined, our objective was to compare the population composition of nodule occupants recovered from a variety of annual and perennial clover species planted into this soil. Findings were made which lead to another line of investigation which involved examining the structure within certain serotypic subpopulations using multi-locus allozyme electrophoresis.

MATERIALS AND METHODS

Trifolium species. The choice of clover species used in this study was based upon both agricultural importance and on establishing continuity with a previous study from this laboratory (Dashti N., M.S. thesis,1989). Seeds of crimson clover (*T. incarnatum, L.,*.cv. *Tibbee,* lot# 70868), arrowleaf clover (*T. vesiculosum, Savi*, cv. *Yuchi,* lot# 70374), white clover (*T. repens L.* cv. Dutch white), red clover (*T. pratense L. cv.* Florie), and alsike clover (*T. hybridum L.*) were obtained from Seed Certification Laboratory of the Department of Crop and Soil Sciences, Oregon State University, Corvallis, Oregon. Seeds of subterranean clover (*T. subterraneum L.* cv. Nangeela) were obtained from G. Evers, Texas A&M University, Agricultural Experiment Station, Angleton, Texas. Seeds of *T. parviflorum,* Ehrh (PI 314397, USSR) and *T. patens,* Schreber (PI 284286, Spain) were obtained from Dr G. Lovell, US Department of Agriculture, Agricultural Research Service, Southern Regional Plant Introduction Station, Griffin, GA 30223-1797.

Soil. Surface samples (10 to 12 cm in depth) of a silty clay loam of the Abiqua series were used. The soil was under a permanent pasture in which subclover was the legume component. The soil is acidic, low in extractable phosphate,

of high cation exchange capacity and organic C, and has been described in detail elsewhere (Dughri and Bottomley, 1983; Almendras and Bottomley, 1987). Samples of soil were taken along a transect which ran diagonally across the field for approximately 100 m. Samples were mixed thoroughly and sieved (< 2 mm). To correct for known nutrient deficiencies, 1.92 mg of $Na_2MoO_4.2H_2O$ and 184.6 mg of KH_2PO_4 were added to 1 kg of soil to achieve final concentrations of 1mg Mo/kg and 55mg P/kg respectively. Since 57.5 ml of water were required to raise 1 kg of soil to a water potential of approximately -30 kPa, the molybdate and phosphate salts were dissolved in the water supplement and mixed thoroughly into the soil prior to potting and planting the seeds.

Set-Up of Pots. A layer (1.5 cm) of sterile gravel was placed in the bottom of each of the 15 cm diameter pots. A length (12 cm) of PVC pipe, sealed at the bottom and with holes drilled along the length was placed in each pot. Into each pot was placed 250 g of a sterile mixture of perlite and vermiculite (1:1 v/v) soaked with 900 ml of a plant nutrient solution (pH 6.5) containing in grams per liter: $CaSO_4.2H_2O$, 0.26; $CaCl_2.2H_2O$,0.006; K_2SO_4, 0.044; $MgSO_4.7H_2O$, 0.123; K_2HPO_4, 0.044; KH_2PO_4, 0.034; Fe-citrate, 0.005; 2.5 ml of a trace element solution(Evans, 1974). The vermiculite/perlite mixture was packed around the watering tube which was centered in the pot. Finally, soil (1 kg) supplemented with Mo and P was added to each pot.

Preparation of clover seeds and recovery of R. leguminosarum bv.trifolii from root nodules. The experiment, was carried out in two parts. Experiment(i) included sub-, crimson, arrowleaf, red, white and alsike clovers. Experiment (ii) included subclover again, *T. parviflorum*, and *T. patens*. Seeds of each of the *Trifolium* species were surfaced sterilized as follows. Approximately 100 seeds of each species were placed separately into sterile 125 ml Erlemeyer flasks, and 25 ml portions of 95% (v/v) ethanol were added and swirled for 30 seconds. The ethanol was decanted and 20 ml of 25% (v/v) Chlorox bleach was added and swirled for 10 minutes. The seeds were washed six or seven times with sterile distilled water with changes occurring at two minute intervals. To encourage the seed to imbibe water, the larger seeds were allowed to soak for 1 hour in the last water wash. Surface sterile seeds were germinated on water agar plates at room temperature in the dark for two days, and 10 seeds of each species were sown into three replicate pots for each species. After two weeks, seedlings were thinned to three per pot and placed in a growth chamber with daylength of 14 hours, air temperature of 22 to 27°C and illumination provided by 16 F48T12VHO daylight fluorescent lamps and six 25 W incandescent bulbs. After eleven weeks of growth, plants were harvested, root systems were washed in distilled water containing 0.1%(v/v) Tween 80, followed by four washes in sterile distilled water. Approximately sixty

nodules (20 nodules per replicate) were recovered from the root systems of plants from each species and surface sterilized. Nodules were placed in lengths of glass capillary tubing (5x1 cm) which were closed at both ends with pieces of nylon stocking. After surface sterilization by the same procedure described above for seeds, the nodules were crushed onto plates of yeast extract mannitol agar (YEM) supplemented with 50 mg/liter of cycloheximide (Vincent, 1970) and incubated at 27°C. After development of growth , cultures were streaked to purity on plates of YEM, single colonies were picked onto YEM agar slants, grown for two or three days, and stored at 4°C.

Serological Analysis (Gel-Immune-Diffusion). The gels were prepared using sterile 0.8% (wt/v) agarose (Sigma) in 0.85%(wt/v) saline containing 0.025%(wt/v) sodium azide as a preservative. The gel layer, approximately 5 mm in depth was contained in small plastic dishes (50 by 9 mm). A hexagonal array of wells (5 mm diameter) were cut in the agarose around a central well (4 mm diameter). Cells of each isolate of *R. leguminosarum* bv. *trifolii* were grown in a defined mannitol-glutamate medium containing in grams per liter: mannitol, 10; glutamic acid, 1; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; Na_2EDTA, 0.01; $FeCl_3.6H_2O$, 0.004; $CaCl_2.2H_2O$, 0.08; vitamin mixture, 0.5 ml; trace elements, 10 ml(Evans, 1974). Cells were harvested by centrifugation at 15,000rpm for 15 minutes, washed in 0.85% (wt/v) saline,

resuspended in 1 ml of saline, transferred to preweighed Eppendorf microfuge tubes and centrifuged for 5 minutes at 12,535 x g. The supernatant was decanted, the cell pellet weighed, and sufficient saline added to produce a final concentration of 60 mg of cell dry weight/ml. Portions of non-diluted antisera (35 *ul*) and cells (65 *ul*) were pipetted into center wells (4 mm diameter) and outer wells (5 mm diameter) respectively. The plates were incubated at room temperature in a sealed container under a humidified atmosphere. The development of precipitin lines was monitored over a two or three day period. Data were recorded both as sketch drawings and by photography using dark field indirect illumination. Technical pan film (TP135, Kodak) was used and exposures at the f setting between 5.6 and 8.0 and at a shutter speed of 1/2 second were found to produce satisfactory results.

Analysis by Multi-locus allozyme electrophoresis.

Preparation of cell lysates : Isolates representing the various serotypes were grown for 48 h on an orbital shaker at 30°C in 100-ml of the defined glutamate-mannitol medium described earlier. Cells were harvested by centrifugation at 10,000 rpm for 15 minutes. After supernatants were discarded, pellets were resuspended in 1.2 ml of ice-cold buffer (10 mM Tris-HCl, 5 mM Na_2EDTA, pH7.6), transferred to 1.5 ml Eppendorf microfuge tubes and 0.03 ml of 100 mM

dithiothreitol and 0.02 ml of freshly-prepared lysozyme (50 mg/ml) were added. The cell suspensions were incubated on ice for 1 hour with occasional mixing. Each sample was sonicated on ice twice for 10 second intervals with a Branson Sonifier 200 equipped with a stepdown microtip (power setting=6, 50% duty cycle). The sonicated suspensions were then centrifuged for 20 minutes at 12,535 x g and the supernatants were transferred to new microfuge tubes and stored at -60°.

Preparation of starch gels. To prepare a gel, a 17.1 g portion of starch (potato, Sigma #S-4501) was suspended in 150 ml of appropriate gel buffer (Table 2.1.) in a 1-liter Erlenmeyer flask. The suspension was heated over a Bunsen burner to just beyond the boiling point with constant vigorous swirling. The suspension was aspirated for about 1 min (until very large bubbles appear) and immediately poured into a Plexiglass gel mold 130 X 100 X 8 mm deep. After a gel has cooled at room temperature for 1 hour, it is wrapped in plastic film to prevent desiccation, stored at 4°C and used within 24 h of preparation.

Loading a starch gel. Cell lysates are removed from the freezer only for enough time to get a modest amount of supernatant to thaw sufficient to load the gel. Individual strips of Whatman no.# 3 mm filter paper (12 by 6 mm) are loaded with portions (15 ul) of each sample of thawed cell

lysate. These strips are then inserted at 5 mm intervals into a slit cut along the length (130 mm) of the gel about 2 cm from one end. Routinely, up to 14 lysates can be electrophoresced on a single gel. Pieces of filter paper dipped in amaranth dye (Sigma) are inserted at both ends of the gel to mark the electrophoretic front. After loading is completed, the lower end of the gel is then pushed back toward the paper strips to clamp them firmly in place. After 10 minutes of allowing the proteins to migrate into the gel under the specific voltage and current of the specific buffer system (see Table 1), the paper strips are removed and the gel covered with plastic film.

Electrophoresis. A horizontal electrophoresis apparatus (Biorad) was operated under constant voltage conditions with voltage and current values dependant upon the buffer system (see Table 2.1.). The gel was kept cool at 2°C by using a Brinkman constant temperature water circulator. The running times ranged from 1.5 to 2.5 h depending largely on the buffer system. Following electrophoresis, the gel is removed from the horizontal apparatus and set up so that four horizontal slices (1 to 2 mm thick) can be cut from each gel with a length of six pound test nylon fishing line tied between two S-hooks. Each slice is designated for a particular enzyme assay and placed in a plastic container 15 x 10 x 2 cm.

Enzyme assay conditions. The appropriate reaction mixtures are added to the appropriate containers and the gel slices are incubated in the dark at room temperature (27°C) until bands appear. Incubation times ranged from 10 to 30 minutes for the majority of the enzymes. Only nucleoside phosphorylase (NSP) required prolonged incubation and was left to stain overnight for bands to develop. The staining solution is then poured off, and the gel slice is rinsed with distilled water before fixing in a 1:5:5 (v/v) mixture of acetic acid, methanol and water. Data were recorded by photography using Ecktochrome 200 and exposures at an f setting of 8 and at a shutter speed of 1/125 second were found to produce satisfactory results. The specific enzyme reaction mixtures are given in Table 2.2. with quantities sufficient to assay a single gel slice.

Data interpretation. Comparisons are made of the mobilities of the allelic variants of specific enzymes from different isolates. Since there is a finite number of alleles per locus for each enzyme (1 to 6), it is relatively straight forward to compare unknowns with standard extracts which are run on the same gels. For each enzyme, distinctive electromorphs are numbered in order of decreasing anodal migration ie."1" moves fastest towards the anode. Each isolate is characterized by its own combination of allelic variants of the enzymes assayed. A distinct electromorph profile is referred to as an electrophoretic type or ET.

Analysing data. The Biosys' 1 program was used to determine the genetic similarity between ETs (Swofford, D.L., 1989, a computer program for analysis of allelic variation in population genetics and biochemical systematics. Release 1.7, Illinois National History Survey, Champaign, Il).

Determination of symbiotic effectiveness. Surface sterile seeds of *T. vesiculosum,* Savi, cv.Yuchi, were prepared as described above, germinated on water agar and transplanted to large (30 x 3 cm) cotton- stoppered test tubes containing five grams of vermiculite/perlite(1:1) soaked in 20 ml of the plant nutrient solution described earlier. Each of five replicate seedlings were inoculated with 1 ml (approximately $5X10^8$ cells) of selected isolates recovered originally from *T. vesiculosum*. The isolates were chosen to represent all of the serotypes found in nodules, namely, AG4, AS21, AS6/27, AS6/21/27, AS36, AS27, AR23, AS6 and unknowns. Five uninoculated seedlings were included to serve as zero nitrogen controls. Another five seedlings were included for supplementation with 3 ml of 18mM KNO_3 at 14 and 28 days after planting to serve as "maximum yield" controls. The plants were grown under greenhouse conditions for approximately 6 weeks. Shoots were removed, dried at 55°C for 5 days, and dry weights obtained. Means and standard deviations were calculated and the symbiotic

effectiveness categories of the isolates were ranked relative to nitrate-supplemented and uninoculated plants.

Table 2.1. Buffer systems for electrophoresis of bacterial enzymes.

Electrode Buffer	Gel Buffer	Voltage & Amps	Running time(h)	Enzymes
Tris-citrate (pH 8.0) (83.2g of Tris [T1378]. 33.09g of anhydrous citric acid, 1.0 liter deionized H_2O)	Tris-citrate (pH 8.0) electrode buffer diluted 1:29	130V 40-55mA/gel	2-2.5	IDH BGA G6PD MDH PGI PEP XDH NSP
Tris-citrate (pH 6.3) (27.0g of Tris,16.52g of anhydrous citric acid 1.0 liter H_2O, pH adjusted with NaOH)	Tris-citrate (pH 6.7) 0.97g of Tris, 0.58g anhydrous citric acid, 1.0 liter of H_2O; pH adjusted with NaOH	150V 30mA/gel	1.5-2.0	6PG HBD SOD
Borate (pH 8.2) (18.5g of boric acid, 2.4g of NaOH, 1.0 liter of H_2O)	Tris HCl(pH 8.5) (1.21g of Tris, 1.0 liter of H_2O pH adjusted with HCl)	250V 15-25mA/gel	approx.2.0	ADK ACP PGM

Enzyme Name	Abbreviation	Enzyme Name	Abbreviation
1. Isocitrate dehydrogenase	IDH	8. Nucleoside phosphorylase	NSP
2. ß-Galactosidase	BGA	9. 6-Phosphogluconate dehydrogenase	6PG
3. Glucose 6-phosphate dehydrogenase	G6PD	10. 3-Hydroxybutyrate dehydrogenase	HBD
4. Malate dehydrogenase	MDH	11. Superoxide dismutase	SOD
5. Phosphoglucose isomerase	PGI	12. Adenylate kinase	ADK
6. Peptidase	PEP	13. Acid phosphatase	ACP
7. Xanthine dehydrogenase	XDH	14. Phosphoglucomutase	PGM

Table 2.2. Staining solutions for the enzymes.

Enzyme	EC no.	Substrate and coupling enzymes (amt)	Assay buffer and salt supplements(amt)	Dye and catalyst (amt)
IDH	1.1.1.42	0.1M isocitric acid[a] (1ml) NADP[b] (1ml)	0.2M Tris HCl[c] (pH8.0) (50ml) 0.1M $MgCl_2$[d] (2ml)	MTT[e](M2128)(1ml) PMS[f](P9625)(0.5ml)
BGA	3.2.1.23	6-Bromo-2-naphthyl-B-D-galactopyranoside 10mg dissolved in 5ml methanol	0.2M Phosphate, 0.1M citrate[g] (8.5ml) Water (30ml)	Tetrazotized-O- dianisidine(30mg) dissolve in 30ml of water, than add to gel
G6PD	1.1.1.49	Glucose 6-phosphate (G7250)(50mg) NADP(1ml)	0.2M Tris HCl(pH8.0)(50ml) 0.1M $MgCl_2$ (1ml)	MTT(1ml) PMS(0.5ml)
MDH	1.1.1.37	2.0M malic acid[h] (3ml) NAD[i](2ml)	0.2M Tris HCl(pH8.0)(40ml)	MTT(1ml) PMS(0.5ml)
PGI[j]	5.3.1.9	Fructose 6-phosphate (10mg) Glucose 6-phosphate dehydrogenase(3U) NADP(0.6ml)	0.2M Tris HCl(pH8.0)(25ml) 0.1M $MgCl_2$(0.3ml)	MTT(1ml) PMS(0.5ml)
PEP[j]	3.4.x.x (undefined)	Peptide[k] Gly-Leu (G2002) (20mg) Peroxidase(P8125)(10mg) Snake Venom(V7000)(10mg) (Source of L amino acid oxidase)	0.2M Tris HCl(25ml) 0.25M $MnCl_2$(0.5ml)	O-Dianisidine dihydrochloride(10mg) add in powder form together with others.
XDH	1.2.3.2	Hypoxanthine(100mg) NAD(2ml)	0.2M Tris HCl(50ml)	MTT(1ml) PMS(0.5ml)
NSP[j]	2.4.2.1	Inosine(I4125)(20mg) Xanthine oxidase(33ul)	8mM Sodium phosphate[m] (pH7.0)(25ml)	MTT(1ml) PMS(0.5ml)
6PG	1.1.1.44	6-Phosphogluconic acid(p7627)(10mg) NADP(1ml)	0.2M Tris HCl(20ml) 0.1M $MgCl_2$(10ml)	MTT(1.0ml) PMS(0.5ml)

Table 2.2. (Continued)

HBD	1.1.1.30	DL-B-Hydroxybutyric acid(H6501)(50mg) NAD(2ml)	0.2M Tris HCl(20ml) 0.1M $MgCl_2$(10ml)	MTT(1.0ml) PMS(0.5ml)
SOD	1.15.1.1	Assayed most conveniently in the NSP system		
ADK[j]	2.7.4.3	Glucose(100mg) ADP(50mg) Hexokinase(17 u) Glucose 6-phosphate dehydrogenase(15U) NADP(0.1ml)	0.2M Tris HCl(25ml) 0.1M $MgCl_2$(1ml)	MTT(1.0ml) PMS(0.5ml)
ACP	3.1.3.2	αNaphthyl acid phosphate (50mg)	0.05M sodium acetate[n] (50ml)	Black K salt(20mg) swirl in H_2O ,then add to gel
PGM	2.7.5.1	Glucose 1,6-diphosphate(5mg) Glucose 6-phosphate dehydrogenase(50U) Glucose 1-phosphate(G1259)(5mg) NADP(0.1ml)	0.2M Tris HCl(5ml) 0.1M $MgCl_2$(5ml) Water(25ml)	MTT(1.0ml) PMS(0.5ml)

a. Isocitric acid solution(0.1M): 2.94g of DL isocitric acid in 100ml of H_2O.

b. NADP (0.01M): 1.0g disodium NADP (Boehringer 128-058) in 100ml of water.

c. 0.2M Tris HCl: 24.2g of Tris in 1 liter of water, adjust pH with HCl to pH8.0.

d. $MgCl_2$ (0.1M):.2.03g of $MgCl_2.6H_2O$ in 100ml of water.

e. MTT (30mM): 1.25g of dimethylthiozol tetrazolium in 100ml of water.

f. PMS (30mM): 1.0g of phenazine methosulfate in 100ml of water.

Table 2.2. (Continued)

g. 0.2M Phosphate 0.1M citrate buffer: Mix 10.2ml of 1.0M phosphoric acid, 10.2ml of 2.0M NaOH and 1.03g of citric acid monohydrate in 26.9ml of water (pH adjusted to 7.8 with saturated $NaHCO_3$ solution). To stain for BGA, incubate gel slices in the substrate solution at 37°C for 15 minutes, then stain with a solution containing 30mg of dye in 30ml of water.

h. 2.0M Malic acid: 26.8g of DL-malic acid and 16.0g of NaOH in 100ml of water. (Caution: exothermic reaction.)

i. NAD (0.02M): 1.0g of NAD-free acid (Boehringer 129-981) in 100ml of water.

j. Agarose overlay: 500mg of agarose in 25 ml of 0.2M Tris HCl buffer (pH8.0). The assays for PGI, PEP, NSP, and ADK require an agarose overlay.

k. Peptide: Gly-Leu G-2002

l. $MnCl_2$ (0.25M): 4.90 g of $MnCl_2.4H_2O$ in 100ml of water.

m. Sodium Phosphate (8mM, pH7.0): Mix equal parts of 27.6g of $NaH_2PO_4.H_2O$ in 1 liter of water and 53.6g of $Na_2HPO_4.7H_2O$ in 1 liter of water, then dilute mixture 1:25 with water.

n Sodium acetate (0.05M): 6.8g of sodium acetate.$3H_2O$ in 1 liter of water, adjust pH to 5.0 with HCl.

RESULTS

Analysis of nodule occupancy by Immunodiffusion.

Immunodiffusional analysis of isolates recovered from nodules of five annual (*T. subterraneum, T. incarnatum, T. vesiculosum, T. parviflorum, T. patens*) and three perennial (*T. pratense, T. repens*, and *T. hybridum*) species of clover reveals that the serotypic composition of the natural population of *R. leguminosarum* bv. *trifolii* in Abiqua soil is almost completely known (Table 2.3.). With antisera to 14 antigenically distinct serotypes at our disposal, only 19 of 272 isolates recovered from these eight clover species were antigenically unknown. Several aspects of these data are worthy of comment. The composition of nodule occupancy on the majority of the annual clover species (*T. subterraneum, T. incarnatum, T. parviflorum* and *T patens*) ranged between three and five serotypes. In contrast, nodule occupancy on the perennial clover species (*T. pratense, T. repens* and *T. hybridum*) was serotypically more diverse with eight to nine serotypes being represented. Arrowleaf clover (*T. vesiculosum*) was the exceptional annual clover with seven serotypes being identified in the nodules.

In all cases of serotype-restricted annual hosts, serotype AS6 occupied high percentages (38-75%) of nodules. In contrast, serotype AS6 was present either as a minor or

an average nodule occupant on perennial clovers and arrowleaf clover. Serotype AG4 appeared consistently within 20 to 41.2% of nodules formed on those host species where AS6 was not dominant. Serotype AS/AR21 was the only subpopulation consistently recovered in nodules on all plant species tested. With the exception of AS6, AS/AR21, and AG4, the other eleven serotypes were minor, and often inconsistent nodule occupants on all host species examined.

Several observations were made which might explain why arrowleaf clover was an exception to the rule of serotype AS6 dominance on annuals. Firstly, in contrast to the other clovers, soil-grown plants of arrowleaf clover were somewhat chlorotic in appearance, and carried significantly more nodules than other clover species (370 per plant versus 100-230). The majority of isolates which were recovered from arrowleaf were completely ineffective on the latter regardless of their serotype affiliation (Table 2.4.). In addition, isolates of serotype AS6 recovered from subclover were completely ineffective on arrowleaf clover (data not shown). From these observations I conclude that the low occurence of isolates from serotype AS6 on arrowleaf clover is due either to their absence from nodules because of complete ineffectiveness, or to the fact that the nodules they had formed were so small to have been overlooked during sampling and sterilization.

Although a large percentage of isolates reacted with antiserum AS6, they were neither antigenically identical

with the parent antiserum strain nor with each other (Figure 2.1.). Furthermore, they were further subdivided by their serological reactions of non-identity with antiserum AS27 or with both antisera AS27 and AS21 (Figures 2.2.,and 2.3.,respectively). Table 2.5. shows the distribution of these serotypes in nodules of the different clover species. Isolates reacting only with AS6 were the dominant representatives of serocluster AS6 both on *T. subterraneum* and *T. incarnatum* from experiment 1. In contrast, serotype AS6-27 contributed significantly to the occupancy by serocluster AS6 on each of the annual species used in experiment 2. Given the serological heterogeneity observed among isolates of serocluster AS6, and the sharing of antigens by some members of AS6 with different serotypes, questions arose about the relatedness among representatives of AS6 recovered from either the same or different hosts. In addition, I was concerned about which serotype (AS6, AS21, or AS27) were these antigenically complex organisms most closely related?

Multi-locus allozyme electrophoretic typing (MLAE) of isolates representing serocluster AS6 and serotype AG4.

Different electrophoretic types (ETs) represented the isolates that we chose to analyze from serocluster AS6 (Table 2.6). Three ETs (4,14 and 61), representing isolates from serotypes AS6, AS6-27, and AS6-21-27 differed only at

one out of 14 loci and are closely related enough to be considered subclones of the same clonal line. In contrast, many allelic differences were apparent between isolates of AS6-21-27 and ETs representing serotypes AS21 and AS27. For example, ET13, which represented serotype AS21 isolates recovered from *T. repens*, *T incarnatum*, and *T. vesiculosum*, differed at 7 of 14 loci from AS6-21-27. ETs representing serotype 27 had an even greater number of differences (9 to 10) from AS6-21-27. The allozyme electrophoretic analysis of isolates from serotype AS6 showed that the majority of isolates were of ET 2 and dominated root nodules of both subclover and crimson clover. Furthermore, this analysis confirmed other data from our laboratory that serotype AS6 isolates represented by ET2 have been consistently the dominant root-nodule representatives of serocluster AS6 recovered from subclover plants sampled from laboratory and field experiments over a period of five years (Leung and Bottomley, unpublished observations).

Since members of serotype AG4 made substantial contributions to the nodule occupants on each of the perennial species and arrowleaf clover, a MLAE analysis of representatives from this serotype was undertaken. Our ET results show that serotype AG4 is composed of isolates represented by seven ETs (Table 2.7.). In contrast to AS6, however, "genotypic distance" between the 7 ETs representing AG4 was often large. The ETs were represented by three distinct clonal lines which clustered only at a similarity

level of 0.6. For example, ET19 and ET23 differed at 6 of 14 loci whereas ET 19 , 60, 21 and 22 differed at only 2 of 14 loci. Nonetheless, serotype AG4 isolates represented by the same ET were recovered from different hosts. For example, ET 19 was found on *T. pratense*, *T. repens* and *T. hybridum*. Furthermore, many of the serotype AG4 isolates recovered in a previous study from 1988 (N collection) were represented by the same ETs (18, 19, and 20) identified in this study (K collection). Indeed, two of the ETs (21 and 22) recovered in the earlier study are subclones of ETs 19 and 60 which were identified in this study.

Finally, the overall relationship between the ETs of serocluster AS6 and serotypes AS21, AS27, and AG4 are revealed in a dendrogram (Figure 2.4.). With the exception of ET33, isolates representing serocluster AS6 are rooted at a similarity of 0.82 and cluster with the other three serotypes (AG4, AS21, and AS27) only at a similarity of 0.37. An anomalous example of a closer relationship existing between an AS6 member and another serotype is revealed by ET33 (serotype AS6-27) and ET15 (serotype AS21) clustering at a similarity of 0.86. These two ETs were not common ETs of their respective serotypes as each was represented by only one isolate. Like serotype AG4, both serotypes AS21 and AS27 consist of isolates that are somewhat distantly related to each other. The similarity between ETs of these two serotypes vary from 0.78 to 0.4 in AS21, and 0.85 to 0.55 in AS27.

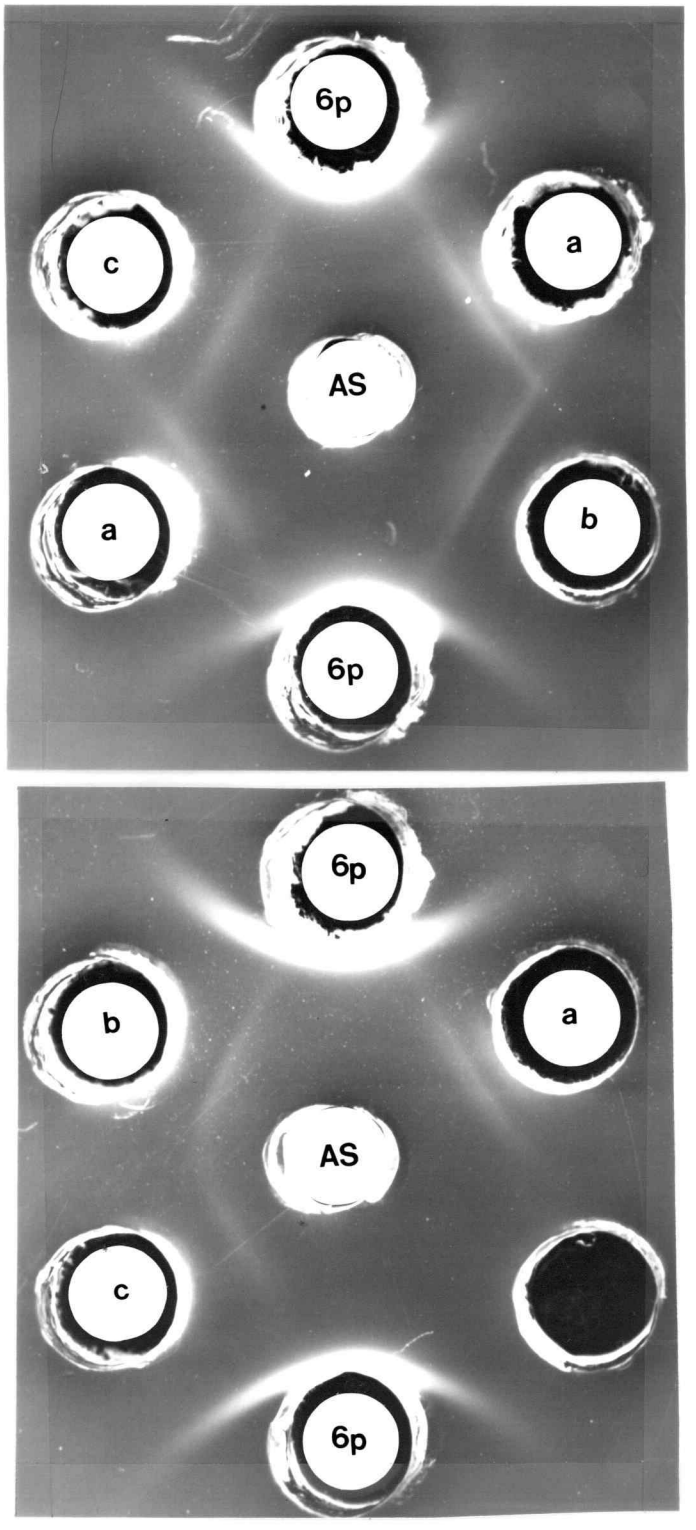

FIG. 2.1. Gel-immune-diffusion reactions of isolates representing serotypes AS6, (a); AS6-27, (b); and AS6-21-27, (c); when challenged against antiserum AS6. Antiserum (AS) is in the center well. Antigens are in the outer wells. Parent strain of AS6 is designated 6p.

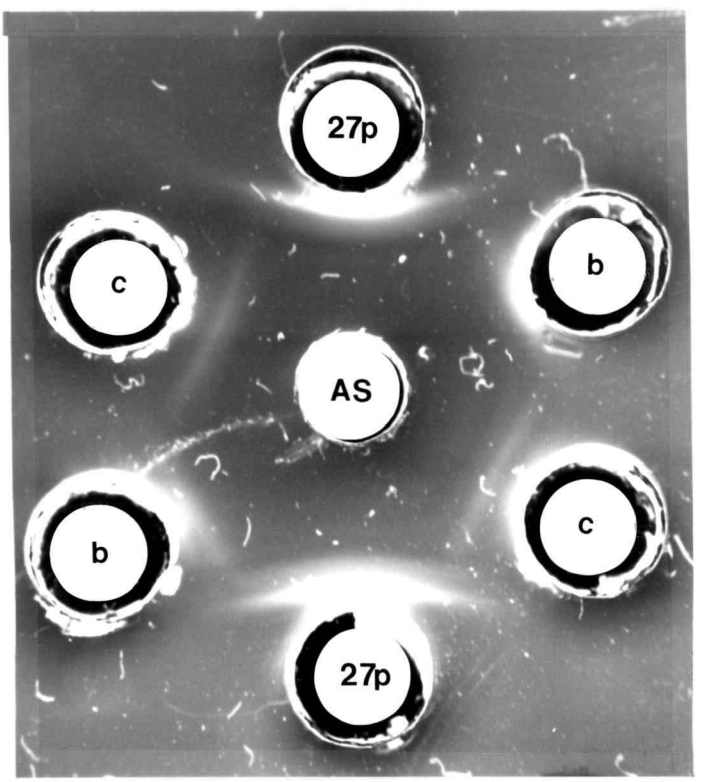

FIG. 2.2. Gel-immune-diffusion reactions of isolates representing serotypes AS27, AS6-27, (b); and AS6-21-27, (c); when challenged against antiserum AS27. Antiserum (AS) is in the center well. Antigens are in the outer wells. Parent strain of AS27 is designated 27p.

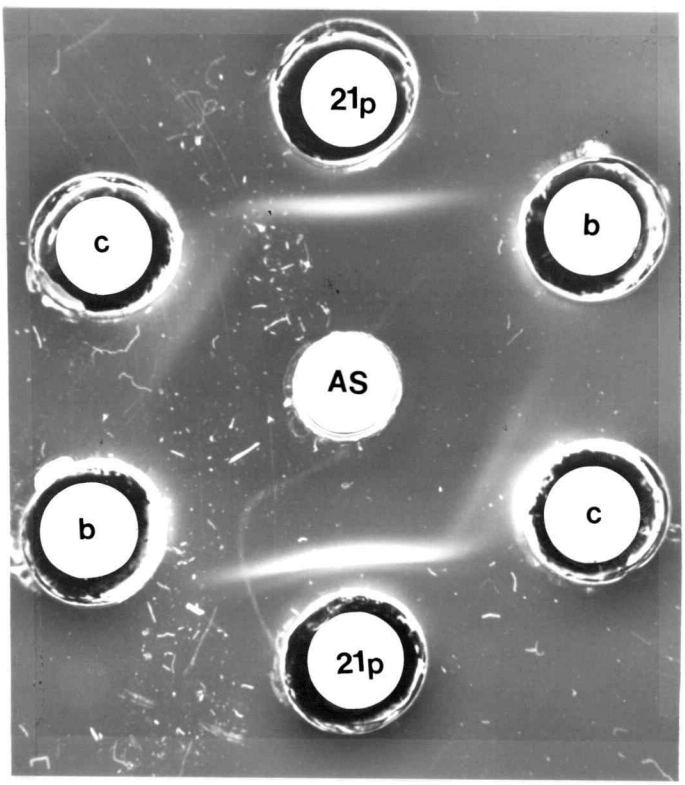

FIG. 2.3. Gel-immune-diffusion reactions of isolates representing serotypes AS21, AS6-27, (b); and AS6-21-27, (c); when challenged against antiserum AS21. Antiserum (AS) is in the center well. Antigens are in the outer wells . Parent strain of AS21 is designated 21p.

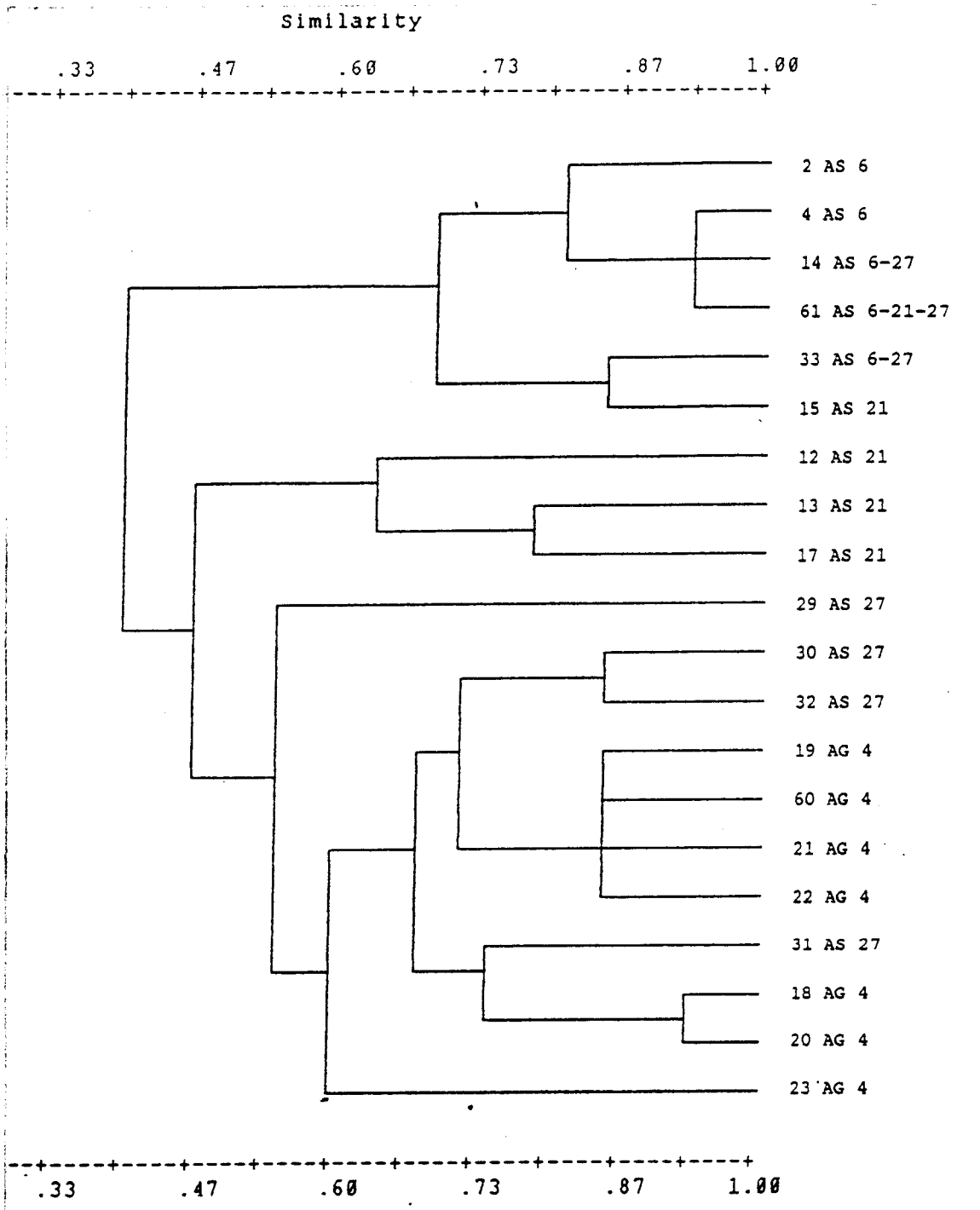

FIG. 2.4. Relatedness among subpopulations of *R. leguminosarum* bv. *trifolii* based on electrophoretically detectable allelic variation at 14 enzyme loci (Table 2.6. and 2.7.)

Table 2.3. Nodule occupancy by indigenous serotypes of Rhizobium leguminosarum bv. trifolii on six annual and three perennial species of Trifolium sown uninoculated into Abiqua soil.

Host Species	[b]Number of Isolates	[a]Serotype (% nodule occupancy)									
		[c]AS6	[d]AS/AR21	[e]AS27	AP17	AG4	AR23	AS16	AS36	[f]Others	[g]Uk
T. subterraneum(i)	28	66.7	3.7	0.0	14.8	0.0	0.0	0.0	0.0	3.7	11.1
T. subterraneum(ii)	32	75.0	15.5	0.0	9.4	0.0	0.0	0.0	0.0	0.0	0.0
T. incarnatum(i)	24	66.7	16.6	0.0	12.5	0.0	4.2	0.0	0.0	0.0	0.0
T. vesiculosum(i)	32	28.1	25.0	3.1	0.0	21.9	3.1	0.0	6.3	3.1	9.3
T. parviflorum(ii)	31	58.2	9.7	0.0	0.0	12.9	6.5	3.0	0.0	0.0	9.6
T. patens(ii)	31	37.9	51.8	0.0	0.0	0.0	0.0	3.5	0.0	0.0	6.9
T. pratense(i)	30	3.3	33.4	10.0	3.3	26.7	3.3	3.3	3.3	6.6	6.6
T. repens(i)	30	20.7	17.3	10.4	3.3	20.0	6.9	3.5	0.0	3.3	13.8
T. hybridum(i)	34	0.0	2.9	17.6	5.9	41.2	17.6	3.0	2.9	3.0	5.9

[a] Refers to the proportion of isolates which gave antigenic reactions against a specific antiserum.

[b] Refers to the total number of nodules analyzed from 3 replicates per host species. An average of 8-14 nodules were analyzed per replicate per host species.

[c] Serocluster AS6 consists of all isolates that formed antigenic reactions against antiserum AS6 (including those cross-reacting with antisera AS21 and AS27).

[d] Refers to the number of isolates that reacted against antisera AS21 and AR21 exclusive of those in serocluster AS6.

[e] Refers to the number of isolates that reacted against antiserum AS27 exclusive of those in serocluster AS6.

[f] Refers to serotypes AP11, AP1, AV5, AW1 & AR6.

[g] Refers to the isolates which did not cross react with any antisera.

Note (i) and (ii) refer to separate experiments carried out on different occasions.

Table 2.4. Symbiotic effectiveness of the isolates from different serotypes on Arrowleaf clover (Trifolium vesiculosum).

Serotype	No. of Isolates	Effectiveness ranking E1	E2	E3
AG4	1	0	1	0
AS6	2	2	0	0
AS6-27	3	3	0	0
AS6-21-27	2	1	1	0
AS21	3	3	0	0
AS27	1	0	1	0
AR23	1	0	0	1
AS36	2	0	2	0
Unknown	2	1	1	0

E1 = Isolates producing shoot dry weights not significantly different from uninoculated plants (4.6-8.4mg).

E2 = Isolates producing shoot dry weights significantly greater than uninoculated plants and less than plants supplemented with nitrate (22.7-45.3mg).

E3 = Isolates producing shoot dry weights not significantly different from plants supplemented with nitrate (47.9-67.34mg).

Table 2.5. Distribution among hosts of serotypes within serocluster AS6.

Host Species	[a]Serotypes (% occupancy) 6	6-27	6-21-27	Total
T. subterraneum(i)	48.2	18.5	0.0	66.7
T. subterraneum(ii)	25.0	25.0	25.0	75.0
T. incarnatum(i)	58.3	8.3	0.0	66.6
T. parviflorum(ii)	32.0	23.0	3.2	58.2
T. patens(ii)	10.3	27.5	0.0	37.9
T. vesiculosum(i)	3.1	15.6	9.4	28.1
T. pratense(i)	3.3	0.0	0.0	3.3
T. repens(i)	6.9	13.8	0.0	20.7

[a] Refers to the proportion of isolates that formed antigenic reactions against antisera AS6 alone, AS6 and AS27, or AS6, AS21 and AS27.

Note (i) and (ii) refer to separate experiments carried out on different occasions.

Table 2.6. Allelic profiles at 14 enzyme loci of isolates from serocluster AS6, and serotypes AS21 and AS27.

Serotype	[a]ET	No. of Isolates	Enzyme locus (allelic variants) [b]IDH	BGA	G6PD	MDH	PGI	PEP	XDH	NSP	PGI	HBD	ADK	ACP	PGM	SOD	[c]Δ
AS6-21-27	61	4	1	5	2	1	1	4	7	4	2	7	1	2	1	2	0
AS6	4	1	1	5	2	1	1	4	7	4	2	7	1	5	1	2	1
AS6-27	14	2	1	5	2	1	1	4	7	4	2	7	1	7	1	2	1
AS6	2	10	1	5	2	1	1	4	7	4	1	7	1	5	1	1	2
AS6-27	33	1	1	5	2	1	1	4	2	3	2	8	1	2	1	2	3
AS21	15	1	1	5	2	1	1	4	3	3	2	8	1	7	1	2	4
AS21	13	5	2	3	2	1	1	4	3	3	2	8	1	7	1	2	7
AS21	12	1	1	5	2	1	1	4	6	1	4	10	2	4	1	2	8
AS21	17	1	3	3	2	1	1	2	5	1	2	5	2	1	1	2	8
AS27	31	6	3	1	2	1	1	2	3	2	2	5	2	1	1	1	9
AS27	29	1	3	4	2	1	3	2	4	2	4	1	1	1	1	1	10
AS27	30	2	3	2	2	1	1	1	4	2	4	5	2	1	1	3	10
AS27	32	1	3	2	2	1	1	1	4	2	4	1	2	1	1	1	10

[a] ET: Electrophoretic type. Isolates of the same ET have an identical allelic profile.

[b] See Table 2.1. of Materials and Methods for the full name of the enzymes.

[c] Refers to the number of enzyme loci at which the alleles differ from that of AS6-21-27.

Table 2.7. Allelic profiles at 14 enzyme loci of the serotype AG4 isolates.

Collection (No. of isolates)			Enzyme locus (allelic variants).													
[a]K	[b]N	[c]ET	IDH	BGA	G6PD	MDH	PGI	PEP	XDH	NSP	6PG	HBD	ADK	ACP	PGM	SOD
2	8	18	3	2	2	1	1	1	3	2	2	6	2	1	1	2
3	2	19	3	2	2	1	3	1	3	2	4	5	1	1	1	2
1	1	20	3	2	2	1	1	1	3	2	2	6	2	1	1	1
3	0	60	3	2	2	1	1	1	3	2	2	5	1	1	1	2
0	1	21	3	2	2	1	1	1	3	1	4	5	1	1	1	2
0	2	22	3	2	2	1	1	1	4	2	4	5	1	1	1	2
0	1	23	3	1	2	1	1	5	3	1	2	6	1	1	1	2

[a] Refers to the isolates of serotype AG4 which were recovered from the studies described in this thesis.

[b] Refers to the isolates of serotype AG4 which were recovered in a previous study from this laboratory (Dashti, N., M.S. thesis, 1989).

[c]ET number : Same definition as previous page.

DISCUSSION

We believe that all of the indigenous serotypes of *R. leguminosarum* bv. *trifolii* in Abiqua soil that are major and minor nodule occupants of *Trifolium* spp. have been identified. In this study, which involved nine species of clover and soil sampled on two occasions in 1989 and 1990, only 14 of 178, and five of 94 isolates, respectively, could not be placed into the fourteen recognizable serotypes. Since several of the serotype acquisitions make only inconsistent and minor appearances in nodules (Table 2.3.), they are quite interesting from both the symbiotic and ecological perspective. They may represent subpopulations which are inherently non-competitive regardless of their soil population densities or the plant species they are exposed to. Alternatively, they may be either residential or transients in the soil but existing at densities too low to consistently compete for nodulation sites, or, they are residential and present at high densities but distributed only in rare "hot spots".

Transiency, irregular distribution, and differences in population size are poorly understood concepts of soil microbial ecology. A similar phenomenon has been observed previously in studies of *E. coli* in the human gut. While a large diversity of *E. coli* clones was recovered at any one sampling time from an individual human male, only a few

clones showed evidence of being permanent residents. Many clones behaved like transients since they could be recovered on no more than a few consecutive days before disappearing permanently (Sears, Brownlee, and Uchiyama,1950; Mason and Richardson,1981; Caugant et al.,1981;1984; Selander et al.,1987). Many of the rhizobial serotypes are definitely soil residents since their populations have been detected repeatedly by immunofluoresence (Dughri and Bottomley,1989; Bottomley and Maggard,1990). Nevertheless, since I have not evaluated all serotype populations by immunofluorescence, and I have established that distantly related clones share common antigens, the issue must be considered far from resolved.

The discovery that isolates representing serocluster AS6 were antigenically complex, and only distantly related to the original antiserum strain is worth discussing in regard to issues about serological identification of rhizobia per se, and to the concept of serotype-clonal relationships in bacteria in general. In the case of rhizobia, it has been known for a long time that isolates belonging to the same serotype can possess dissimilar biotypic characteristics (Vincent,1954 ,1962). In recent years this issue has been raised most often about serogroup USDA123 of *Bradyrhizobium japonicum*. The latter is widely distributed in soils throughout the USA (Johnson and Means, 1963), and occupies a high proportion of the root-nodules that form on field-grown soybeans in the mid-western states

(Damirgi et al.,1967). Members of this group are not only antigenically complex (Date and Decker,1965; Dudman,1971; Gibson et al.,1971; Bezdicek,1972; Schmidt et al.,1986), but they are variable in symbiotically related characteristics such as competitive ability(Bottomley, 1991) and in the host-restricted nodulation phenomenon(Cregan et al., 1991; Sadowsky et al.,1991).

Without evidence that members of a serotype or a serogroup are more closely related to each other than to other serotypes, and that they have similar and unique ecotypic and symbiotic characteristics, it is not surprising that skepticism has arisen about the interpretation of serotyping data (Schofield et al.,1987; Young,1989). These concerns have not only been restricted to rhizobial researchers. For many years, serotyping was the foundation underlying strain categorization of *E. coli* (Orskov and Orskov,1984) and *Salmonella* (Minor,1984). Our multi-locus allozyme electrophoretic findings are somewhat similar to those of Selander and colleagues who examined serotype-clonal relationships in *E. coli* and *Salmonella*. Isolates belonging to the same serotype can be, but do not necessarily have to be of the same clonal line (Ochman and Selander,1984; Caugant et al.,1985; Beltran et al,1988,1991). Obviously, if cross-reactions occur against both genetically similar and distant clonal lines from within the same population, then it is not surprising that discrepancies arise when serotypic population structure is

compared with that generated by other methods such as restriction fragment length polymorphism patterns (Schofield et al., 1987).

Our data should be discussed in regard to the phenomenon of host preference from soil rhizobial populations. Since serocluster AS6 shows superior nodulating ability on all the annuals except for arrowleaf, and appears to be composed of closely related sub-clones, we are now in a position to elucidate the mechanism underlying its nodulating success. In the case of the perennial species, there was no evidence for any overly competitive serotypes. Even the case for AG4 being an effective competitor on perennials was significantly weakened because of the genetic diversity among its members. One might argue that since perennials had never been grown in this soil, insufficient selective pressure had occurred for a competitive type to have been enriched. However, neither had the site grown any of the annual clovers, with the exception of subclover. The data tend to corroborate those of Harrison and co-workers who showed that a diverse rhizobial population was found in white clover nodules regardless of plant cultivar or plant growth condition (Harrison et al., 1987 and 1989b), or location (Harrison et al., 1989a). Researchers interested in the phenomena of host preference and competitive nodulation in clovers should be concerned about their choice of plant species before making data comparisons with others who might have used different hosts.

CHAPTER 3

Development of an Assay to Evaluate the Substrate Responsiveness of Specific Indigenous Serotypes of *R. leguminosarum* bv. *trifolii* as they Reside Within the Soil Community

INTRODUCTION

Although the immunofluorescence technique has allowed microbiologists to detect and enumerate rhizobial subpopulations in soil (Schmidt et al., 1968; Bohlool and Schmidt, 1980), conventional use of the method tells us nothing about the physiological competence of rhizobia in their soil habitat. The nalidixic acid cell elongation assay (Kogure et al., 1979) when combined with immunofluorescence has been used to study the response of soil rhizobia to added substrates (Maggard and Bottomley, 1990). The rationale behind this method lies principally in the action of DNA gyrase inhibitors. These antibiotics inhibit DNA replication (Goss et al., 1964) and prevent cell division while allowing other biosynthetic pathways to operate. The formation of elongated cells not only indicates their viability but also their physiological readiness to respond to the added substrate. This method might have the potential to reveal physiological differences between subpopulations of *R. leguminosarum* bv. *trifolii* which may in turn lead to more insights about rhizobial ecology.

Two issues arose from the previous study (Bottomley and Maggard, 1990). While the majority of cells within most indigenous serotypes of *R. leguminosarum* bv. *trifolii* elongated in response to yeast extract and nalidixic acid,

it was shown that a low percentage (≤ 20%) of the population of serotype AR23 elongated. Secondly, while nalidixic acid (10mg/liter) prevented cell proliferation of most indigenous rhizobial serotypes, it was noted, on occasion, that higher concentrations were required to control proliferation of serotype AS21. Furthermore, in recent reports, up to 100mg/liter of nalidixic acid was required to control proliferation of an aquatic bacterial population (Liebart and Barklay, 1988) and varying concentrations of the antibiotic were required for populations of enteric bacteria in drinking water depending upon whether or not the bacteria were chlorine-damaged (Singh et al., 1989,1990).In this study experiments were carried out with the following objectives:(i) to compare nalidixic acid with other DNA-gyrase inhibitory antibiotics such as ciprofloxacin and norfloxacin for the elongation assay of soil rhizobia, and (ii) to compare the cell elongation characteristics of indigenous serotypes AR23 and AS6 populations when exposed to substrates other than yeast extract.

MATERIALS AND METHODS

Evaluation of cell elongation assay on serotype representatives of R. leguminosarum bv.trifolii.

(i) Preparation of nalidixic acid. Nalidixic acid (150 mg/100 ml) was dissolved in 0.01 M NaOH and filter sterilized. The pH of the medium in which the bacteria were incubated did not significantly change after the addition of portions of the nalidixic acid stock solution sufficient to provide final concentrations up to ≤30 ug/ml (pH 6.5-7.0). A fresh stock solution of nalidixic acid was prepared at 2 week intervals.

(ii) Preparation of norfloxacin and ciprofloxacin. Norfloxacin (150 mg/100 ml) and ciprofloxacin (150 mg/100 ml) were dissolved in 0.01 M NaOH, filter sterilized, and were further diluted 10-fold in 0.01 M NaOH before use.

(iii) Comparison of growth response of serotype representatives of R. leguminosarum bv.trifolii (pure culture) to different concentrations of yeast extract. Isolates of *R. leguminosarum* bv. *trifolii* representing serotypes AS6, and AS21 were cultured in large test tubes (20 by 2.5 cm), each containing 30 ml portions of yeast extract mannitol (YEM) medium (Vincent, 1970). Growth was followed in a temperature controlled water bath (27° C), and the cultures were continuously aerated with a supply of filter-sterilized air. Mid-log phase cells were inoculated

to a final density of approximately 1 x 10^6 to 3 x 10^6 cells/ml into the mineral salts component of YEM medium containing filter-sterile yeast extract (50, 100, 200, and 500 mg/liter) as the sole source of carbon and nitrogen. The optical densities of the cultures were monitored at 3 h intervals under the growth conditions described above.

(iv) Influence of different concentrations of either nalidixic acid, norfloxacin, or ciprofloxacin on the kinetics of cell proliferation and cell elongation. Cells grown in complete YEM were used as inocula. Portions of the cultures of AS6 and AS21 were transferred back into growth tubes containing mineral salts and yeast extract (400 mg/liter) supplemented a) with or without nalidixic acid (final concentrations of 10, 20, 25 and 30 mg/l); b) with or without norfloxacin (final concentrations of 0.25, 1.0, 1.5, 2.0, and 4.0 mg/l) and c) with or without ciprofloxacin (final concentrations of 0.25, 0.5, and 1.0 mg/l). At 3 h intervals, portions (1.9 ml) of the cultures were removed from each tube, and formalin (final concentration, 2% (vol/vol) was added. Samples were maintained at 4°C until they were processed for microscopy.

(v) Experimental protocol for soil rhizobial populations. Three gram portions of field-moist Abiqua soil were dispensed into 160 ml milk dilution bottles, each of which contained a layer of glass beads (diameter, 3 mm) and 27 ml of filter-sterilized (0.2 *um* pore size) 0.15 M NaCl. The bottles were shaken vigorously by hand for 10 minutes,

and the soil suspension was allowed to settle for 5 minutes. A portion (10 ml) was recovered from the upper layer of suspension in each bottle and was added to 190 ml of filter-sterilized deionized water to give a final soil dilution of approximately 200-fold. The diluted soil suspension was filtered sequentially through 47 mm-diameter polycarbonate membrane filters with pore sizes of 8.0 and 3.0 um, respectively. For cell elongation assays, filter sterilized nutrient stock solutions were added to the suspension of soil microorganisms to provide the following concentrations in grams per liter: yeast extract, 0.4 or 0.2; Bacto-tryptone, 0.2; Brain Heart infusion, 0.2; or crimson clover seedling exudates (9 ml of exudate per 20 ml of soil suspension). Portions (30 ml) were dispensed into growth tubes. Nalidixic acid or norfloxacin were added immediately to each growth tube to achieve final concentrations ranging between 5 to 30, or 1.5 to 2.5 mg/liter, respectively. Tubes were incubated at 27°C and aerated as described above over a period of 24 h. The contents of tubes were sacrificed at zero, 16, 20, and 24 h by adding formalin (final concentration, 2% [v/v]), cooled to 4° C and processed for microscopy as follows: Portions (15 ml) were passed through 0.4um pore size polycarbonate, Sudan black-stained filters under 50kPa of negative pressure. Each filter was then stained with 2 drops of a rhodamine-gelatin conjugate (Bohlool and Schmidt, 1968), dried in a 55°C oven for ten minutes, followed by staining for 20-30 minutes with two

drops of fluorescent antibody (25 x diluted in 0.02 M phosphate buffer and filter sterilized through a 0.2 *um* pore size polycarbonate filter) . Destaining involved the passage of 100 ml of 0.02 M phosphate buffer (pH7.2) through the filters under 50 kPa of negative pressure. Each filter was mounted on a microscopic slide and air-dried. 1 drop of mounting fluid containing the fluorescence enhancer p-phenylenediamine (100 mg of p-phenylenediamine in 100 ml of glycerol, pH adjusted to 8.0 with 0.5 M carbonate-bicarbonate buffer) was added to the upper surface of the filter before a cover slip was applied. One drop of immersion oil was applied onto the cover slip before microscopic examination. Included in every experiment were extra tubes containing filtered soil suspension supplemented with yeast extract and incubated in the presence or absence of nalidixic acid or norfloxacin. Growth was followed turbidimetrically in these tubes to evaluate if the antibiotic could control cell proliferation and to monitor the kinetics of appearance of visible growth in the absence of nalidixic acid.

(vi) Microscopic procedures. Enumerations of total cells and elongated cells were carried out by immunofluorescence with the aid of a whipple disk inserted into the eyepiece of an epifluorescence microscope (Zeiss) equipped with a fluorescein filter set. A Zeiss Planachromat 100 X/1.25 oil objective was used to make all observations. At least 30 fields of view per filter were counted at 1000X

magnification for assessment of both the total number and the proportion of cells that were elongated (≥4.2 um)

Preparation of crimson clover (Trifolium incarnatum L.) seedling exudate. Crimson clover seeds (6 g) were surface sterilized as described in Chapter 2, and approximately 1 g portions of seeds were enclosed in each of six stainless steel tissue capsules. Six of the capsules were placed in a petri dish (100 x 15 mm) which contained about 40 ml of sterile distilled water. The seeds were left to germinate for 3 to 4 days at room temperature in a dark cupboard after which the seedling exudate solution was collected and filtered sequentially through polycarbonate membrane filters with pore sizes of 3.0 and 0.4 um respectively. The filtered exudates were either used immediately or stored in screw cap tubes at 4°C until required. 9 ml of exudates supplemented with 1 ml of 10x mineral salts were added to each cell elongation assay tube containing 20 ml of soil suspension. Tubes were incubated and processed as described above.

RESULTS

(i) Evaluating nutrient sources for the elongation assay. Yeast extract is not used traditionally as the sole source of carbon and nitrogen for growth of *Rhizobium*. Nevertheless, data in Fig.3.1.and 3.2. show that mineral salts supplemented with 200 to 500 mg per liter of yeast extract was sufficient to support short-term exponential growth of representatives from serotypes AS6 and AS21 with generation times of 3.3 and 3.6h respectively. Furthermore, we determined that exudates from newly germinated seedlings of clover could support exponential growth of representatives from each of six indigenous serotypes at a generation time of about 4h for at least 24h (data not shown). Supplementation of the exudates with either yeast extract or mannitol did not increase the growth rate, thereby attesting to the nutritional completeness of this material and its potential as a "natural substrate" for use in the elongation assay.

(ii) Evaluating the antibiotic concentration required for cell elongation. While 10 mg per liter of nalidixic acid was sufficient to prevent cell proliferation by strain AS6 in the presence of 400 mg per liter of yeast extract, at least 20 mg per liter was required to prevent multiplication by strain AS21. (Table 3.1.). Between 60 and 85% of the cells of strain AS21 were elongated to ≥4 um after 20 h of

incubation in the presence of 20 to 30 mg per liter of nalidixic acid. A somewhat lower percentage of elongated cells was observed at 10 mg per liter; this observation was not surprizing since the latter concentration did not completely block cell division.

(iii) Comparison of Norfloxacin and Ciprofloxacin with Nalidixic acid. The strains were much more sensitive to these inhibitors of DNA gyrase than to nalidixic acid. Cell proliferation of strain AS21 was prevented completely by norfloxacin and ciprofloxacin at 1.5 and 0.25 mg per liter,respectively. The greater sensitivity of the strains to these inhibitors was also revealed by the results of cell elongation. In contrast to nalidixic acid, which allowed cell elongation to occur at concentrations above that required to stop cell proliferation, concentrations only slightly above MIC values (0.5 mg per liter ciprofloxacin and 4.0 mg per liter norfloxacin) significantly reduced the proportion of elongating cells (Table 3.2.). Cells of strain AS6 were also capable of elongating in 1.5 to 2.0 mg per liter of norfloxacin. Likewise, concentrations greater than 2.0 mg per liter were toxic to cells and reduced the proportion of elongating cells to 30% (Data not shown).

(iv) Response of soil populations to the antibiotics. The high degree of sensitivity to norfloxacin, and the narrow window of concentration tolerance for cell elongation observed in pure cultures was even more exaggerated in soil populations. While 2 mg per liter of norfloxacin was

sufficient to allow 90% of cells of strains AS6 and AS21 to elongate in pure culture after 12h of incubation, this concentration resulted in significantly lower percentage values of elongated cells (35 to 46%) when indigenous serotype populations were evaluated over a 24h period (Table 3.3.). Slightly higher concentrations (2.5 mg per liter) completely prevented cell elongation of both serotypes. Despite 10 mg per liter of nalidixic acid being insufficient to prevent cell proliferation of strain AS21 in pure culture, this concentration was very effective on the indigenous population of AS21. (Table 3.4.). However, just as with norfloxacin, concentrations of nalidixic acid >20 mg per liter either delayed or completely inhibited the indigenous soil population from elongating.

(v)Differences in elongation response by two indigenous serotype populations. Although the soil population densities of serotypes AS6 and AR23 are similar and are the highest among the serotypes in Abiqua soil, the appearance of elongated cells of AR23 was significantly delayed relative to AS6. Furthermore, ≤ 20% of the cells of AR23 became elongated after a 24-h exposure to yeast extract. Additional studies were carried out to evaluate other nutrient sources. Regardless of nutrient type, or concentration, the percentage of elongated cells of AR23 remained low (<16%) after 24-h of incubation. There were some interesting differences in the rate of appearance of elongated cells in response to different nutrient sources (Fig. 3.3., 3.4., and

3.5.). While yeast extract showed the largest difference between the two serotypes, the response to seedling exudates was rather similar (Fig.3.4). This was due to the response of AS6 being delayed relative to yeast extract while AR23 was unaffected. Relative to yeast extract, exposure to brain-heart infusion or tryptone caused the appearance of elongated cells to be significantly delayed in both serotypes, and resulted in a lower total percentage (<20%) of elongated cells boing produced. (Fig.3.5.).

(vi)Diversity within serotype AR23. In chapter 2 we discussed the issue of serotype heterogeneity and the genetic relatedness of isolates from within the same serotype. Data in Table 5 show that representatives of serotype AR23 were also diverse with the parent isolate being different at 7 of 14 loci from isolates represented by the most dominant ET. It is possible, therefore, that cells elongating within the soil population may represent a specific subpopulation of this serotype.

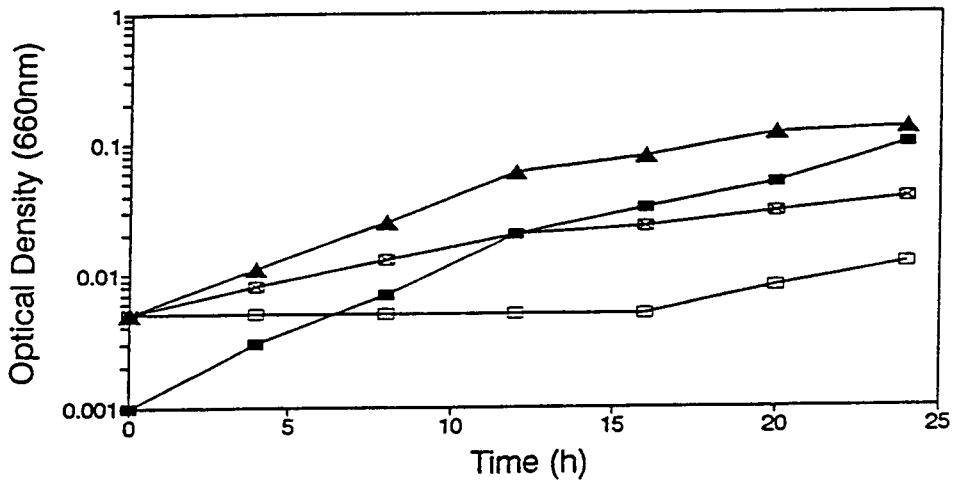

FIG. 3.1. Growth characteristics of *R. leguminosarum* bv. *trifolii*, strain AS6 from serotype AS6 in varying concentrations of yeast extract (mg/l): ☐ 50, ⊠ 100, ■ 200,and ▲ 500.

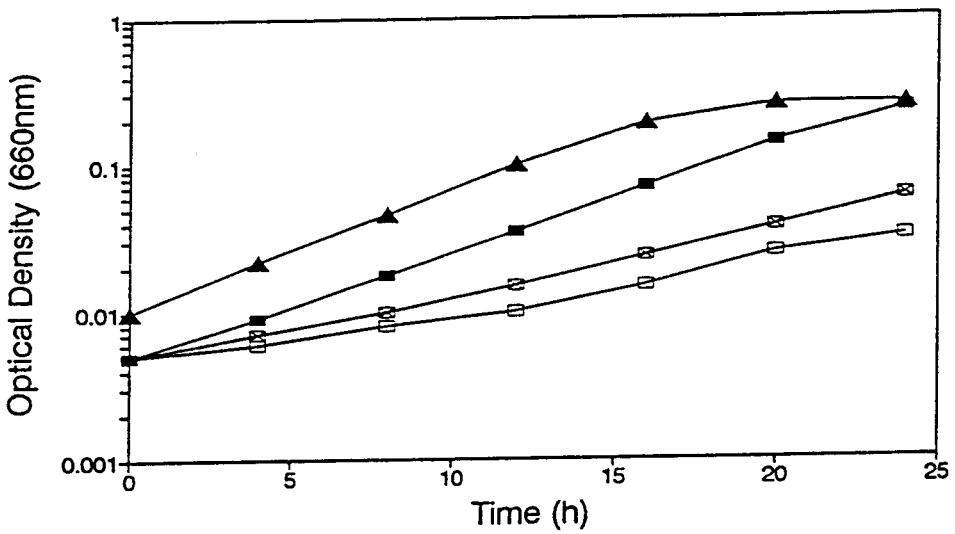

FIG. 3.2. Growth characteristics of *R. leguminosarum bv. trifolii*, strain AS21 from serotype AS21 in varying concentrations of yeast extract (mg/l): □ 50, ⊠ 100, ■ 200, and ▲ 500.

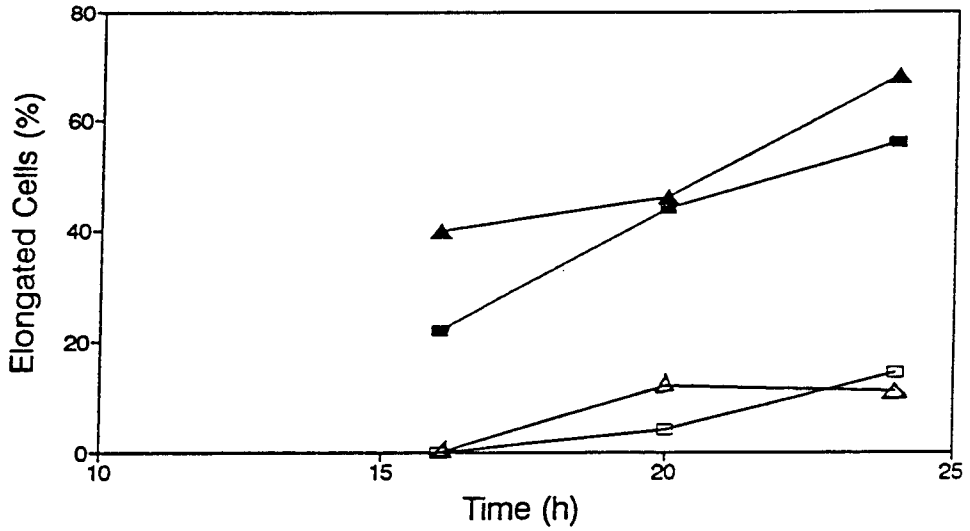

FIG. 3.3. Kinetics of cell elongation by indigenous serotypes AS6 (closed symbols) and AR23 (open symbols) incubated in the presence of nalidixic acid (10 mg/liter) and yeast extract at 200 (□, ■) or 400 (△, ▲)mg/liter. Note abscissa commences at t=10h.

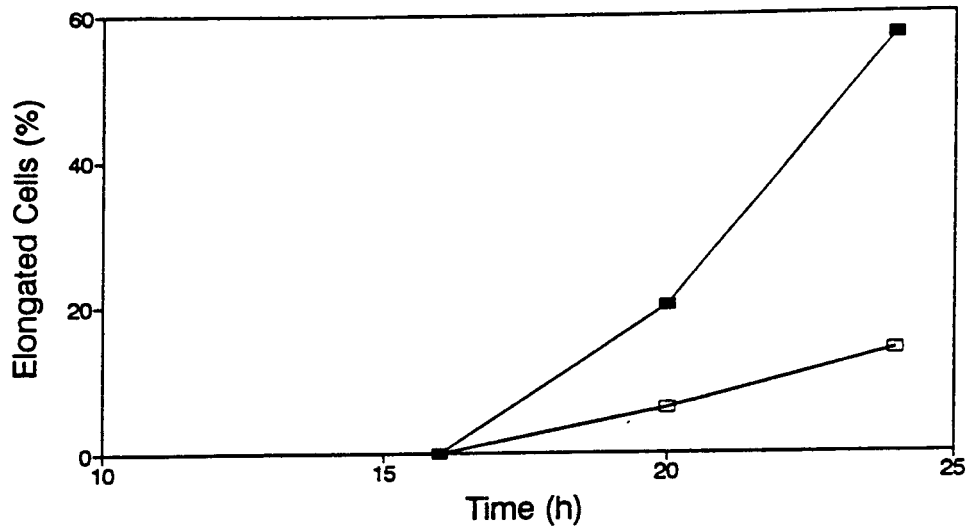

FIG. 3.4. Kinetics of cell elongation by indigenous serotypes AS6 (closed symbols) and AR23 (open symbols) incubated in the presence of crimson clover seedling exudates. Note abscissa commences at t=10h.

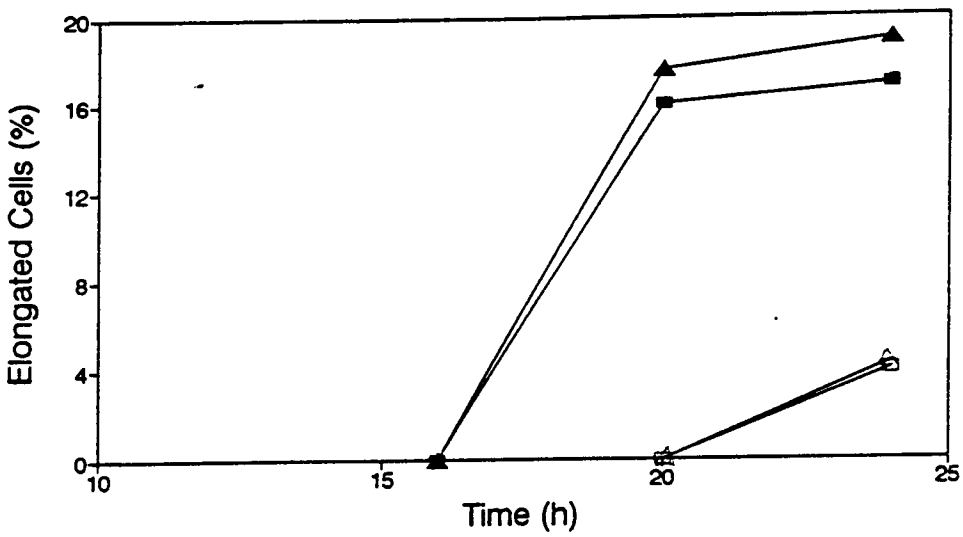

FIG. 3.5. Kinetics of cell elongation by indigenous serotypes AS6 (closed symbols) and AR23 (open symbols) incubated in the presence of nalidixic acid (10 mg/liter) and tryptone (■, □) and brain heart infusion (▲, △).
Note abscissa commences at t=10h.

Table 3.1. Kinetics of cell elongation by a representative of *R. leguminosarum* bv. *trifolii* serotype AS21 incubated in the presence of yeast extract and different concentrations of nalidixic acid.

NAL (mg/l)	$^{a}N_{24}/N_{o}$	Elongated cells(%) at different times(h)		
		12	20	24
10	30.00	40	48	50
20	1.30	52	60	60
25	0.94	44	79	69
30	0.70	49	85	80

a. Density of cells at time = 24h relative to density of cells at time = 0h.

Table 3.2. Kinetics of cell elongation by a representative of *R. leguminosarum* bv. *trifolii* serotype AS21 incubated in the presence of YEM and different concentrations of norfloxacin (Norf) and ciprofloxacin (Cipro).

Antibiotic (mg/l)	[a] N_{24}/N_0	Elongated cells(%) at different times(h) 12	24
Norf (0.25)	18.00	NT	13
Norf (1.0)	1.20	25	55
Norf (1.5)	0.25	71	70
Norf (2.0)	0.20	92	69
Norf (4.0)	0.80	NT	27
Cipro (0.25)	0.63	49	76
Cipro (0.5)	0.60	NT	28
Cipro (1.0)	1.00	28	27

a. See Table 3.1.

Table 3.3. Kinetics of cell elongation by indigenous serotypes AS21 and AS6 in the presence of yeast extract (400mg/l) and different concentrations of norfloxacin (Norf).

Serotype	Norf (mg/l)	$^{a}N_{24}/N_{o}$	Elongated cells(%) at different times(h) 16	20	24
AS6:	1.5	0.9	12.5	37	57
	2.0	0.6	10	44	46
	2.5	1.1	NT	0	0
AS21:	1.5	0.6	11	18	19
	2.0	1.7	17.5	26	35
	2.5	0.9	NT	0	0

a. See Table 3.1.

Table 3.4. Kinetics of cell elongation by indigenous serotype AS21 incubated in the presence of yeast extract (400mg/l) and different concentrations of nalidixic acid (Nal).

Nal (mg/l)	$^{a}N_{24}/N_0$	Elongated cells(%) at diff. times(h) 16	20	24
5	2.7	0	15.7	32
10	2.2	0	12.5	56
20	6.0	31	47	63
25	3.3	0	19	63
30	0.6	NT	0	0

a. See Table 3.1.

Table 3.5. Allelic profiles at 14 enzyme loci of isolates from serotype AR23.

No. of		Enzyme Locus (allelic variant)														
Isolates	ET	[a]IDH	BGA	G6PD	MDH	PGI	PEP	XDH	NSP	6PG	HBD	ADK	ACP	PGM	SOD	[b]
7	25	3	3	2	1	3	5	3	2	2	7	1	1	1	2	0
1	26	4	1	2	1	4	2	3	2	2	7	2	1	1	1	6
1	27	4	1	2	1	1	5	3	1	2	4	1	1	1	2	5
1	28	1	5	2	1	1	4	7	4	2	11	1	7	1	2	8
1	[c]24	4	1	2	1	4	2	5	2	2	5	1	3	1	2	7

a. See Table 2.1. of Material and Methods (Chapter 2) for the full names of enzymes.

b. Refers to the number of enzyme loci at which the alles differ from that of ET25.

c. ET designating the serotype parent AR23.

DISCUSSION

Although the yeast extract-nalidixic acid elongation method (DVC) has been used extensively for aquatic studies, this work represents only the second report of its use in a soil system (Bottomley and Maggard, 1990). As mentioned in the introduction,this research was prompted,in part, by issues left unanswered in the previous study.

Some users of the DVC method have commented upon the difficulty encountered with nalidixic acid in preventing cell proliferation in some bacterial populations (Kogure et al., 1980). In other cases very high concentrations (100 mg per liter) were needed (Liebert and Barklay, 1988), and in others, the effective concentration varied depending upon the "health" of the bacterial population (Singh et al., 1990). Our results with commercially available alternate inhibitors of DNA gyrase, norfloxacin and ciprofloxacin, showed there to be a potential for replacing nalidixic acid in the DVC procedure. Unfortunately, the high degree of sensitivity shown by our rhizobial strains in pure culture and the narrow concentration window shown by the soil populations made it difficult to fine-tune an optimal concentration. These data are in agreement with a generally recognized fact that members of *R. leguminosarum* are highly sensitive to many antibiotics (Josey et al., 1979; Beynon and Josey, 1980). Our findings also agree with others who

have shown clinically important bacteria to be about ten times more sensitive to norfloxacin and ciprofloxacin than to nalidixic acid (Hoogkamp-Korstanje, 1984; Zeiler and Grohe, 1984; Auckenthaler et al., 1986).

Using soil sampled from the same field site about two years later, we observed the same trend in cell elongation behavior of indigenous serotypes AS6 and AR23. We speculated that the unresponsiveness of AR23 might be due to nutritional inadequacies of yeast extract. However, since different nutrient types did not enhance the proportion of AR23 cells which elongated, alternate explanations are needed to explain the lack of response by the majority of this serotype.

The simplest possibility is that most AR23 cells are non-viable and have defied predation because they reside in pores too small for predators to enter. The issue of persistence of non-dividing bacterial cells in soil has been controversial. Although Bohlool and Schmidt (1973) showed that dead rhizobia were cleared from soil rapidly after their introduction on buried slides, more recent studies show that bacteria, including rhizobia,can defy predation and remain intact when they are present in the micro-pore structure of the soil (Vargas and Hattori, 1986; Postma et al., 1989,1990). However, if the explanation was so simple, why do we not see similar results with other indigenous serotypes?

Since serotype AR23 is composed of genetically diverse clonal lines, it is plausible that some lines are in a substrate-responsive state, while others lie in a non-competitive dormant state. Although some strains of pathogenic bacterial species have been shown to enter a non-culturable, substrate-responsive state upon starvation (Rozak and Colwell, 1987), there is some controversy about the fish pathogen, *Aeromonas salmonicida*. While some workers have shown certain strains of this bacterium to be substrate responsive after entering a non-culturable state, (Allen-Austin et al., 1984), substrate-responsiveness has not been shown in other strains after cells became non-culturable (Rose et al., 1990; Morgan et al., 1991). Perhaps different clonal lines of this pathogenic bacterium respond to starvation in different ways?

Another equally plausible explanation for lack of response by AR23 members cannot be overlooked and that lies with the limitations of the fluorescent-antibody technique. There is a precedent for other soil bacteria to cross-react with fluorescent-antibodies prepared to rhizobia (Bohlool and Schmidt, 1970). As a result we cannot discount the possibility that many of the cells cross-reacting with FA AR23 are sufficiently different from rhizobia to have a different range of substrates they respond to. Only by chance do they have an antigenic determinant(s) in common with AR23 of *R. leguminosarum* bv. *trifolii* (Jarvis et al., 1989; Soberon-Chavez and Najera, 1989; Pinero, 1990).

While AS6 responded more rapidly to YE than did AR23, the response of the former serotype was influenced more markedly by nutrient source than was the response of AR23. The delayed and inferior response of members of AS6 to other nutrient sources could be due to their antagonism by antibiotic-producing members of the soil population. Alternatively, the rhizobia might have been simply outcompeted for nutrients by a segment of the microflora activated by BHI, Tryptone, or root exudates but not by YE. While it is tempting to speculate that such interactions play a role in influencing nodulating advantage of one rhizobial population over another, more studies are needed to place this phenomenon upon a firmer foundation.

BIBLIOGRAPHY

Allen-Austin, D., B. Austin, and R.R. Colwell. 1984. Survival of Aeromonas salmonicida in river water. FEMS Microbiol. Lett. 21:143-146.

Almendras, A.S., and P.J. Bottomley. 1987. Influence of lime and phosphate on nodulation of soil-grown Trifolium subterraneum L. by indigenous Rhizobium trifolii. Appl. Environ. Microbiol. 53:2090-2097.

Amarger, N., 1984. Evaluation of competition in Rhizobium spp. In: Current Perspectives in Microbial Ecology (Klug, M.J., and Reddy, C. A., eds.), Washington, D.C, Amer. Soc. Microbiol. pp. 300-305.

Amarger, N., and J.P. Lobreau. 1982. Quantitative study of nodulation competitiveness in Rhizobium strains. Appl. Environ. Microbiol. 44, 583-588.

Auckenthaler, R. M. Michea-Hamzehpou, and J.C. Pechere. 1986. In-vitro activity of newer quinolones against aerobic bacteria. J Anti-microb. Chemother. 17(Suppl B):29-39.

Beltran,P., J.M. Musser, R. Helmuth, R. Farmer, J.J. Frerichs, I.K.Wachsmuth, K. Ferris, A.C. McWharter, J.G. Wells, A. Cravioto, and R.K. Selander. 1988. Toward a population genetic analysis of Salmonella: genetic diversity and relationships among strains of serotypes S. choleraesius, S.derby, S. dublin, S. enteritidis, S. heidelberg, S. infantis, S.newport, and S. typhimurium. Proc. Natl. Acad. Sci. 85:7753-7757.

Beynon, J.L. and D.P. Josey. 1980. Demonstration of heterogeneity in a natural population of Rhizobium phaseoli using variation in intrinsic antibiotic resistance. J. Gen. Microbiol. 118:437-442.

Bezdicek, D.F. 1972. Effect of soil factors on the distribution of Rhizobium japonicum serogroups. Soil Sci. Soc. Am. Proc. 36:305-307.

Bohlool, B.B., and E.L. Schmidt. 1970. Immunofluorescent detection of Rhizobium japonicum in soils. Soil Sci. 110:229-236.

Bohlool, B.B., and E.L. Schmidt. 1973. Persistence and competition aspects of Rhizobium japonicum observed in soil by immunofluorescence microscopy. Soil Sci. Soc. Am. Proc. 37:561-564.

Bohlool, B.B., and E.L. Schmidt. 1980. The immunofluorescence approach to microbial ecology. In: Advances in Microbial Ecology, vol.4 (Alexander,M.,ed.), New York, Plenum Press. pp. 203-241.

Bottomley, P.J. 1991. Ecology of Brayrhizobium and Rhizobium. In: Nitrogen Fixation. (Stacey, G. Evans, H.J.,and Burris, R.H., eds.) Chapman and Hall. New York. pp292-347.

Bottomley, P.J., and M.H. Dughri. 1989. Population size and distribution of Rhizobium leguminosarum bv. trifolii in relation to total soil bacteria and soil depth. Appl. Environ. Microbiol. 55:959-964.

Bottomley, P.J., and S.P. Maggard. 1990. Determination of viability within serotypes of a soil population of Rhizobium leguminosarum bv.trifolii. Appl. Environ. Microbiol. 56:533-540.

Bottomley, P.J., K. Leung, S.R. Strain, K.Yap, N. Dashti, and P. Claycomb. 1990. The ecology of indigenous populations of Rhizobium leguminosarum bvs. trifolii and viceae. In: Nitrogen fixation, Achievements and Objectives. (Gresshoff,P.M., Roth,E., Stacey,G., and Newton, W.E., eds.) Chapman and Hall. New York. pp.371-378.

Brockwell, J., R.R. Gault, M. Zorin, and M.J. Roberts. 1982. Effects of environmental variables on the competition between inoculum strains and naturalized populations of Rhizobium trifolii for nodulation of Trifolium subterraneum L. and Rhizobia persistence in the soil. Aust. J. Agric. Res. 33:803-815.

Bromfield, E.S.P., I.B. Sinha, and M.S. Wolynetz. 1986. Influence of location, host cultivar, and inoculation on the composition of naturalized populations of Rhizobium meliloti in Medicago sativa nodules. Appl. Environ. Microbiol. 51:1077-1084.

Bushby, H.V.A. 1982. Ecology. In: Nitrogen Fixation, vol 2, Rhizobium (Broughton, W.J., ed). Clarendon Press. Oxford. pp. 35-75.

Caugant, D.A., B.R. Levin, I.Orskov, F. Orskov, and R.K. Selander. 1981. Genetic diversity and temporal variation in the E. coli population of a human host. Genetics 8:467-490.

Caugant, D.A., B.R. Levin, I.Orskov, F. Orskov, and R.K. Selander. 1984. Distribution of multilocus genotypes of E.coli within and between families. J. Hyg. 92:377-384.

Caugant, D.A., B.R. Levin, I.Orskov, F. Orskov, C. Svanborg Eden, and R.K. Selander. 1985. Genetic diversity in relation to serotype in Escherichia coli. Infect. Immun. 49:407-413.

Chatel D.L. and R.M. Greenwood, 1973. Differences between strain of Rhizobium trifolii in ability to colonize soil and plant roots in the absence of their specific host plants. Soil Biol. Biochem. 5:809-813.

Cregan, P.B., M.J. Sadowsky, and H.H. Keyser. 1991. Gene-for-gene interaction in the legume-Rhizobium symbiosis. In: The rhizosphere and plant growth, D.L.Keister and P.B. Cregan (eds.). Kluwer Academic Publishers, Netherlands. pp 163-171.

Damirgi, S.M., L.R. Frederick, and I.C. Anderson. 1967. Serogroups of Rhizobium japonicum in soybean nodules as affected by soil type. Agron. J. 59:10-12.

Danso, S.K.A. and G.D. Bowen. 1989. Methods of inoculation and how they influence pattern and nitrogen fixation using two contrasting strains of Bradyrhizobium japonicum. Soil. Biol. Biochem. 21:1053-1058.

Danso, S.K.A., and J.D. Owiredu. 1988. Competitiveness of introduced and indigenous cowpea Bradyrhizobium strains for nodule formation on cowpea Vigna unguiculata L. Walp in three soils. Soil Biol. Biochem. 20:305-310.

Date, R.A., and A.M. Decker. 1965. Minimal antigenic constitution of 28 strains of Rhizobium japonicum. Can. J. Microbiol. 11:1-8.

Date, R.A., and J. Brockwell. 1978. Rhizobium strain competition and host interaction for nodulation. In: Plant relations in pastures (Wilson, J.R., ed), East Melbourne. CSIRO. pp206-216.

Demezas, D.H., and P.J. Bottomley. 1986. Autecology in rhizospheres and nodulating behavior of indigenous Rhizobium trifolii. Appl. Environ. Microbiol. 52:1014-1019.

Dowling, D.N., and W. J. Broughton. 1986. Competition for nodulation of legumes. Ann. Rev. Microbiol. 40:131-157.

Dudman, W.F. 1971. Antigenic analysis of Rhizobium japonicum by immunodiffusion. Appl. Microbiol. 21:973-985.

Dudman, W.F., and J. Brockwell. 1968. Ecological studies of root nodule bacteria introduced into field environments. I. A survey of field performance of

clover inoculants by gel immuno-diffusion. Aust. J. Agric. Res. 19:739-747

Dughri, M.H., and P.J. Bottomley. 1983. Complementary methodologies to delineate the composition of Rhizobium trifolii populations in root nodules. Soil Sci. Soc. Am. J. 47:39-945.

Dughri, M.H., and P.J. Bottomley. 1984. Soil acidity and the composition of an indigenous population of Rhizobium trifolii in nodules of different cultivars of Trifolium subterraneum L. Soil Biol. Biochem. 16:405-411

Ellis, W.R., G.E. Ham, and E.L. Schmidt. 1984. Persistence and recovery of Rhizobium japonicum inoculum in a field soil. Agron. J. 76:573-576.

Evans, H.J. 1974. Symbiotic nitrogen fixation in legume nodules. In: Research experiences in plant physiology, a laboratory manual (T.C. Moore ed.), pp.417-426.

Fuhrmann, J., and A.G., Wollum. 1989. Symbiotic interactions between soybean and competing strains of Bradyrhizobium japonicum. Plant and Soil 119:139-145.

Gaur, Y.D., and W.L.,Lowther. 1980. Distribution, symbiotic effectiveness, and fluorescent antibody reaction of naturalized populations of Rhizobium trifolii in Otago soils. N.Z.J. Agric. Res. 23:529-532.

Gibson, A.H., B.C. Curnow, F.J. Bergersen, J. Brockwell, and A.C. Robinson. 1975. Studies of field populations of Rhizobium: Effectiveness of strains of Rhizobium trifolii associated with Trifolium subterraneum L. pastures in southeastern Australia. Soil Biol. Biochem. 7:95-102.

Gibson, A.H., R.A. Date, and J.A. Brockwell. 1976. A comparison of competitiveness and persistence amongst five strains of Rhizobium trifolii. Soil Biol. Biochem. 8:395-401.

Gibson, A.H., W.F. Dudman, R.W. Weaver, J.C. Horton, and I.C. Anderson. 1971. Variation within serogroup 123 of Rhizobium japonicum. Plant and Soil, special vol.33-37.

Goss, W.A., W.H. Deitz, and T.M. Cook, 1965. Mechanism of action of Nalidixic acid on Escherichia coli. J. Bacteriol. 89:1068-1074.

Ham, G.E., l.R. Frederick, and I.C. Anderson. 1971. Serogroups of Rhizobium japonicum in soybean nodules sampled in Iowa. Agron. J. 63:69-72.

Harrison, S.P., D.G. Jones, and J.P.W. Young. 1989a. Rhizobium population genetics: genetic variation within and between populations from diverse locations. J. Gen. Microbiol. 135:1061-1069.

Harrison, S.P., J.P.W. Young, and D.G. Jones. 1987. Rhizobium population genetics: Effect of clover variety and inoculum dilution on the genetic diversity sampled from a natural population. Plant and Soil. 103:147-150.

Harrison, S.P. J.P.W. Young, and D.G. Jones. 1989b. Rhizobium population genetics: host preference and strain competition effects on the range of Rhizobium leguminosarum biovar trifolii genotypes isolated from natural populations. Soil. Biol. Biochem. 21:981-986.

Holland, A.A. 1966. Serological characteristics of certain root-nodule bacteria of legumes. Antonie Van Leeuwenhoek. 32:410-418.

Holland, A.A. 1970. Competition between soil and seed-borne Rhizobium trifolii in nodulation of introduced Trifolium subterraneum. Plant Soil 32:293-302.

Hoogkamp-Korstanje, J.A.A. 1984. Comparative in vitro activity of five quinolone derivatives and five other antimicrobial agents used in oral therapy. Eur. J. Clin. Microbiol 3:333-338.

Hynes, M.F. and M.P. O'Connell. 1990. Host plant effect on competition among strains of Rhizobium leguminosarum. Can. J. Microbiol. 36:864-869.

Ireland, J.A., and J.M. Vincent. 1968. A quantitative study of competition for nodule formation. Int. Congr. Soil Sci., Trans. 9th (Adelaide Aust.). 2:85-93.

Jarvis, B. D. W., L. J. H. Ward, and E.A. Slade. 1989. Expression by soil bacteria of nodulation genes from Rhizobium leguminosarum bv. trifolii. Appl. Environ. Microbiol. 55:1426-1434.

Johnson, H.W., and U.M. Means. 1963. Serological groups of Rhizobium japonicum recovered from nodules of soybeans (Glycine max) in field soils. Agron. J. 55:269-271.

Johnson, H.W., U.M. Means, and C.R. Weber. 1965. Competition for nodule sites between strains of Rhizobium japonicum applied as inoculum and strains in the soil. Agron. J. 57:179-185.

Jones, D.G., and G. Hardarson. 1979. Variation within and between white clover varieties in their preference for strains of Rhizobium trifolii. Ann. Appl. Biol. 92:221-228.

Kapusta G. and D.L. Rouwenhorst. 1973. Influence of inoculum size on Rhizobium japonicum serogroup distribution frequency in soybean nodules. Agron. J. 65:916-919.

Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415-420.

Kogure, K., U. Simidu, and N. Taga. 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. 26:318-323.

Liebert, C. and T. Barkay. 1988. A direct viable counting method for measuring tolerance of aquatic microbial communities to Hg^{2+}. Can. J. Microbiol. 34:1090-1095.

Lynch, D.L., and O.H. Sears. 1952. The effect of inoculation upon yields of soybean in treated and untreated soils. Soil. Sci. Soc. Am. J. 16:214-216.

Mason, T.G., and G. Richardson. 1981. A review: Escherichia coli and the human gut: some ecological considerations. J. Appl. Bacteriol. 1:1-16.

Masterson, C.L., and M.T. Sherwood. 1974. Selection of Rhizobium trifolii strains by white and subterranean clovers. Ir. J. Agric. Res. 20:827-841.

McDermott, T.R., and P.H. Graham. 1989. Bradyrhizobium japonicum inoculant motility, nodule occupancy, and acetylene reduction in the soybean root system. Appl. Environ. Microbiol. 55:2493-2498.

McLoughlin, T.J., L.M. Bordeleaux, and L.K. Dunican. 1984. Competitiveness studies with Rhizobium trifolii in a field experiment. J. Appl. Bacteriol. 56:131-135.

Miller R.H. and S. May. 1991. Legume inoculation: success and failures. In: The rhizosphere and plant growth, (D.L. Keister and P.B. Cregan Eds.). Kluwer Academic Publishers, Netherlands. pp. 123-134.

Minor, L. 1984. Salmonella. In: Bergey's Manual of Systematic Bacteriology (eds Kreig, N.R. & Holt, J.G. eds). Williams & Wilkins, Baltimore. Vol:1 pp.427-458.

Moawad, H., and B.B. Bohlool. 1984. Competition among Rhizobium spp. for nodulation of Leucaena leucocephala

in two tropical soils. Appl. Environ. Microbiol. 48:5-9.

Morgan, J.A.W., P.A. Cranwell, and R.W. Pickup, 1991. Survival of Aeromonas salmonicida in lake water. Appl. Environ. Microbiol. 57:1777-1782.

Ochman, H., and R.K. Selander. 1984. Evidence for clonal population structure in Escherichia coli. Proc. Natl. Acad. Sci. USA. 81:198-201.

Orskov, F. and Orskov, I. 1984. Serotyping in Escherichia coli. In: Methods in Microbiology. Academic Press, London. Vol 14:43-112.

Parker, C.A., M.J. Trinick, and D.L. Chatel. 1977. Rhizobia as soil and rhizosphere inhabitants. In: A Treatise on Dinitrogen Fixation, (Hardy, R.W.F., and Gibson, A.H., eds.), Wiley, New York, pp. 311-352.

Pinero, D., E. Martinez, and R.K. Selander. 1988. Genetic diversity and relationships among isolates of Rhizobium leguminosarum biovar phaseoli. Appl. Environ. Microbiol. 54:2825-2832.

Postgate, J.R. The nitrogen-fixing bacteria. 1982. In: The fundamentals of nitrogen fixation (Ed. Postgate, J.R.), Cambridge University Press, Cambridge. pp.5-20.

Postma, J., J.A. van Veen, and S. Walter. 1989. Influence of different initial soil moisture contents on the distribution and population dynamics of introduced Rhizobium leguminosarum bv. trifolii. Soil Biol. Biochem 18:601-606.

Robinson, A.C. 1969. Host selection for effective Rhizobium trifolii by red clover and subterranean clover in the field. Aust. J. Agric. Res. 20:1053-1060.

Rose, A.S., A.E. Ellis, and A.L.S. Munro. 1990. Evidence against dormancy in the bacterial fish pathogen Aeromonas salmonicida subsp. salmonicida. FEMS Microbiol. Lett. 68:105-108.

Roughley, R.J., W.M. Blowes, and D.F. Herridge. 1976. Nodulation of Trifolium subterraneum by introducing Rhizobium in competition with naturalized strains. Soil Biol. Biochem. 8:403-407.

Rozak, D.B., and R.R. Colwell, 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51:365-379.

Schmidt, E.L., and F.M. Robert. 1985. Recent advances in the ecology of Rhizobium. In: Nitrogen Fixation Research Progress. (H.J. Evans, P.J. Bottomley, and W.E. Newton, eds.) pp. 379-385.

Schmidt, E.L., M.J. Zidwick, and H.M. Abebe. 1986. Bradyrhizobium japonicum serocluster 123 and diversity among member isolates. Appl. Environ. Microbiol. 51:1212-1215.

Schmidt, E.L., R.O. Bankole, and B.B. Bohlool. 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. 95:1987-1992.

Schofield, P.R., A.H. Gibson, W.F. Dudman, and J.M. Watson. 1987. Evidence for genetic exchange and recombination of Rhizobium symbiotic plasmids in a soil population. Appl. Environ. Microbiol. 53:2942-2947.

Sears H.J., I. Brownlee, and J. Uchiyama. 1950. Persistence of individual strains of Escherichia coli in the intestinal tract of man. J. Bacteriol. 59:293-301.

Selander, K.R., D.A. Caugant, and T.S. Whittam. 1987. Natural populations of Escherichia coli genetic structure and variation. In: Escherichia coli and Salmonella typhimurium. (J.L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter and H.E. Umbarger, eds.), ASM. Washington D.C. 2:1625-1648.

Selander, R.K., D.A. Caugant, H. Ochman, J.M. Musser, M.N. Gilmour, and T.S. Whittam. 1986. Methods of multi-locus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51:873-884.

Singh, A., B.H. Pyle, and G.A. McFeters. 1989. Rapid enumeration of viable bacteria by image analysis. J. Microbiol. Methods. 10:91-101.

Singh, A., F.P. Yu, and G.A. McFeters. 1990. Rapid detection of chlorine-induced bacterial injury by the direct viable count method using image analysis. Appl. Environ. Microbiol. 56:389-394.

Soberon-Chavez, G., and R. Najera. 1989. Isolation from soil of Rhizobium leguminosarum lacking symbiotic information. Can. J. Microbiol. 44:330-333.

Triplett, E.W. 1990. The molecular genetics of nodulation competitiveness in Rhizobium and Bradyrhizobium. Molec. Pl. Micr. Interac. 3:199-206.

Valdivia, B., M.H. Dughri, and P.J. Bottomley. 1988. Antigenic and symbiotic characterization of indigenous Rhizobium leguminosarum bv. trifolii recovered from root nodules of Trifolium pratense L. sown into subterranean clover pasture soils. Soil Biol. Biochem. 20:267-272.

Vargas R., T. Hattori 1986. Protozoan predation of bacterial cells in soil aggregates. FEMS Microbiol. Ecol. 38:233-242.

Vincent, J.M. 1954. The root-nodule bacteria of pasture legumes. Proc. Linn. Soc. N.S.W. 79:4-32.

Vincent, J.M. 1962. Australian studies of the root-nodule bacteria. A review. Proc. Linn. Soc. N.S.W. 87:8-38.

Vincent, J.M., and L.M. Waters. 1953. The influence of host on competition amongst clover-root nodule bacteria. J. Gen. Microbiol. 9:357-370.

Weaver R.W., and L.R. Fredrick. 1974. Effect of inoculum rate on competitive nodulation of Glycine max L. Merrill. II Field Studies. Agron. J. 66:233-236.

Weaver, R.W., D. Sen, J.J. Coll, C.R.Dixon, and G.R.Smith. 1989. Specificity of arrowleaf clover for rhizobia and its establishment in soil from crimson clover pastures. Soil Sci. Soc. Am. J. 53:731-734.

Young, J.P.W. 1985. Rhizobium population genetics: enzyme polymorphism in isolates from peas, clover, beans and lucerne grown at the same site. J. Gen. Microbiol. 131:2399-2408.

Young, J.P.W. 1989. The Population Genetics of Bacteria. In: Genetics of Bacterial Diversity. 1989. (D.A. Hopwood and K.F. Chater eds.) Academic Press Inc., London. pp.417-436.

Young, J.P.W., L. Demetriou, and R.G.Apte. 1987. Rhizobium population genetics: enzyme polymorphism in Rhizobium leguminosarum from plants and soil in a pea crop. Appl. Environ. Microbiol. 53:397-402.

Young, J.P.W., and M. Wexler. 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of Rhizobium leguminosarum. J. Gen. Microbiol. 134:2731-2739.

Zeiler H.J., and K. Grohe. 1984. The in vitro and in vivo activity of ciprofloxacin. Eur. J. Clin. Microbiol. 3:339-343.