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Title: Novel Characteristics (Intra and Ex Planta) of Indigenous Serogroups of Rhizobium meliloti

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This thesis presents novel findings regarding symbiotic, physiological, and antigenic characteristics of representatives from an indigenous soil-borne population of Rhizobium meliloti. Nodules were sampled from 'Vernal', 'Anchor', and 'Saranac' alfalfa, and the isolates analyzed serologically. Rhizobium meliloti serogroup 31 was identified as a dominant nodule occupant, with a greater and more consistent nodule occupancy rate on Vernal (60%) compared to Anchor (24%) or Saranac (36%). The symbiotic effectiveness of the parent isolate of serogroup 31 was evaluated on each cultivar over four harvests in a greenhouse study. Only inoculated Vernal responded with an increase in shoot dry weight and N_2 assimilated relative to N supplemented plants between harvests two and three. Field isolates of serogroup 31 from nodules on Vernal

produced homogeneous, effective responses on Vernal and Anchor. In contrast, serogroup 31 isolates from Anchor nodules were heterogeneous in effectiveness on the parent host, with poorly effective isolates shown to be substantially more effective on Vernal.

Isolates of Rhizobium meliloti serogroups 31 and 17 were grown in yeast extract mannitol broth (YEM) containing NaCl or polyethylene glycol (PEG). At water potentials below -0.5 MPa imposed by PEG, strain 31 had a lower specific growth rate than strain 17 which was accompanied by irregular cell morphology. In contrast, neither strain was affected significantly over the range of water potentials created by NaCl. Upshock of water stressed cells (-1.0 MPa PEG) into normal YEM (-0.15 MPa) resulted in a faster recovery of growth by strain 31 than strain 17. Strain 31 also required significantly fewer days to nodulate alfalfa compared with strain 17 when transferred from YEM at -1.0 MPa PEG onto the roots of alfalfa seedlings in plant growth medium (-0.1 MPa). Supplemental calcium (0.1mM) reduced the differences between strains in their response to water stress.

Several novel phenomena were shown to hinder the production of fluorescent antibody conjugates specific to serogroup 31. An indirect fluorescent methodology, using the biotin-avidin system, was developed to successfully detect and specifically discriminate members of serogroup

31 from other strains of R. meliloti. Using this technique, members of serogroup 31 were found to comprise 17% of the total R. meliloti population in soil samples with a recent history of alfalfa cultivation.

Novel Characteristics (*Intra* and *Ex Planta*) of Indigenous Serogroups of *Rhizobium meliloti*

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	Introduction	1
II.	Nitrogen fixing characteristics of alfalfa cultivars nodulated by representatives of an indigenous *Rhizobium meliloti* serogroup.	13
	Abstract	14
	Introduction	16
	Materials and Methods	18
	Results	22
	Discussion	33
	Acknowledgments	37
III.	Growth and nodulation responses of *Rhizobium meliloti* to water stress induced by permeable and impermeable solutes.	38
	Abstract	39
	Introduction	41
	Materials and Methods	43
	Results	48
	Discussion	59
	Acknowledgments	63
IV.	Unusual serological phenomena encountered during the development of serogroup and strain specific immunofluorescent conjugates for *Rhizobium meliloti*.	64
	Abstract	65
	Introduction	67
	Materials and Methods	69
	Results and Discussion	76
CONCLUSIONS		87
BIBLIOGRAPHY		90

LIST OF TABLES

Table		Page
1.	Distribution of R. meliloti serogroup 31 in root nodules of alfalfa cultivars within the field site.	26
2.	Comparison of herbage dry weight and nodulation characteristics of alfalfa cultivars over successive harvests when inoculated with strain 31 (-N) or grown with NH_4NO_3 (+N).	27
3.	Nitrogen content of herbage from three alfalfa cultivars over successive harvests when inoculated with strain 31 (-N) or grown with NH_4NO_3 (+N).	28
4.	Symbiotic effectiveness ratings of indigenous R. meliloti strains in the plant tube system.	29
5.	Yield characteristics of Anchor alfalfa inoculated with strains of R. meliloti representing isolates from within and outside of serogroup 31.	30
6.	Yield characteristics of Vernal alfalfa when inoculated with field isolates representing serogroup 31 and with commercial inoculant strains of R. meliloti.	31
7.	Comparison of symbiotic effectiveness of two serogroup 31 isolates on Vernal and Anchor alfalfa.	32
8.	Influence of permeable and impermeable solutes on the cation content of R. meliloti strains 17 and 31 grown in YEM.	55
9.	Viability of strains 17 and 31 following an increase in water potential from -1.0 MPa PEG to -0.15 MPa.	58
10.	Bacteria used to determine species specificity of the fluorescein labeled immunoglobulin conjugate to R. meliloti serogroup 31.	83
11.	Cross-reaction of the fluorescein labeled conjugate prepared from antiserum 31 using direct immunofluorescence.	84

12. Agglutination titer of antiserum 31 following dialysis against buffers of varying ionic strength. 85

13. Population densities of R. meliloti and R. meliloti serogroup 31 in soils with different cropping histories. 86

LIST OF FIGURES

Figures		Page
1.	Growth response of strains 17 and 31 to water stress imposed by increasing concentrations of either NaCl or PEG.	53
2.	Growth response of strains 17 and 31 in -1.0 MPa PEG to the addition of 0.1 mM calcium.	54
3.	Growth response of strains 17 and 31 to a water potential increase from -1.0 to -0.15 MPa.	56
4.	Nodulation kinetics of strains 17 and 31 following a water potential increase.	57

Novel Characteristics (*Intra* and *Ex Planta*) of Indigenous Serogroups of *Rhizobium meliloti*

CHAPTER I

Introduction

Nitrogen fixation is an intricate biological process responsible for the transformation of dinitrogen gas to a biologically available form. Current global demands for N input rely extensively on this process, and in particular on the symbiotic association between plants of the Leguminosae family and soil-borne bacteria of the genera *Rhizobium* and *Bradyrhizobium*. As a result, considerable research effort has been focused on furthering the scientific understanding of this symbiosis, with the goal of improving both the effective range of leguminous plants and the current levels of N_2 fixation. While advances in such diverse research areas of nitrogen fixation as enzymology, host-plant and bacterial physiology, and genetics have been significant (for reviews, see Quispel, 1974; Hardy *et al.*, 1977; Hardy and Gibson, 1977; Hollaender *et al.*, 1977; Newton *et al.*, 1977; Newton and Orme-Johnson, 1980; Gibson and Newton, 1981; Veeger and Newton, 1984; Evans *et al.*, 1985), corresponding improvements in agricultural productivity have been more difficult to achieve. Obviously, to speculate on the factors contributing to the failure of technology transfer from the basic to the applied science is difficult.

Nevertheless, one related factor receiving greater attention in recent years concerns the role of indigenous soil populations of rhizobia and bradyrhizobia. The inability of symbiotically preferred, inoculant strains to compete with the indigenous population for nodule sites has been noted in numerous studies over the past twenty years (Johnson and Means, 1963; Ham et al.,1971; Hardarson et al., 1982; Bromfield et al., 1986), much to the dismay of the rhizobia inoculant industry. Legume yields, in turn, rely on native rhizobial strains of unknown symbiotic effectiveness. Until a more complete understanding is achieved of both the symbiotic character of indigenous Rhizobium and Bradyrhizobium and the factors which contribute to their competitive nature, the need to introduce improved inoculant strains will remain controversial and their success will remain unpredictable.

This review will concentrate on two components of nitrogen fixation which directly involve indigenous Rhizobium populations; i) the contribution of the indigenous populations to nitrogen fixation and agronomic yield, and ii) soil factors which influence the survival and competitive ability of indigenous rhizobia. The emphasis of this review will focus on the symbiotic association between alfalfa (Medicago sativa L.) and Rhizobium meliloti. Relevant examples from other symbioses will be included to provide additional background information when applicable.

Contribution of indigenous populations to nitrogen fixation by alfalfa.

Alfalfa is a perennial forage legume extensively cultivated in Oregon (Miles, 1984) and world-wide (Bolton et al., 1972). Among the reasons for its agronomic importance include the unsurpassed nutritional value for livestock feed, the adaptability to a wide range of climatic conditions, and the ability of the Rhizobium-alfalfa symbiosis to supply a large percentage of the total plant N content. Reports show the symbiosis capable of contributing up to 400 kg N/ha/yr given optimum growth conditions (Hardarson et al., 1988). Heichel et al. (1984) provided a critical analysis of the percentage of total plant N derived from N_2 fixation for alfalfa growing in Minnesota soils over a four year period. When grown in a pure alfalfa stand, N_2 fixation accounted for 25% of the total plant N during the establishment year. Decreases in soil N reserves in the subsequent seasons resulted in N fixation accounting for 66% of the total N by the fourth year. Recent studies of mixed alfalfa-grass pastures identified N_2 fixation contributing between 88 and 91% of the N content of alfalfa (West and Wedin, 1985; Danso et al., 1988). The percentage of N derived from N_2 fixation should vary according to the soil N content. For example, N_2 fixation accounted for only 40% of soybean N in fertile midwest soils (Weber, 1966). In contrast, estimates for

southeastern soils, where the availability of soil N is lower, ranged from 60 to 91% (Matheny and Hunt, 1983; Hunt et al., 1985; Thurlow and Hiltbold, 1985). Legumes grown in central and eastern Oregon soils, which are typically shallow and contain low organic N, would also be expected to depend on N_2 fixation for a large percentage of their total N requirements.

Given the dependence of alfalfa production on the N fixation process, it is imperative to address the relative contributions by inoculant and native strains of R. meliloti to the levels of N_2 fixation. Historically, the need for introduction of inoculant strains dates to observations of rhizobia free soils and soils sparsely populated with symbiotically inferior strains (Vincent, 1954; Date, 1969; Caldwell and Vest, 1970). The early choices for inoculum strains, quite surprisingly, were not always screened for symbiotic effectiveness (Date, 1969). Steady improvements in cultivar potential over the past thiry years, however, have lead researchers to continually evaluate and identify symbiotically improved inoculant strains (Caldwell and Vest, 1970; McGregor et al, 1983; Mytton et al., 1984). General interest in the role of indigenous rhizobia in legume production was first stimulated following documented failures of B. japonicum inoculation in midwest soybean plots (Johnson et al., 1965; Damirgi et al., 1967; Ham et al., 1971). These studies identified that soybean nodules were dominated by

members of the indigenous B. japonicum population regardless of inoculant strain or technology of application. An addition problem is faced when discussing nodulation of alfalfa. Since alfalfa is a perennial crop, inoculant R. meliloti strains must possess the ability to survive over several growing seasons. In this regard, van Rensburg and Strijdom (1982) found inoculant strains of R. meliloti occupied a high percentage of nodules during the establishment year but were unable to compete for nodulation during the following season. Hardarson et al. (1982) observed an increase in the percentage of nodules occupied by indigenous R. meliloti even during the first year of production. Similar results were also indicated in earlier studies in which poorly nodulated plants in uninoculated alfalfa plots became well nodulated by the native population in subsequent seasons (Bell and Nutman, 1971; Brockwell, 1971).

Obviously, if the native population is symbiotically effective then the interest or need for detailed studies regarding their ecology and competitiveness would be academic. By the same token, if their effectiveness is suboptimal then the potential impact on crop yield could be significant. To date, several studies have documented that indigenous populations of R. meliloti are inferior in symbiotic effectiveness to commercial strains (Brockwell and Hely, 1961; Brockwell and Hely, 1966; Brockwell, 1971; Bottomley and Jenkins, 1983). Most symbiotic effectiveness

testings associated with small seeded legumes are conducted in small containers over a relatively restricted time periods. Whether this method accurately estimates the symbiotic response expected over one or more growing seasons is unclear. For this reason, it is important to gain more information regarding the symbiotic characteristics of indigenous R. meliloti relative to long-term alfalfa production.

Survival and competitive ability of indigenous rhizobia.

Conflicting evidence has been presented in regard to the overall saprophytic competence of R. meliloti in soil. Vincent (1954) suggested that members of the species are not effective soil colonizers since their numbers were depleted at field sites not cultivated to Medicago or Melilotus spp. The inability of R. meliloti to colonize the rhizosphere of non-host plants in Australian soils (Robinson, 1967) supports this claim. In comparison, Bergersen (1970) found survival of R. meliloti strains following an extended absence of the host legume. Hely and Brockwell (1961) also identified R. meliloti survival in soils with no previous cropping history of alfalfa. Much of the confusion regarding the ability of R. meliloti to colonize soil stems from the lack of appropriate methodologies to study the species. Autecological studies

of rhizobia and bradyrhizobia have relied on the techniques of immunofluorescence (Schmidt et al., 1968) to monitor specific populations and provide quantitative results of population dynamics. The majority of studies, however, have monitored Bradyrhizobium and only certain species of Rhizobium. To date there have been no studies of indigenous R. meliloti in soils using the immunofluorescence approach. This shortcoming can be traced, in part, to the anomalous and troublesome nature of R. meliloti serology which has been described in several reports (Dudman, 1964; Gibbins, 1967; Humphrey and Vincent, 1975; Wilson et al, 1975; Kishinevsky and Gurfel, 1980; Fuquay et al., 1984).

The persistence of R. meliloti in a given soil depends on a complex set of environmental parameters. Recent studies have indicated that several factors including soil pH, soil moisture, soil temperature, solar radiation, phosphorus retention, cation exchange capacity, organic carbon, and the presence of the host legume influence the occurrence and density of rhizobial populations (Lawson et al., 1987; Yousef et al., 1987; Woomer et al., 1988). Survival and proliferation of Rhizobium cells in the soil environment requires their ability to remain in thermodynamic equilibrium with the surrounding soil water potential (Brown, 1972; Griffin, 1978). Temporal shifts in water potential from drying or wetting events must result in corresponding changes in cellular water potential to

ensure survival. The internal mechanisms responsible for maintaining this dynamic equilibrium with the environment include regulation of internal osmotic and turgor potentials (Harris, 1981). The immediate response to a decrease in water potential from dessication involves the loss of cell water, with a concurrent reduction of cellular volume and turgor (Wohdringh et al., 1981; Koch, 1984; Baldwin et al., 1988). A longer-term rebalancing of internal osmolytes by osmotic adjustment follows, allowing cells to maintain adequate cellular water content and avoid plasmolysis. By comparison, the response of microbial cells to an increase in water potential following a re-wetting process depends predominently on the inherent strength of the cell wall to avoid plasmoptysis due to excessive turgor. According to the classification scheme of Harris (1981), all bacteria can be separated in four categories based on their abilities to regulate osmotic and turgor potentials. Gram-negative bacteria, including rhizobia and bradyrhizobia, are grouped as Class II organisms as a result of their moderate sensitivity to water stress. Although they are capable of osmoregulation through the production of internal solutes, the weaker cell walls of gram-negative organisms restricts the turgor potential range necessary to adapt to downshock and upshock extremes.

With specific regard to the indigenous rhizobia population, it is unknown whether individual strains have

developed unique strategies to respond to water stress. It is clear, however, that osmoregulation is the key process which dictates the adaptive ability of cells to shifting water potentials (LeRudulier et al., 1984). Most organisms respond to water stress by accumulation of high intracellular concentrations of osmotic solutes such as potassium (Epstein, 1986), glutamate (Csonka, 1988; Measures, 1975), proline (Measures, 1975), trehalose (Giaever et al., 1988), and glycine betaine (Sutherland et al., 1986; Jovanovich et al., 1988). In addition to lowering the internal osmotic potential necessary to maintain cell turgor, these solutes are compatible with the functioning of intracellular enzymes. Evidence for the role of B-glucans, outer membrane oligosaccharides, has also been implicated during osmotic stress (Kennedy, 1987; Miller and Kennedy, 1986). Consistent with these general trends for gram negative bacteria, studies directly involving R. meliloti have shown accumulation of both intracellular potassium (Yelton et al., 1983) and glutamate (Botsford, 1984). Selected strains have also been shown to accumulate glycine betaine if supplied in the growth medium (Bernard et al., 1986). Differences between R. meliloti and Escherichia coli with respect to glycine betaine metabolism when exposed to water stress have also been reported (Smith et al., 1988).

Results from applied studies consistently show water stress to affect both the saprophytic growth of rhizobia

(Bushby and Marshall, 1977a and b; Mahler and Wollum, 1981) as well as the processes of nodulation and nitrogen fixation (Sprent, 1984). Zahran and Sprent (1986) found nodulation events including root hair curling and infection thread formation were sensitive to water stress at potentials which had no effect on rhizosphere colonization by R. leguminosarum. In support of the above, Worrall and Roughley (1976) identified a complete inhibition of nodulation as the soil water potential decreased from -0.036 to -0.36 MPa even though the number of rhizobia present remained unaffected. These findings do not suggest that the influence of water stress is only relevant in the presence of the host, however. The effect of water stress on the saprophytic growth of rhizobia is a critical selective pressure on strain survival. As an example, studies evaluating Rhizobium population dynamics under dessicating conditions have shown considerable variation in the survival rate of Rhizobium strains. Van Rensburg and Strijdom (1980) and Bushby and Marshall (1977a) both found significant differences in the survival rate of R. meliloti strains at low water potentials. Other studies have shown that the impact of water potential on Rhizobium population dynamics can vary with soil texture (Mahler and Wollum, 1981), additions of montmorillonite clay (Bushby and Marshall (1977b), and cycles of wetting and drying (Pena-Cabriales and Alexander, 1979).

From the studies of rhizobia and bradyrhizobia described above it is my intent to emphasize the need for a better understanding of the biotic and abiotic factors which influence indigenous populations. These concerns are of particular relevance to studies of the Rhizobium-alfalfa symbiosis where a lack of autecological information exists. A set of objectives were developed based upon the extensive characterization of the indigenous R. meliloti population from a central Oregon field site (Jenkins and Bottomley, 1984, 1985a, 1985b). Although the indigenous population was extremely diverse, serogroup 31, an antigenically distinct group, dominated nodule occupancy. The identification of a competitive, serologically unique group provided the opportunity to study serogroup 31 from a variety of perspectives. The initial objective was to determine the extent of nodule occupancy by serogroup 31 on several field-grown alfalfa cultivars, and to evaluate the impact of serogroup isolates on the symbiotic effectiveness of the representative cultivars over several harvests. These findings are reported in chapter 2. Given the superior nodulating ability of serogroup 31, the next question became whether any environmental factors could be elucidated which might contribute to the nodule dominance. To address this question, the second objective was to compare the responses to water stress by serogroup 31 with those of a non-competitive serogroup. These findings are

described in chapter 3. Parallel with these objectives I sought to develop a specific fluorescent antibody to R. meliloti serogroup 31 for use in autecological studies. The latter task resulted in the discovery of anomalous serological behavior which is described in chapter 4.

CHAPTER II

Nitrogen Fixing Characteristics of Alfalfa Cultivars Nodulated by Representatives of an Indigenous *Rhizobium meliloti* serogroup

ABSTRACT

Studies were conducted to evaluate whether field-grown cultivars of alfalfa (Medicago sativa L.) nodulate differentially with members of a soil population of Rhizobium meliloti, and to determine the influence of the dominant nodule occupants on N_2 dependent growth of the same cultivars under greenhouse conditions. Nodules were sampled from four replicate plots of 'Vernal', 'Anchor', and 'Saranac' alfalfa, and the isolates analyzed serologically. Results from agglutination tests identified serogroup 31 as a dominant nodule occupant. A significant cultivar effect was observed, with a greater and more consistent occupancy rate by serogroup 31 across the replicates on Vernal (60%) compared to Anchor (24%) or Saranac (36%). The symbiotic effectiveness of the parent isolate of serogroup 31 was evaluated on each cultivar over four successive harvests in a greenhouse study. Significant cultivar x N source interactions for herbage dry weight resulted following the second harvest. Of the three cultivars, only inoculated Vernal responded with an increase in shoot dry weight and N_2 assimilated relative to N supplemented plants between harvests two and three. In separate greenhouse experiments, field isolates of serogroup 31 from nodules on Vernal produced homogeneous, effective responses on Vernal and Anchor. In contrast, serogroup 31 field isolates from Anchor nodules were

highly heterogeneous in effectiveness on the parent host, with poorly effective isolates being substantially more effective on Vernal. The data indicate that attention be given to the potential impact of the indigenous R. meliloti population upon cultivar ranking at specific field locations, and also to strain-cultivar idiosyncracies when carrying out physiological studies of regrowth characteristics.

INTRODUCTION

Failure of inoculant strains of R. meliloti to successfully occupy a significant proportion of nodules on alfalfa has been documented in soils already containing resident rhizobia populations (Hardarson et al., 1982; Van Rensburg and Strijdom, 1982; Bromfield et al., 1986). As a result, it is reasonable to suggest that agronomic yields of this perennial legume will become increasingly reliant on fixed N provided by microsymbionts of unknown composition and effectiveness as soil N levels are depleted (Heichel et al., 1981; 1984; 1985). This concern draws even more significance following the results from surveys (Barber, 1980; Bottomley and Jenkins, 1983), and detailed field studies at specific locations (Jenkins and Bottomley, 1984; Eardly et al., 1985), which have revealed the majority of field isolates of R. meliloti from Oregon soils are unable to supply N to alfalfa seedlings in quantities comparable to nitrate supplements.

The question arises whether such intermediate symbiotic effectiveness traits are confounded by the complex nature of the alfalfa germplasm in agricultural use. Although selection of alfalfa lines for improved N_2 fixing characteristics has been achieved (Phillips et al., 1985; Teuber et al., 1984; Teuber and Phillips, 1988; Viands et al., 1981), such advances are confronted by repeated observations of variability in cultivar

performance when challenged with different Rhizobium strains (Burton and Wilson, 1939; Erdman and Means, 1953; Gibson, 1962; McGregor et al., 1983; Miller and Sirois, 1982; Mytton et al., 1984; Tan and Tan, 1986). Error in cultivar selection for regional use could occur if the symbiotic effectiveness of the dominant nodule occupants at the trial site is inconsistent between the plant germplasm being evaluated.

Rhizobium meliloti serogroup 31, an antigenically homogeneous group, was found to dominate nodule occupancy within a diverse indigenous population at a central Oregon field site (Jenkins and Bottomley, 1985a and b). Toward obtaining a better understanding of the significance of serogroup 31 in relationship to cultivar effectiveness, the following objectives were formulated: i) to determine the extent of nodule occupancy by serogroup 31 on three uninoculated cultivars of alfalfa growing under field conditions, ii) to determine whether differences in symbiotic effectiveness are expressed between alfalfa cultivars, over successive harvests, when inoculated with representatives of serogroup 31 and other members of the indigenous R. meliloti population.

MATERIALS AND METHODS

Field sampling. Alfalfa plants were sampled in September 1984 from a three year-old variety trial at the central Oregon Agricultural Experiment Station, Powell Butte, OR. The field site was adjacent to the original plots where serogroup 31 was first identified. The soil was a Deschutes sandy loam (xerollic Camborthid), with a cropping history of Vernal alfalfa and small grain cereal rotations, and was described in detail previously (Jenkins and Bottomley, 1985a). The experimental design consisted of 36 varieties replicated in a randomized complete block design which allowed for sampling of nodules from plants distributed throughout the entire field plot (38 x 53 m). Two plants, inclusive of their root systems to a depth of 0.4 m, were sampled within each of four replicate plots of Anchor, Vernal, and Saranac alfalfa, and were stored at 4^{o}C. Nodule isolates were purified by standard procedures (Vincent, 1970), and cultures originating from single colonies were stored on yeast extract mannitol agar slants under 20% (v/v) glycerol at -30^{o}C until needed.

Identification protocols. Each isolate was challenged against antiserum raised to R. meliloti isolate 31 in whole cell, somatic agglutination tests (Fuquay et al., 1984). Two-fold serial dilutions, ranging from 1/4 to 1/1024, were made in microtiter plates, and reactions were

compared with those of the parent 31 strain after 2 hours of incubation at 39°C.

Symbiotic effectiveness of the serogroup parent, strain 31, on three cultivars of alfalfa. Anchor, Vernal, and Saranac alfalfa were grown in Leonard jar assemblies (Vincent, 1970) over four successive harvests from February to May under greenhouse conditions described previously (Bottomley and Jenkins, 1983). Plants were arranged in a factorial treatment design consisting of the three cultivars in combination with two N sources: either inoculated with the parent isolate of serogroup 31 (-N), or grown with ammonium nitrate (+N) as a source of mineral nitrogen. Six replications were included per treatment. Seedlings grown without either mineral N or inoculation were included to evaluate the possibility of Rhizobium contamination, but were not included in the data analysis. Seeds were surface sterilized, germinated for 24 h on water agar plates, and placed in sterilized Leonard Jars, each containing 100 g of vermiculite. Cells for inoculum were grown in a defined liquid medium (Dughri and Bottomley, 1983), harvested at late log phase, diluted to provide approximately 10^5 cells/ g vermiculite, and a 5 ml portion inoculated into each assembly immediately following planting. Plant nutrient solution was added to the lower half of the assemblies, and contained in mMoles per liter; $CaSO_4 \cdot 2H_2O$ (1.5); $MgSO_4 \cdot 7H2O$, (0.5); K_2SO_4,

(0.25); K_2HPO_4, (0.25); KH_2PO_4 (0.25); Fe citrate·$5H_2O$ (0.015), $CaCl·2H_2O$ (0.04), and 2.5 ml/L of a micronutrient solution (Moore, 1974). Mineral N treatments received the equivalent nutrient solution containing 8 mM NH_4NO_3 commencing 13 d after planting. Seedlings were thinned to two per jar 18 d after planting, and additional paraffinized sand (Vincent, 1970) was added to provide a hydrophobic layer approximately 2.5 cm in depth over the vermiculite surface. Nutrient solutions were monitored routinely for both transpirational loss and pH decrease, and replaced with sterile nutrient solution as necessary. Plants were harvested 4 cm above the crown after 7 weeks of growth. Successive harvests were taken at approximately 24 d intervals when 20% of the total plants had flowered. All herbage samples were analyzed for dry weight and Kjeldahl N content. Following the fourth harvest, whole plants were harvested and nodule number and dry weights determined.

Data were subjected to an analysis of variance. Symbiotic effectiveness, defined as the proportional dry weight of inoculated versus N supplemented plants, was determined by the F test ($P \leq 0.05$) for N source main effects. Comparisons of strain 31 effectiveness on the different cultivars were determined by the cultivar x N source interaction.

Symbiotic effectiveness of field isolates from within and outside of serogroup 31 on Anchor and Vernal.

a) Representatives of serogroup 31, including 8 isolates originally recovered from Anchor nodules and 9 from Vernal, were tested for symbiotic effectiveness on the host cultivar from which they originated in standard plant tube effectiveness tests (Bottomley and Jenkins, 1983). An additional 12, non-serogroup 31 isolates originally recovered from Anchor nodules, were also included in the experiment. Each isolate was challenged on 10 replicate seedlings, with a single harvest following 35 d of growth. b) Symbiotic effectiveness was also examined over successive harvests in open Leonard jar assemblies. Six serogroup 31 strains, and 4 non-serogroup 31 strains originally recovered from Anchor nodules, were challenged on the same host over four harvests from April to July under greenhouse conditions. c) Six serogroup 31 strains from Vernal, two strains from a commercial inoculant manufacturer (102F34 and 102F28), and two serogroup 31 isolates originally recovered from Anchor, and shown to be of mediocre effectiveness on the latter by the plant tube test, were evaluated in open Leonard jar assemblies over three harvests from Aug. to Oct. on Vernal alfalfa. Experimental conditions followed the protocols listed above, except that Kjeldahl N was determined on samples composited over harvest.

RESULTS

Low numbers of nodules were found on plants throughout the field site (data not shown), with a total of 94 isolates recovered into pure culture. Isolates belonging to serogroup 31 were identified from nodules in 11 out of the 12 plot areas sampled, confirming the extensive distribution by serogroup 31 in soil and root nodules at the Powell Butte site (Table 1). Within the 11 plots, nodule occupancy of serogroup 31 ranged from 14 to 75%, with a mean value of 44%. A significant cultivar effect was observed since nodule occupancy by serogroup 31 was approximately two to three fold greater on Vernal compared to Anchor and Saranac. Occupancy on Vernal was consistently high across the four replicate plots. There was a trend for variation between replicates to be greater for the other two cultivars. Inconsistency in the number of isolates recovered from each replicate, however, prevented solid inferences from being drawn.

Under the vigorous growth conditions afforded by the Leonard jar assemblies, the parent strain of serogroup 31 was moderately effective when challenged on the three cultivars (Table 2). Total herbage yield of inoculated treatments ranged between 28.9 and 43.1% of N supplemented plants. There were no differences in symbiotic effectiveness between cultivars over the first two harvests, as seen by the non-significant cultivar x N

source interactions. Significant differences were revealed commencing with the third harvest, however, and resulted in a significant cultivar x N source interaction based on total herbage dry weight. In effect, the Vernal-strain 31 combination resulted in 28 and 31% greater yields respectively than Anchor or Saranac combinations even though the yield of N supplemented Vernal was only 90% of N supplemented Anchor and Saranac. Enhanced symbiotic effectiveness of Vernal plants was accompanied by significantly fewer nodules of larger average weight than the other two cultivars.

Nitrogen content by harvest showed even more emphatic differences between cultivars when inoculated with parent strain 31 (Table 3). Only in the case of Vernal did the amount of N_2 fixed increase significantly between the second and third harvests. Neither Saranac nor Anchor showed any significant increase from the second through the fourth harvest. As a result, Vernal plants, despite assimilating only 86 and 84% as much N as N supplemented Anchor and Saranac, respectively, fixed 33 and 39% more N_2 than the two respective cultivars.

The regrowth characteristics between harvests 2 and 3 identified a critical difference between the development of N supplemented Anchor and Vernal alfalfa (Table 2). A 100% increase in herbage dry weight and N content was observed between the two harvests for Anchor, and resulted in a growth response approximating exponential character

over the first three harvests. In contrast, the growth response of Vernal was less dramatic, with 40 and 34% increases in herbage dry weight and N content, respectively, representing a nearly linear growth response over the first three harvests.

Field isolates of serogroup 31, obtained originally from Vernal nodules, showed a uniform and effective response when challenged to Vernal seedlings in short-term, plant tube effectivness tests (Table 4). In contrast, serogroup 31 isolates from Anchor nodules produced a heterogeneous response which ranged from highly effective to poorly effective when evaluated on Anchor. Additional analysis of 12 non-serogroup 31 isolates failed to identify any organisms which were more effective than serogroup 31 representatives on Anchor seedlings.

The variability in effectiveness among members of serogroup 31 was also observed when evaluated over successive harvests in the more rigorous Leonard jar system. Herbage dry weight and total N deviated significantly with strain on Anchor over all harvests (Table 5). For example, isolates AD3 and AD5 produced 61% of the average yield of the other four serogroup 31 field isolates. Interestingly, the parent isolate of serogroup 31 also fell into this low yield category. The performance of non-31 isolates was consistent over the four harvests with respect to the plant tube effectiveness results, and also illustrated heterogeneity in effectiveness character

on Anchor. For example, strains AA2 and AA3, previously classified as poorly effective, remained in this category over all four harvests, and strains AB8 and AC4, classified as moderately effective, were of intermediate effectiveness throughout the experiment.

A Leonard jar experiment confirmed that all six serogroup 31 isolates originally recovered from Vernal nodules were of uniform effectiveness on Vernal alfalfa over three successive harvests (Table 6). Poor yield by plants inoculated with isolate VA9 during the second harvest resulted in the only non-uniform response in this experiment. Two commercial inoculant strains, 102F28 and 102F34, used extensively by other researchers in alfalfa studies, failed to produced a response significantly different from the indigenous strains.

Evidence was obtained to suggest that the expression of N_2 fixing heterogeneity by serogroup 31 on Anchor was host influenced. Data in Table 7 show that isolates AD3 and AD5 fixed 73 and 51% more nitrogen, respectively, with Vernal than with Anchor as host. In comparison, representative isolates from Vernal nodules, which were uniformily effective in plant-tube experiments on the parent host remained nonsignificantly different from each other in their effectiveness when challenged on Anchor (data not shown).

Table 1. Distribution of R. meliloti serogroup 31 in root nodules of alfalfa cultivars within the field site.

Cultivar		Plot Replicate A	B	C	D	Mean Percent[b]
Vernal	Total isolates	8	8	10	9	60 ± 11
	Serogroup 31[a]	4	6	6	5	
Anchor	Total isolates	7	10	10	6	24 ± 21
	Serogroup 31	1	0	3	3	
Saranac	Total isolates	4	8	10	4	36 ± 11
	Serogroup 31	2	3	3	1	
				lsd (0.05)		20.0

a Isolates which reacted in whole cell agglutination with antiserum 31 to a titer identical to parent isolate 31.

b Statistical analysis based on log transformed data.

Table 2. Comparison of herbage dry weight and nodulation characteristics of alfalfa cultivars over successive harvests when inoculated with strain 31 (-N) or grown with NH_4NO_3 (+N).

Cultivar	N source	Herbage dry weight					Nodule Character	
		Harvest						
		1	2	3	4	Total	Number	Dry weight
		g/pot					#	mg
Anchor	+ N	1.14	2.18	4.33	3.96	11.61	--	--
	- N	0.51	0.92	1.18	0.99	3.60	191.5	84.8
Vernal	+ N	1.34	2.58	3.60	3.20	10.72	--	--
	- N	0.66	1.20	1.45	1.31	4.62	101.5	77.1
Saranac	+ N	1.14	3.02	4.38	3.68	12.22	--	--
	- N	0.59	0.89	1.08	0.96	3.53	195.1	76.5
LSD (0.05)								
Cultivar	(a)	NS	NS	NS	NS	NS	59.2	NS
N source	(b)	0.11	0.42	0.35	0.35	0.73		
a x b		NS	NS	0.60	0.60	1.27		

Table 3. Nitrogen content of herbage from three alfalfa cultivars over successive harvests when inoculated with strain 31 (-N) or grown with NH_4NO_3 (+N).

Cultivar	N source	Kjeldahl N					LSD (0.05)
		Harvest					
		1	2	3	4	Total	
		mg/pot					
Anchor	+ N	60.7	101.8	204.5	232.2	599.2	37.9
	- N	22.6	39.2	44.5	46.5	152.8	7.7
Vernal	+ N	57.9	118.1	157.8	179.2	513.0	31.0
	- N	25.9	48.6	62.7	65.5	202.7	11.2
Saranac	+ N	59.2	143.8	199.3	211.6	613.9	34.2
	- N	23.1	37.3	38.5	46.7	145.6	12.0

Table 4. Symbiotic effectiveness ratings of indigenous R. meliloti strains in the plant tube system.

Host Cultivar	Serogroup	No. of isolates	Effectivess rating[a]		
			E1	E2	E3
			No. of isolates		
Vernal	31	9	0	0	9
Anchor	31	9	3	2	3
Anchor	non-31[b]	12	2	10	0

[a] E1 = shoot dry weights not significantly different ($P < 0.05$) from uninoculated plants.
E2 = shoot dry weights significantly greater than uninoculated plants and less than nitrate supplemented plants.
E3 = shoot dry weights not significantly different from nitrate supplemented plants.

[b] Representative isolates chosen outside of serogroup 31, based on differences in their intrinsic antibiotic resistance characteristics.

Table 5. Yield characteristics of Anchor alfalfa inoculated with strains of *R. meliloti* representing isolates from within and outside of serogroup 31.

Serogroup Identity	Isolate	Herbage dry weight					Total Kjeldahl N
		Harvest					
		1	2	3	4	Total	
		g/pot					mg/pot
31	AD1	0.54	0.87	1.68	1.54	4.63	175.1
	AC6	0.60	0.95	1.70	1.42	4.67	175.0
	AC9	0.55	0.87	1.58	1.21	4.21	161.2
	AC8	0.61	0.86	1.60	1.20	4.27	165.8
	AD3	0.31	0.39	0.96	0.95	2.61	104.8
	AD5	0.32	0.46	0.97	1.04	2.78	119.0
	31 (parent)	0.38	0.56	0.86	1.13	2.93	123.9
unknown	AA2	0.11	0.15	0.47	0.36	1.09	43.0
	AA3	0.14	0.20	0.45	0.47	1.26	52.3
	AB8	0.37	0.59	1.00	0.82	2.78	109.4
	AC4	0.42	0.63	1.30	1.14	3.51	140.4
LSD (0.05)		0.14	0.21	0.34	0.19	0.64	31.3

Table 6. Yield characteristics of Vernal alfalfa when inoculated with field isolates representing serogroup 31 and with commercial inoculant strains of *R. meliloti*.

Serogroup identity	Isolate	Herbage dry weight				Total Kjeldahl N
		Harvest 1	Harvest 2	Harvest 3	Total	
		g/pot				mg/pot
31	VA9	0.40	0.57	0.99	1.96	79.3
	VA6	0.39	0.74	1.10	2.23	88.6
	VD3	0.32	0.70	1.08	2.10	85.3
	VB1	0.40	0.84	1.21	2.45	101.1
	VA7	0.43	0.86	1.25	2.54	101.6
	VD2	0.38	0.76	1.06	2.20	87.6
	31 (parent)	0.42	0.83	1.27	2.56	109.9
Commercial	102F28	0.30	0.73	1.31	2.34	96.7
	102F34	0.49	0.83	1.04	2.36	101.8
LSD (0.05)		NS	NS	NS	NS	NS

Table 7. Comparison of symbiotic effectiveness of two serogroup 31 isolates on Vernal and Anchor alfalfa.

Host	Serogroup 31 Isolate	Total Kjeldahl N			
		Harvest			
		1	2	3	Total
		mg/pot			
Anchor	AD3	12.7	18.8	34.1	65.6
	AD5	11.6	20.6	41.2	73.4
Vernal	AD3	20.0	36.3	57.2	113.5
	AD5	23.7	33.4	53.4	110.5

DISCUSSION

Serogroup 31 represents a highly competitive, naturalized group within a diverse R. meliloti population. Over a period of four years, members of this serogroup have now been identified as dominant nodule occupants on different cultivars, and shown to be more widely associated thoughout the soil at the field site than previously described (Jenkins and Bottomley, 1985a and b). Bromfield et al. (1986) have also observed that alfalfa nodules at particular field sites can be dominated by specific R. meliloti groups within diverse soil populations, and that nodule occupancy is influenced by alfalfa cultivars. Both studies confirm the possibility of identifying competitive, indigenous R. meliloti for comparison with well-characterized laboratory and inoculant strains.

In our study, Vernal possessed the highest level of dominance by serogroup 31 and also had the least plant to plant variation. It has been suggested recently that inter-plant variation in nodule occupancy of Medicago and Melilotus decreases when either genus is sown in soil with an homologous cropping history (Thurman and Bromfield, 1988). It is therefore of interest to note that Vernal has been the cultivar of choice on this particular site for the past twenty-five years as a result of its winter hardiness traits. Traditionally it has been the standard

against which other cultivars are evaluated in field trials. In addition to the nodule occupancy data, the uniformity in effectiveness response by serogroup 31 on Vernal, in contrast to Anchor, provided further evidence for superior control by Vernal over the symbioses established with this particular soil population of R. meliloti. Evidence for host control of N_2 fixation was established when representatives of serogroup 31 originally recovered from Anchor, and of intermediate effectiveness on the latter, showed improved N2 fixing potential on Vernal. Furthermore, since the serogroup 31 isolates originating from Vernal nodules expressed uniform effectiveness on both cultivars, the data also support the possibility that Vernal, in contrast to Anchor, was infected under field conditions with the more effective subpopulation of serogroup 31.

The inability of inoculated plants to produce the equivalent dry matter yield and N content of N supplemented plants was evident for all cultivars. Previous studies have also found R. meliloti strains incapable of producing yields equivalent to mineral N controls under greenhouse and controlled environment conditions (Bottomley and Jenkins, 1983; Kapulnik et al., 1986; McGregor, 1983; Phillips et al., 1982; Phillips et al., 1985; Teuber and Phillips, 1988). Positive responses to N fertilization have also been reported for field grown

alfalfa during the second and third harvests of the growing season (Jenkins and Bottomley, 1984). In comparison, Fishbeck and Phillips (1981) found that alfalfa plants supplemented with mineral N had increased dry matter yields over symbiotically dependent plants during the first two harvests. Subsequent differences between N supplemented and inoculated treatments, however, diminished and were not significantly different by the fourth harvest. Our results failed to detect such large scale improvement in effectiveness over successive harvests. This was especially evident in the inability of strain 31 to meet the increasing host demand for N between harvests 2 and 3 with Anchor and Saranac. Strain 31 inoculated Vernal was capable of a greater response at this growth stage, and provided the only evidence of a trend similar to that reported by Fishbeck and Phillips (1981).

An additional concern raised in this study is the wide-spread use of plant-tube systems in determination of R. meliloti strain effectiveness. Gibson (1967) showed plant growth in this system became carbon limited rather rapidly. Our data also suggest that N supplemental control plants do not express their true growth potential in such a system and therefore the symbiotic effectiveness of isolates is overestimated. Comparisons between the closed test-tube system and the more rigorous Leonard jar

assembly, however, suggest the strength of the closed system relies on its ability to cluster or separate strains based on their relative effectiveness.

The findings of this study present practical implications for both laboratory and field studies. For example, new cultivars might not produce to their yield potential under field conditions if the dominant nodule occupying rhizobia show inferior effectiveness per se, or if they are not in physiological synchrony with the plant in its demand for N. These findings obviously raise questions about the criteria for choosing strain/cultivar combinations and suggest that more physiology and biochemistry should be focused on the symbiosis after the second harvest has been taken.

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CHAPTER III

Growth and Nodulation Responses of *Rhizobium Meliloti* to Water Stress Induced by Permeable and Impermeable solutes

ABSTRACT

Isolates of Rhizobium meliloti, representing antigenically distinct indigenous serogroups 31 and 17, were grown in yeast extract mannitol broth (YEM) containing NaCl or polyethylene glycol (PEG) to provide external water potentials ranging from -0.15 to -1.5 MPa. Several differences were found between representatives of the two groups in their ability to adapt to water stress induced by the impermeable solute, PEG. At potentials below -0.5 MPa, strain 31 had a lower specific growth rate than strain 17 which was accompanied by irregular cell morphology. In contrast, neither growth nor cell morphology of either strain was affected significantly over the same range of water potentials created by the permeable solute, NaCl. Despite strain 17 showing superior growth at low water potentials imposed by PEG, upshock of water stressed cells (-1.0 MPa PEG) into normal YEM (-0.15 MPa) resulted in a faster recovery of growth by strain 31 than strain 17. Different responses of the two strains to a water potential increase were also revealed in nodulation studies. Strain 31 required significantly fewer days to nodulate alfalfa compared with strain 17 when transferred from YEM at -1.0 MPa PEG onto the roots of alfalfa seedlings in plant growth medium (-0.1 MPa). The addition of supplemental calcium (0.1mM) to -1.0 MPa PEG

growth medium reduced the differences between strains in their response to water stress. The severe growth restriction and morphological abnormalities shown by strain 31 were corrected, and the prolonged recovery time shown by strain 17 upon transfer to normal YEM was shortened. The latter strain also nodulated earlier and more rapidly after growth at -1.0 MPa PEG in the presence of supplemental calcium ions. These results indicate that the efficacy of osmoregulation can vary between strains of the same species, and that the mechanisms of osmoregulation differ depending on the nature of the water stress.

INTRODUCTION

The majority of physiological studies of osmoregulation by heterotrophic bacteria use permeable solutes to lower the water potential of the suspending growth medium (Yancey et al., 1982; Le Rudulier et al., 1984). Osmotic adjustment under these conditions depends, in part, on external solute uptake to regain water potential equilibrium. Under non-saline soil conditions, water potential is controlled predominantly by capillary phenomena. As a result, soil bacteria must osmotically adjust to shifts in water potential without significant assistance from external, permeable solutes (Papendick and Campbell, 1981; Harris, 1981). Plant and soil scientists have recognized a need to study water relations under conditions where water stress is induced in a manner realistic of that imposed under soil conditions. Polyethylene glycol (PEG) has been used in plant (Kaufmann and Eckard, 1971; Fisher, 1985; Zahran and Sprent, 1986) and bacterial (McAneney et al., 1980) studies based on its ability as an impermeable solute to lower the external water potential without penetrating the cell wall.

Several studies have shown the capability of Rhizobium sp. to survive at low water potential (Worrall and Roughley, 1976; van Rensburg and Strijdom, 1980; Mahler and Wollum, 1981; Al-Rashidi et al., 1982; Mary et al., 1986; Fuhrman et al., 1986). Few studies, however, have

addressed the importance of a rapid water potential increase brought about by a re-wetting process (Bushby and Marshall, 1977; Salema et al., 1982). Kieft et al. (1987) have recently identified a significant turn-over of microbial biomass during re-wetting of field soils. It is unknown what impact a rapid water potential change might have on soil Rhizobium populations both under free-living conditions and during the period of establishment of the symbiosis. The objectives of this research were to compare the growth responses of R. meliloti isolates to decreases in water potential imposed by PEG or NaCl, and to evaluate the influence of a rapid increase in water potential on both the growth characteristics and nodulating kinetics of the isolates on alfalfa seedlings.

MATERIALS AND METHODS

Rhizobium strains and growth media. Representative isolates of R. meliloti serogroups 31 and 17 were chosen for study based on their antigenic differences (Fuquay et al., 1984) and their competitive (serogroup 31) and non-competitive (serogroup 17) capabilities in nodule occupancy of field-grown alfalfa (Jenkins and Bottomley, 1985a). The strains were maintained as described previously (Jenkins and Bottomley, 1985b), and were routinely grown in yeast extract mannitol broth (YEM) to serve as inoculum for the water stress experiments.

Response of R. meliloti to decreased water potential. i) Growth. A range of water potentials was established in YEM broth with the addition of either NaCl or PEG 4000 (J.T.Baker, lot # A42733). Metal contamination in the PEG was found to be insignificant after a sample was ashed and analyzed for cation content using an inductively coupled argon plasma spectrometer. The osmolality values of NaCl and PEG solutions were determined with a vapor pressure osmometer (Westcor model 5100C, Logan, Utah) and converted to water potential values using the Van't Hoff equation. Water potential values for PEG 4000 were considered approximations due to the failure of PEG solutions to specifically adhere to the Van't Hoff equation (Steuter et al., 1982). The final potentials were chosen to reflect a

range of realistic soil water potentials under which leguminous seeds might germinate and be susceptible to nodulation. Values included were -0.15 (YEM broth minus solute), -0.25, -0.50, -0.75, -1.0, and -1.5 MPa. All PEG solutions were autoclaved separately from YEM. Three replicate growth tubes (20 by 2.5 cm), each containing 35 ml of YEM were included for each isolate at each water potential, and the experiments were repeated at least twice. Growth was followed at 30^{o} C in a temperature controlled water bath, with cultures receiving a continuous supply of filter sterilized air. Inoculum densities were 10^{6} cells/ml, and turbidity of the cultures was determined at 3 h intervals at 660 nm until late exponential phase. Treatments which failed to enter exponential growth were monitored for at least 72 h and then plated on YEM supplemented with NaCl (-1.0 MPa) to determine viability. Cultures grown at -1.0 MPa PEG or NaCl were back transferred into isotonic YEM and growth rates measured to determine whether the growth observed might have resulted from enrichment of spontaneous mutants. The growth response of isolates to water stress was similar at 25^{o} C, and also when KCl was substituted for NaCl.

ii) Cell morphology. Strains 17 and 31 were observed microscopically to determine whether exposure to low water potentials had resulted in modified cell morphology.

Cultures were grown in YEM at -1.0 MPa PEG or NaCl for 72 h, and aliquots were diluted with isotonic NaCl solutions to provide 50-100 cells per microscopic field when viewed at one thousand times magnification. Two ml subsamples were stained for 5 min with 0.05% (w/v) acridine orange, filtered onto 0.2 um pore size filters (Nuclepore, Pleasanton, CA), and examined by epifluorescence microscopy (Demezas and Bottomley, 1986). Cell dimensions were compared with those from cultures grown in YEM without additional solutes.

iii) Influence of organic and inorganic supplements on growth response under water stress. Cultures growing under PEG water stress failed to achieve the cell densities of control cultures at stationary phase. As a result, supplements were added to YEM broth to determine if inadequate energy or other nutritional factors were contributing to the limited growth response under water stress. Substrates compared in separate experiments included mannitol (10, 15, and 20 g per liter); $CaCl_2 \cdot 2H_2O$ (0.025, 0.1, 0.25, 0.5 mM); $MgSO_4 \cdot 7H_2O$ (0.8, 1.6, 2.4 mM); K_2HPO_4 (2.3, 4.6 mM). The ability of the osmoprotectant glycine betaine to assist in osmoregulation during permeable and impermeable solute stress was evaluated. Strains 17 and 31 were grown in YEM, with or without glycine betaine (1mM), at low water potentials created by PEG (-1.0 MPa) or NaCl (-2.0 MPa). It was

necessary to reduce the water potential of the NaCl treatment to -2.0 MPa to obtain conditions which sufficiently restricted the growth rate of the isolates. Inoculum densities and monitoring of growth rates were as described above.

iv) Mineral cation content of bacterial cells. The K^+, Ca^{2+}, and Mg^{2+} contents of cells of strains 17 and 31 were measured when grown under PEG-stressed, NaCl stressed, or non-stressed conditions to determine if the growth differences observed between strains might correlate with different nutrient uptake. A minimum of 12 separately grown cultures per treatment were combined to provide sufficient dry weight for analysis of K^+, Ca^{2+}, and Mg^{2+} by atomic absorption spectrophotometry. Since strain 31 showed limited growth at -1.0 MPa PEG, four liters of culture were grown. All cultures were washed twice in isotonic saline solution and cell pellets dried at 55^oC for 36 h. Replicate samples of each treatment were retained for dry weight determinations.

R. meliloti growth response to an increase in water potential. The growth responses of strains 17 and 31 were determined following an increase in water potential from -1.0 to -0.15 MPa. Cultures were grown in YEM at -1.0 MPa created by either PEG or NaCl and inoculated into YEM

(-0.15 MPa) at final densities of 10^6 cells/ml. The inoculum densities were based on the total number of viable cells as determined by dilution plating on isotonic YEM medium solidified with 1.5% w/v Bacto agar. Growth was monitored turbidimetrically every 3 h following the upshock of cells.

Nodulation kinetics of R. meliloti following an increase in water potential. Strains 17 and 31 were grown in the following media: i) YEM, ii) YEM plus PEG (-1.0 MPa), and iii) YEM plus PEG (-1.0 MPa) and $CaCl_2 \cdot 2H_2O$ (0.1mM). Cultures were diluted with sterile, isotonic NaCl solutions, plated on YEM medium to determine viability, and 1.0 ml portions were inoculated onto roots of 'Vernal' alfalfa seedlings to provide approximately 10^4 viable cells/seedling. The seedlings were grown in large test tubes (30 x 3.2 cm) containing 25 ml of plant nutrient agar (Bottomley and Jenkins, 1983) at a water potential of -0.1 MPa solidified with 1% w/v Bacto agar. Seven seedlings were included per replication, with 3 replications of each treatment arranged in a completely randomized experimental design and grown under greenhouse conditions as previously described (Bottomley and Jenkins, 1983). Kinetics of nodulation were monitored at daily intervals and the experiment was repeated twice under similar experimental conditions.

RESULTS

Influence of low water potential on growth kinetics.

The specific growth rates of isolates of serogroups 17 and 31 in YEM were dependent on the solute used to create the range of water potentials. Lowering the water potential with NaCl to -1.5 MPa resulted in only moderate declines in specific growth rates of either serogroup (Fig 1). In contrast, low water potentials created by the impermeable solute, PEG, resulted in a more dramatic and differential decrease in the growth rates of both serogroups (Fig. 1). As water potentials decreased, serogroup 17 isolates showed consistently higher growth rates than members of serogroup 31, with the differences between the serogroups most pronounced at -1.0 MPa. Although neither isolate was able to grow at -1.50 MPa PEG, cells from the initial inoculum of both serogroups were found to have maintained their viability after 72 h of water stress when plated on YEM agar with supplemental NaCl (-1.5 MPa). Backtransfer of cultures from -1.0 MPa PEG into the same medium resulted in identical growth kinetics, indicating that growth under water stress was not the result of enrichment of spontaneous mutants during the initial exposure to water stress (data not shown).

Influence of low water potential on cell morphology.

Microscopic observation of cultures grown at -1.0 MPa PEG

indicated that cells of strain 31 had undergone gross morphological change. Individual cells were irregular in shape, and their average length increased from 1.7 um for non-stressed cells to 3.9 um for PEG stressed cells. In contrast, strain 17 showed minor change in morphology, increasing from 1.5 um for non-stressed to 2.0 um for stressed cells. Growth of either strain at -1.0 MPa NaCl resulted in negligible changes in cell size (1.4 um).

A role for supplemental calcium in adaption to water stress. I consistently observed that the optical densities of cultures of water-stressed cells at stationary phase were substantially less than those of non-stressed cells. I suspected an inadequacy of the YEM medium to meet the growth requirements of the cells under PEG water-stressed conditions. In support of this possibility, cells of both strains reached high optical densities at -1.0 MPa PEG when grown in a defined, glutamate-mannitol medium. Supplemental mannitol, glutamate, and K^+ were ruled out in separate growth rate experiments as the causative agents (data not shown). Calcium, which is supplemented into defined medium at a concentration of 0.5 mM, in comparison with a concentration of 60uM in YEM (supplied by yeast extract), was found to be the critical element. The addition of supplemental calcium to YEM medium at concentrations as low as 0.1 mM increased the growth rate of strain 31 at

-1.0 MPa PEG (Fig. 2). The improvement in growth for strain 17 was less dramatic, and resulted in comparable growth rates for the two strains. In addition, strain 31 cells, grown at -1.0 PEG with 0.1mM supplemental Ca^{2+}, were not enlarged or distorted as previously observed. A general divalent cation effect was ruled out since magnesium ion could not substitiute for the Ca^{2+} response by strain 31 (data not shown).

The calcium contents of strains 17 and 31 were similar when grown in normal YEM, or in YEM with PEG or NaCl (Table 1). The calcium content was greater in cells of both strains when grown at -1.0 MPa PEG supplemented with 0.1mM calcium than in cells grown in normal YEM with the same quantity of supplemental calcium. Both strains increased their K^+ concentrations when stressed with NaCl. In contrast, PEG stressed cells did not accumulate K^+ to greater concentrations than control cells (Table 1). To further emphasize the differences in osmoregulatory adjustment to permeable and impermeable solutes, glycine betaine shortened the generation times of both strains an average of 34% when they were grown at -2.0 MPa NaCl, yet failed to influence their growth rates at -1.0 MPa PEG (data not shown).

Growth recovery following a water potential increase.

Strain 31 responded to an increase in water potential 10 to 12 h faster than strain 17 (Fig. 3a). The presence of

supplemental Ca^{2+} during the water stress growth period had little effect on strain 31 recovery rate, but reduced the lag period of strain 17 by approximately 15 h (Fig. 3b). No differences between strains were found in their growth response following an increase in water potential from -1.0 MPa NaCl (data not shown).

Influence of growth at low water potential on nodulation. Only slight differences in the kinetics of nodule formation were observed between the two strains when inocula were grown under non-stressed conditions. A slightly higher percentage of seedlings were nodulated at day 7 by strain 17 compared with strain 31. In contrast, PEG stressed cells of strain 31 nodulated the seedlings 48 h earlier than strain 17 following the increase in water potential, and subsequent nodulation developed more rapidly (Fig. 5). The presence of supplemental calcium during growth of strain 17 at -1.0 MPa PEG was sufficient to correct this delay. Of interest was a consistent observation that cells of strain 31, grown initially at -1.0 MPa PEG with or without supplemental Ca^{2+}, nodulated seedlings approximately 24 h faster than normal YEM-grown cells.

The viability of strain 31 was not influence by an increase in water potential, whereas viability of strain 17 decreased ten fold in response to an increase in water potential (Table 2). Similar results were observed in a

plant bioassay (data not shown). This loss in viability could have influenced both the delay in growth and nodulation following upshock of strain 17. The inoculum size used in the nodulation experiment ensured a minimum of 10^3 viable cells per seedling following the water potential increase. However, the delay in nodulation could not be attributed solely to a loss of cell viability since nodulation by strain 17 was delayed to a similar extent when inoculum size ranges from 10^1 to 10^4 per ml were utilized (data not shown).

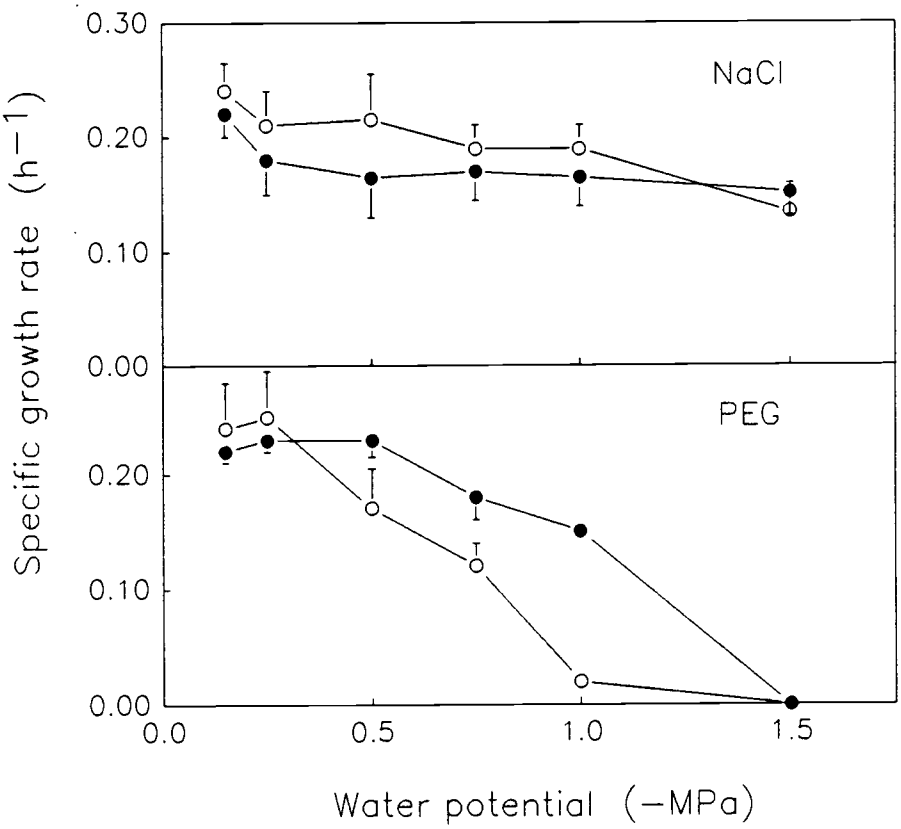

Figure 1. Growth response of strains 17 and 31 to water stress imposed by increasing concentrations of either NaCl or PEG. Symbols: (●) strain 17; (○) strain 31.

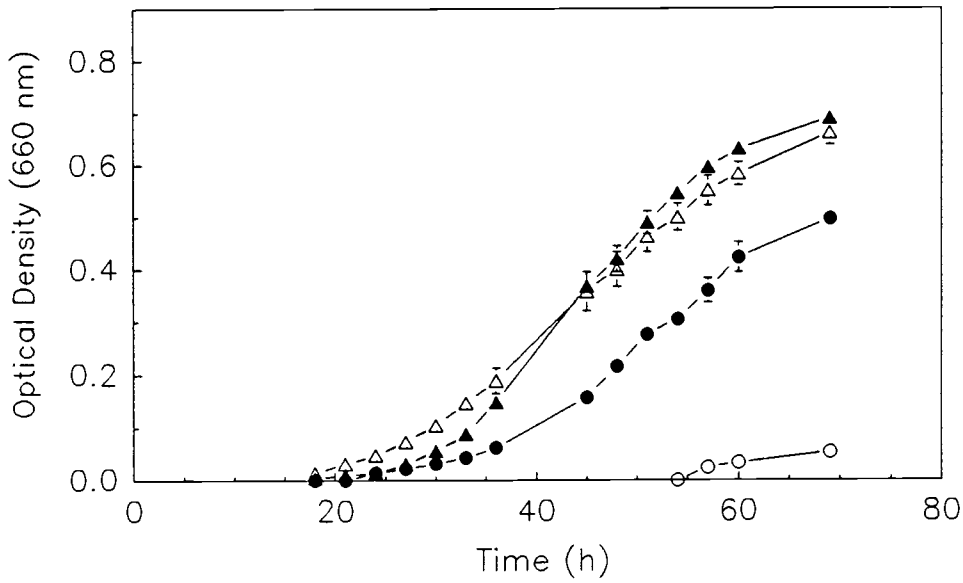

Figure 2. Growth response of strains 17 and 31 in -1.0 MPa PEG to the addition of 0.1mM calcium. Symbols: (●) 17; (Δ) 17 + calcium; (○) 31; (▲) 31 + calcium.

Table 8. Influence of permeable and impermeable solutes on the cation content of R. meliloti strains 17 and 31 grown in YEM.

Strain	YEM supplement[a]	K+	Ca++	Mg++
		g of cation (kg dry wt)$^{-1}$		
17	none	3.5	0.4	1.3
	calcium	4.9	0.6	1.2
	PEG	1.7	0.5	0.4
	PEG + Ca^{2+}	5.3	1.0	0.6
	NaCl	9.3	0.5	0.8
31	control	4.8	0.4	1.3
	calcium	5.0	0.6	1.3
	PEG	5.0	0.4	m.d.[b]
	PEG + Ca^{2+}	3.7	1.1	0.7
	NaCl	8.6	0.5	1.0

[a] Calcium was supplemented to YEM at 0.1 mM. Both PEG and NaCL were added to provide water potentials of -1.0 MPa.

[b] Missing data.

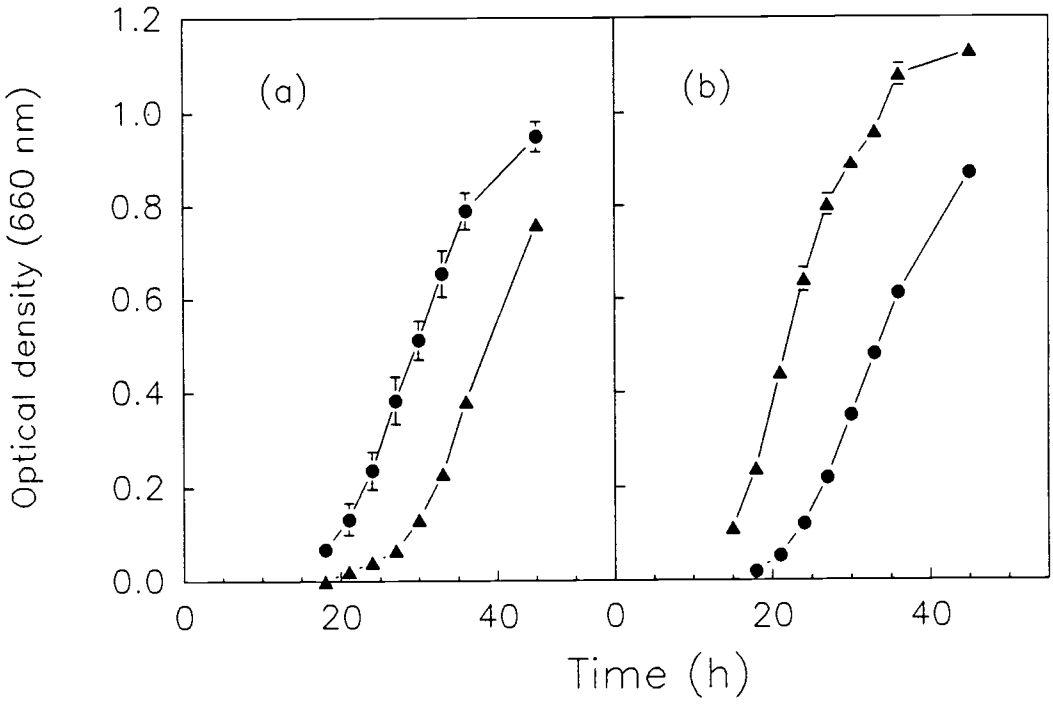

Figure 3. Growth response of strains 17 and 31 to a water potential increase from -1.0 to -0.15 MPa. Cultures were grown initially at -1.0 MPa PEG in the absence (a) or presence (b) of 0.1mM calcium. Symbols: (▲)strain 17; (●) strain 31.

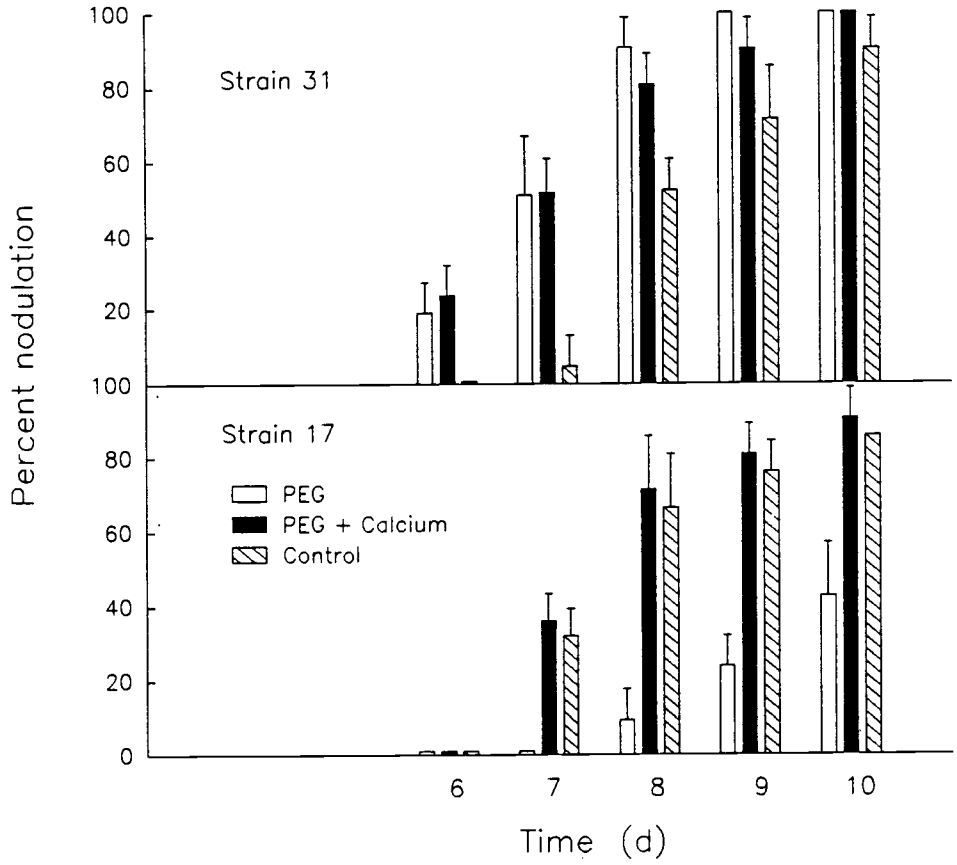

Figure 4. Nodulation kinetics of strains 17 and 31 following a water potential increase. Inocula were grown at -1.0 MPa PEG in the presence or absence of 0.1mM calcium and transferred onto alfalfa seedlings growing in mineral salts agar (-0.1 MPa).

Table 9. Viability of strains 17 and 31 following an increase in water potential from -1.0 MPa PEG to -0.15 MPa.

	Plate count[a]	
Strain	YEM (-1.0 MPa)	YEM (-0.15 MPa)
	x 10^6 cells/ml	
17	2.3 ± 1.1	0.2 ± 0.1
31	5.1 ± 1.4	4.8 ± 1.5

[a] Cultures were plated following 72 h growth on either YEM (-0.15 MPa, unprotected) or YEM + 0.22 M NaCl (-1.0 MPa, protected).

DISCUSSION

The data presented in this manuscript have implications both to the importance of water relations in the success of microsymbionts in the free-living state and during the establishment of a symbiosis. Furthermore, they have general applicability to the study of osmoregulation by gram negative bacteria. Most studies on the molecular mechanisms of osmoregulation by procaryotes evaluate responses to water stress induced by solutes freely permeable through the cell wall (Le Rudulier et al., 1984; Yancey et al., 1982). In addition, a few well characterized laboratory strains of Enterobacteriaceae have received the majority of attention. The ability of isolates of both serogroups 17 and 31 to grow at low water potentials induced by NaCl was not unexpected since Rhizobium meliloti is credited with growth in medium containing 2% NaCl (Jordan, 1984). Additionally, both the increase in intracellular K^+ content (Table 1) and stimulation of growth rate by glycine betaine are in agreement with other observations on osmoregulatory responses by gram negative bacteria to NaCl induced water stress (Epstein and Schultz, 1965; Epstein, 1986; Landfald et al., 1986; Sutherland, 1986; Smith et al., 1988; Jovanovich et al., 1988). However, our findings that growth under water stress induced by an impermeable solute is inferior to growth in the presence of a permeable

solute, in conjuction with the inability of either glycine betaine to remediate the growth rate or for cellular K^+ content to increase under PEG induced water stress supports the suggestions of Harris (1981) that more attention be focused on osmoregulatory mechanisms relevant to water stress conditions associated with the soil environment. Furthermore, the failure of different serogroups within a species to respond uniformily to an impermeable solute induced water-stress suggests caution is needed in the choice of strain utilized in studies where the modeling of osmoregulation is the goal.

The role of calcium in osmoregulation by R. meliloti merits comment. Although Vincent (1962) showed that calcium requirements for Rhizobium growth were low (25 uM), several reports have shown that calcium requirements can be much higher for nodulation (Loneragan and Dowling, 1958; Lowther and Loneragan, 1968; Munns, 1970), growth in the presence of low phosphate (Beck and Munns, 1985), and complete expression of surface antigens (Humphrey and Vincent, 1965; Vincent and Humphrey, 1968; Fuquay et al., 1984). Our findings suggest water stress from an impermeable solute increased the absolute requirement of calcium for growth of strain 31 and for improving the tolerance of strain 17 to a water potential increase. Although we did not localize the site of calcium accumulation, Vincent and Humphrey (1963) found that calcium was more concentrated in the cell wall of R.

trifolii than in the cytoplasm, and Vincent and Colburn (1961) found calcium deficient cells of R. trifolii lost cell wall rigidity which was associated with swollen cell morphology. Consistent with these findings, strain 31 showed modifications in cell wall morphology when grown without supplemental calcium. More recently, a specific role for calcium has been proposed in stabilizing outer membrane architecture in gram negative bacteria (Berenguer et al., 1988; Wee and Wilkinson, 1988). Taken together these findings indicate more attention be given to the interactions of calcium and cell wall architecture in studies of the osmoregulatory response by R. meliloti to impermeable solutes.

In conclusion we would like to discuss our findings in relation to competitive nodulation between strains of Rhizobium. It is well documented that rhizobia are capable of survival under low water potential (Mahler and Wollum, 1982; Fuhrmann et al., 1986). However, as Kieft et al. (1987) pointed out, water potential increases, coincidental with the return of favorable growth conditions for plants and bacteria, are likely to have a more important effect on bacteria which inhabit surface soils. Our data revealed that strain 17 took 48h longer than strain 31 to nodulate alfalfa seedlings when exposed to a water potential increase. This time difference is of particular interest since data from split-root experiments have shown that seedlings of soybean (Kosslak et al.,

1983; Pierce and Bauer, 1983; Kosslak and Bohlool, 1984) and subclover (Sargent et al., 1986) inoculated with a primary strain 48 h prior to a second strain suppress nodulation by the delayed inoculant. Perhaps the superior ability of members of serogroup 31 to respond to an increase in soil water potential contributes to their ability to dominate nodule occupancy of alfalfa under field conditions (Jenkins and Bottomley, 1985a).

ACKNOWLEDGMENTS

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CHAPTER IV

Unusual Serological Phenomena Encountered During the Development of Serogroup and Strain Specific Immunofluorescent Conjugates for *Rhizobium meliloti*

ABSTRACT

The findings reported in this study verify the difficulties and unique characteristics encountered when studying R. meliloti serology. Standard procedures, successfully used with other bacteria to produce intra-species specific fluorescent conjugates, were not applicable to antiserum raised to R. meliloti serogroup 31. Several novel phenomena, previously unreported in studies of Rhizobium serology, were shown to hinder the production of specific, functional conjugates. Precipitation and denaturation of agglutinating specific immunoglobulins occurred during dialysis against low ionic strength buffers used for separation of immunoglobulin from other serum proteins on DEAE Affigel Cibacron Blue, and at other stages in the procedure. Loss of antigen-antibody affinity occurred after FITC conjugation to the agglutinating immunoglobulin. Commercially available goat anti-rabbit immunoglobulins targeted to immunoglobulin classes M and G did not effectively recognize the agglutinating antibody bound to cells, suggesting that either the agglutinating antibody did not belong to these immunoglobulin classes, or had poor affinity with the commercial products tested. An indirect fluorescent methodology, using the biotinylated agglutinating antibody and fluorescein labeled avidin was developed which successfully detected and discriminated members of

serogroup 31 from other strains. Using this technique, members of serogroup 31 were found to represent approximately 17% of the total R. meliloti population in soil samples taken from fields with a recent history of alfalfa cultivation. Although bacteroids in nodules of 17 d old seedlings reacted strongly in immunofluorescence, bacteroids on 28 d old plants failed to react with the biotinylated antibody, suggesting modification, substantial reduction, or loss of the agglutinating antigen occurred during bacteroid maturation.

INTRODUCTION

Immunofluorescence techniques have provided invaluable insight in the study of soil-borne populations of Bradyrhizobium and Rhizobium in regard to both their saprophytic and competitive nodulating abilities on leguminous plants (Schmidt, 1974; Robert and Schmidt, 1983; Moawad et al., 1984; Demezas and Bottomley, 1986). The first preparations of fluorescent antibodies to somatic antigens of B. japonicum (Schmidt et al., 1968) involved the use of procedures already well established from studies of pathogenic microorganisms (Coons et al., 1942; Moody et al., 1963; Lewis et al., 1964). Subsequently, these procedures were found to be successful with other Rhizobium species, including R. leguminosarum (Bezdicek and Donaldson, 1980), Rhizobium sp. (Leucaena) (Moawad and Bohlool, 1984), Rhizobium sp. (chickpea) (Kingsley and Bohlool, 1983), R. trifolii (Trinick, 1969; Jones and Russell, 1972), R. fredii (Sadowsky et al., 1987), and R. phaseoli (Robert and Schmidt, 1983). In contrast, serological methods have been consistently troublesome to researchers of R. meliloti. Problems have been reported in the production of strain specific antiserum (Humphrey and Vincent, 1975), high variability of animal immune system response (Kishinevsky and Gurfel, 1980), instability of specific antigens during storage and growth of cultures (Dudman, 1964; Wilson et al., 1975;

Fuquay et al., 1984), and anomalous behavior of antigen-antibody reactions in standard serological tests (Gibbins, 1967; Fuquay et al., 1984).

Fuquay et al. (1984) identified serogroup specific, whole-cell agglutinating antibodies in antisera raised to R. meliloti serogroup 31. Evidence for the existence of common antigens shared with non-agglutinating R. meliloti strains was revealed by immunoprecipitin reactions. Previous reports also suggested that agglutinating antibodies of R. meliloti can be strain specific (Dudman, 1964; Humphrey and Vincent, 1975; Olsen et al., 1982). At the outset, my primary objective was to prepare a fluorescein labeled, immunoglobulin conjugate which was specific for R. meliloti serogroup 31. As the research progressed, certain novel characteristics were observed that I wish to share with other Rhizobium researchers who might have encountered anomalous problems when following standard serological procedures. Furthermore, I report on an indirect immunofluorescence procedure involving biotinylated immunoglobulin and fluorescein-avidin which holds promise for immunofluorescence studies of R. meliloti in soil systems.

MATERIALS AND METHODS

Bacterial strains and media: R. meliloti serogroup 31, which includes strains 21, 27, and 31, was chosen as the test serogroup based on its highly competitive nodulating capabilities on field-grown alfalfa (Jenkins and Bottomley, 1985a). All bacterial strains used in this study are listed in Table 1. With the exception of the miscellaneous strains, which were cultured on brain heart infusion or nutrient agar slants, all strains were maintained on yeast extract-mannitol (YEM) agar slants and grown and prepared for serological tests as described previously (Dughri and Bottomley, 1983).

Serological tests: Gel-immune diffusion and whole cell, somatic agglutination tests were used throughout this study to verify the specificity of various antibody preparations. Serological methods followed the procedures of Fuquay et al. (1984).

Production of a fluorescein-labeled, immunoglobulin conjugate from unadsorbed, whole antiserum to R. meliloti strain 31: Antiserum, previously raised to serogroup 31 and described in detail elsewhere (Fuquay et al., 1984), was confirmed to be agglutination specific. A fluorescein-labeled, immunoglobulin conjugate was produced by the procedures outlined elsewhere (Banner, et al., 1982).

To evaluate the species and strain specificity of the conjugate, cultures of Rhizobium, Bradyrhizobium, and Agrobacterium were grown to late exponential phase in YEM broth, washed twice with 0.15 M phosphate buffered saline (pH 7.2), diluted with distilled water to 10^7 cells per ml, and heat fixed smears were prepared. Duplicate samples of cells were heat treated at 95° C for 40 min. prior to washing and smear preparation, to address the concerns of Sadowsky et al. (1987) who reported inconsistent staining of Rhizobium fredii with fluorescent antibodies unless cells were pre-heated. All smears were pre-stained with gelatin-rhodamine conjugate (Bohlool and Schmidt, 1968) to suppress non-specific fluorescence and allowed to dry at 60° C. The conjugate was diluted eighty-fold in 0.02 M phosphate buffer (pH 7.2), since this dilution was pre-determined to give maximum fluorescence with cells of strain 31. Smears were stained for 30 min followed by destaining for 25 min with 0.02 M phosphate buffer. Smears were evaluated for cross-reaction by the intensity of fluorescence on a 0-4 scale (0 = no reaction, 4 = maximum fluorescence) with a Zeiss Standard microscope equipped with a tungsten halogen lamp operating in the epifluorescent mode.

Adsorption studies: Due to the lack of serogroup specificity of the fluorescent conjugate, experiments were conducted to remove cross-reactive antibodies from the

antiserum. Cultures of the non-agglutinating, cross precipitating R. meliloti strain 17 were grown in 100 ml of glutamate-mannitol broth, washed 3x in 0.15 M PBS, and resuspended in 4 ml of antiserum. The suspension was incubated for 15 min. at 37° C, and the antiserum was recovered by centrifugation at 3,000 rpm for 10 min. Following the fifth adsorptive cycle the antiserum was filter sterilized through 0.4 um polycarbonate filters (Nuclepore, Pleasanton, CA) and stored at -20° C. Adsorption with sonicated and heat treated cells (95° C for 40 min) of strain 17 was also evaluated. Adsorbed and unadsorbed antisera were compared in both whole-cell agglutination and gel-immune-diffusion tests to evaluate the heterologous and homologous antibody reactions.

As a result of the above experiments, a large sample (15 ml) of antiserum 31 was adsorbed eight times with sonicated cells of strain 17, and a fluorescent conjugate prepared. An alternate approach was also taken whereby the modified procedure of Robert and Schmidt (1985) was used to adsorb the fluorescein-labeled conjugate prepared previously from unadsorbed antiserum. A small volume (0.1 ml) of the conjugate was diluted ten-fold with 0.02 M PB, incubated with the equivalent of 100 ul packed cell volume of sonicated cells of strain 17 for 15 min. at 20° C, and separated by centrifugation. The adsorptive cycle was repeated until the heterologous cross-reaction was no

longer detected by immunofluorescence on smears of strain 17.

Loss of agglutination titer: Neither conjugate produced with adsorbed or unadsorbed antiserum possessed agglutinating activity against whole cells of strain 31. The possibility was raised that the specific agglutinating antibody had been lost during the separation of immunoglobulin from other serum proteins. To identify the factor(s) responsible for these observations, the agglutination titer of antiserum 31 was monitored during each step of the procedure. Samples (1.0 ml) of whole antisera to strain 31 were dialyzed against the four buffers recommended for use at various stages in the standard procedure. These included 0.02M Tris-HCl, pH 8.2, containing either 0.028 or 0.15M NaCl; 0.02M sodium phosphate, pH 6.5; and 0.15M sodium bicarbonate/carbonate, pH 9.0. After 24 h of dialysis with four changes of buffer (1 liter total), the agglutinating titer was compared with that of non-dialyzed antiserum. The effect of dialysis on the agglutinating titer of antisera from other members of serogroup 31 (strains 21 and 27) and from other agglutinating specific R. meliloti strains (17 and 41) was also evaluated.

Separation of agglutinating antibody from other serum proteins. Alternative chromatographic procedures were used

in an attempt to separate the specific agglutinating antiserum component from cross-reactive antibodies. Antiserum 31 was dialyzed for 24 h at 4° C with 0.02 M Tris buffer containing 0.15 M NaCl, and chromatographed consecutively on columns of Sephadex G-200 (158 ml column size) and Sepharose 4B (129 ml). Protein elution profiles were monitored and those fractions which corresponded with the elution of immumoglobulin standards were concentrated separately to original volume (4.1 ml). Agglutinating activity of the fractions within the protein peaks was tested, and an immunofluorescent conjugate was prepared from those fractions showing high agglutinating activity.

Indirect immunofluorescence: Significant loss in homologous antibody titer followed FITC conjugation, indicating that direct immunofluorescence techniques would not be successful. Three indirect procedures were compared in attempt to circumvent this problem.

i) Goat anti-rabbit IgM (anti-IgM). Anti-IgM (PEl-freez, Rogers, AR) containing 4.4 mg protein/ml was dialyzed with bicarbonate-carbonate buffer (pH 9.0) for 24 h. FITC was added at 40 mg/ml protein and conjugation allowed to occur for 1 h at 20° C. Unbound FITC was separated from the conjugated immunoglobulin on a Sephadex G-25 (fine) column. The final fluorescein/protein ratio was 2.63. Smears of strain 31 were stained with rhodamine-gelatin,

flooded with a dilution series of either adsorbed or unadsorbed whole antiserum 31 for 20 min, rinsed with 0.02M phosphate buffer for 30 min, and then incubated with fluorescein labeled, anti-IgM for 20 min. The smears were evaluated by epifluorescence after destaining with 0.02M phosphate buffer for 1 h.

ii) Goat anti-rabbit IgG (anti-IgG). Staining procedures were identical to those for anti-IgM except that commercially available, fluorescein labeled anti-IgG (Sigma Co., St. Louis, MO) was used in place of fluorescein labeled anti-IgM as the second antibody. Comparisons of adsorbed and unadsorbed whole antisera were made, along with the response of the agglutinating fraction recovered from Sephadex G-200.

iii) Biotin-Avidin. Biotinylation of antibodies raised to strain 31 was carried out using a modification of the procedure of Guesdon et al. (1983). Antiserum 31 was dialyzed against 0.02M Tris buffer, pH 7.2, containing 0.15 M NaCl for 24 h at 4° C, and 3.7 ml were applied to a Sephadex G200 column (60 ml bed volume). Fractions which contained agglutinating activity were concentrated to 9.4 mg/ml, dialyzed for 24 h against 0.15 M NaHCO3 (pH 8.2), and incubated for 1 h at 20° C with 57 ul of 0.1 M biotin-N-hydroxysuccinimide (Sigma Co., St. Louis, MO) per ml of protein. After dialysis against 0.1 M PBS for 24 h at 4° C, the biotinylated antibodies were stored at -20° C in an

equal volume of glycerol. Smears from cultures and from alfalfa nodules were incubated with the biotinylated antibody for 20 min, washed with 0.1 M PBS for 25 min, and stained for 20 min with FITC labeled avidin (Sigma Chem. Co., St. Louis, MO) diluted to 0.04mg/ml in 0.1 M PBS . After destaining with 0.1 M PBS for 25 min, specimens were observed by epifluorescence. Smears were compared from nodules collected 17, 28, or 120 d after inoculation of alfalfa seedlings with either strain 31 or 17.

Enumeration of indigenous soil-borne populations of R. meliloti: Soil samples were taken from a Deschutes sandy loam (xerollic Camborthid). Locations were chosen to represent soils which were either currently planted to alfalfa, or had a past history of alfalfa, or had never been planted to alfalfa. Rhizobia were separated from the soil, collected on 0.4 um pore size filters, and enumerated by immunofluorescence as described previously (Demezas and Bottomley, 1986). The R. meliloti specific fluorescein labeled conjugate and the serogroup 31 specific biotinylated antibody in conjunction with fluorescein labeled avidin were used to enumerate total R. meliloti and serogroup 31 respectively.

RESULTS AND DISCUSSION

The fluorescein labeled, immunoglobulin conjugate prepared from unadsorbed antiserum 31 had an acceptable end point titer of 1/80 when evaluated on cells of strain 31. Although the original antiserum was serogroup specific in agglutination tests, cross-reactivity of the conjugate was found with R. meliloti strains recovered from a variety of Medicago species originating from diverse geographic areas (Table 2). Heat treatment of cells prior to exposure to the conjugate had no influence on the intensity or on the specificity of the fluorescence reaction per se (data not shown). Despite the extensive cross-reactivity with R. meliloti strains, when the conjugate was challenged to a variety of bacterial species, only Micrococcus luteus was found to cross-react. No cross-reaction was observed with strains of Agrobacterium tumefaciens despite several reports in the literature that members of this genus are often cross-reactive to R. meliloti antisera (Graham and Parker, 1963; Bouzar et al., 1986). In addition, three strains of R. fredii (USDA194, USDA201, AND USDA217) which have been reported to cross-react with R. meliloti antiserum 31 (Sadowsky et al., 1987), failed to cross-react with the conjugate. These findings indicate that a respectable degree of specificity was achieved at the species level by following standard procedures used for other Rhizobium

species (Schmidt et al., 1968; Demezas and Bottomley, 1986).

In studies of rhizobia, antiserum adsorption procedures have been used for many years to remove cross-reactive antibodies (Vincent, 1941; Purchase and Vincent, 1949; Robert and Schmidt, 1985; Demezas and Bottomley, 1986). In the present study, adsorption of antiserum 31 with sonicated cells of strain 17 eliminated all gel-immune diffusion, cross-reactive precipitin bands, with only a 2-fold reduction in specific agglutination titer (data not shown). Although the fluorescent conjugate produced following antiserum adsorption only reacted with serogroup 31 strains, a dramatic decrease in titer, from 1/150 to 1/2, suggested that the majority of agglutinating antibodies had been lost during the separation of immunoglobulins from other serum proteins.

The majority of agglutinating specific antibodies was found to precipitate from whole serum when dialyzed against the standard buffers recommended for use with DEAE-Affigel Cibacron Blue and at other stages in the procedure (Table 2). Dialysis of antisera raised to R. meliloti strains 21, 27, 17, and 41 against 0.02M Tris supplemented with 0.02M NaCl also resulted in 8-fold decreases in their specific agglutinating titers (data not shown), indicating that the phenomenon was not unique to antiserum 31. Although antibody precipitation during dialysis in low ionic strength buffer is not uncommon

(Heide and Schwick, 1973), it was surprising in this study since the buffer concentrations which resulted in precipitation have been used routinely in the production of strain specific fluorescent antibodies to other bacterial species, including Rhizobium (Banner et al., 1982; Demezas and Bottomley, 1986). Use of buffers of high ionic strength throughout the procedure was absolutely necessary to maintain the agglutinating antibody in solution during immunoglobulin separation. As a result of this finding, the DEAE-Affigel Blue procedure, which depends on low ionic strength solutions to separate serum proteins, could not be used with these R. meliloti antisera.

Antiserum 31 was chromatographed consecutively on Sephadex G-200 and Sepharose 4B columns in a further attempt to separate specific and cross-reactive antibodies. Three distinct peaks were eluted from Sephadex G-200 (Fig. 1a), with the agglutinating activity associated with the first peak off the column. This peak was concentrated, dialyzed against Tris buffer (0.02M + 0.15M NaCl), loaded and eluted on the Sepharose 4B column. Two protein peaks were resolved, with agglutinating activity in the second peak which corresponded with the elution profile of a pure sample of immunoglobulin G.

A further complication arose in the preparation of a functional conjugate which has not been previously reported in the Rhizobium serology literature. Conjugation

of the chromatographed immunoglobulin with FITC resulted in the loss of affinity of the specific antibody-antigen reaction in agglutination and immunofluorescence tests. At this time we can only hypothesize that there was conjugation of fluorescein in the binding region of the antibody molecule which reduced the affinity of the antibody-antigen complex, and resulted in the failure of cells to agglutinate or fluoresce.

As a consequence of the above observation, we considered the use of indirect fluorescent antibody procedures. The majority of Rhizobium researchers utilizing indirect procedures use either fluorescein labeled or enzyme linked, goat anti-rabbit immunoglobulin G (Fuhrmann and Wollum, 1985; Ayanaba, et al., 1986). Again, another unusual observation without precedent in Rhizobium literature was made. Only a weak reaction was observed when cells previously exposed to the agglutinating antibody were incubated with FITC-labelled, goat anti-rabbit IgG in an indirect fluorescence test. Presumably the goat antibody did not recognize the agglutinating antibody either because of low affinity for the particular IgG subclass, or, alternatively, because the agglutinating antibody was not of the IgG class. Related to this possibility, we noted a loss of specific agglutinating titer when antiserum 31 was incubated in 0.2M 2-mercaptoethanol. Vincent and Humphrey (1973, 1975) also detected a loss of agglutinating activity following

similar treatment of antisera to R. meliloti and R. trifolii. Because of the sensitivity of IgM to disulfide bond reduction and the resistance of IgG to to such a treatment, these authors suggested the agglutinating antibody was an IgM molecule. In our case, the failure of fluorescein labeled, goat anti-rabbit IgM to react with cells incubated with the agglutinating antibody, or even with unadsorbed whole antiserum, provided no evidence that the specific agglutinating component of antiserum 31 is an IgM molecule.

The biotin-avidin indirect system has become extensively used in immunological studies (Bayer et al., 1976; Berman and Basch, 1980; Kendall et al., 1983; Vilja et al., 1985; Wadsley and Watt, 1987). The binding affinity of biotin and avidin, with a dissociation coefficient of 10^{-15} M, is considered one of the highest among biological compounds (Green, 1963). An added strength of this methodology is that biotin can be covalently linked to immunoglobulins without significantly altering their immunological characteristics (Guesdon et al., 1979). Unlike FITC conjugation, biotinylation of the agglutinating antibody had no affect on the titer or affinity of the antibody-antigen reaction. Maximum immunofluorescence of strain 31 occurred up to a dilution of 1/320 of the biotinylated agglutinating fraction, while all other strains of R. meliloti failed to react.

The biotinylated immunoglobulin was successfully used to detect the presence, and to determine the density of indigenous, free-living cells of strain 31 in soil (Table 2). The population density of strain 31 was compared with the density of total R. meliloti determined with the species specific conjugate. In soil samples from sites with a recent history of alfalfa production, serogroup 31 was found to comprise an average of 17% of the total R. meliloti population. The R. meliloti population density from the site without recent alfalfa production was only 5% of the average of the other three sites, and members of serogroup 31 were below the limits of detection ($\leq 10^3$ per gram of soil). The R. meliloti population densities determined by immunofluorescence were similar to those measured by plant infection/soil dilution procedures. Neither conjugate cross-reacted with bacteria recovered from soil samples taken from nearby rangeland with no history of alfalfa cultivation. In turn, this soil was found to be devoid of R. meliloti as determined by a plant infection/soil dilution procedure.

An interesting observation was made when the biotinylated antibody was evaluated for its ability to detect serogroup 31 in alfalfa nodules. Both bacteroids and undifferentiated cells fluoresced strongly when nodules were sampled 17 days after inoculation. When the nodules were allowed to develop for 28 d or longer, the antibody failed to react with bacteroids even though

undifferentiated cells in the same nodule smears remained strongly reactive. Presumably, modification of the bacteroid surface during nodule maturation had resulted in the loss of the agglutinating antigen. As a consequence, nodule occupancy by strain 31 might be underestimated in nodules of alfalfa plants sampled several weeks after establishment. Differences in the ability of monoclonal antibodies to detect antigens on free-living cells and bacteroids have also been reported for strains of R. leguminosarum (Brewin, et al., 1986).

Table 10. Bacteria used to determine species specificity of the fluorescein labeled immunoglobulin conjugate to R. meliloti serogroup 31.

Species	Strain	Source
R. meliloti:	21, 27, 31, 17, PB3, PB4, PB12, PB18, PB27, PB29, PB32, 10, 41, 8, 18	1
	102F28, 102F34, 102F51, 102F66	2
R. leguminosarum bv. trifolii:	101, 210, W7, W9, W11, 16, 162K10, 162P17A, 127K126, 126P147	1
B. japonicum:	USDA 122	3
R. fredii:	USDA 191, USDA 194, USDA 201, USDA 217	3
Agrobacterium tumefaciens:	A4, K84, I27, T37, A348	4
Miscellaneous:	Klebsiella pneumoniae Pseudomonas micab Streptococcus salivarius Bacillus subtilis Proteus vulgaris Escherichia coli Staphylococcus aureus	5

source: (1) Peter J. Bottomley, personal collection; (2) Nitragin Co., Milwaukie, WI; (3) Harold Keyser, USDA-ARS, Beltsville, MD; (4) Larry Moore, Oregon State Univ.; (5) Microbiology Dept. culture collection, Oregon State Univ.

Table 11. Cross-reaction of a fluorescein labeled conjugate prepared to antiserum 31 using direct immunofluorescence.

Bacterial species	No. of strains	No. of positive reactions[a]
R. meliloti	22	22
R. leguminosarum bv. trifolii	11	0
R. fredii	4	0[b]
B. japonicum	1	0[b]
A. tumefaciens	5	0
Misc.	8	1[c]

a Tested at a dilution of 1/80 which was the highest dilution giving maximum fluorescence with R. meliloti strain 31.

b R. fredii 217 and B. japonicum USDA122 had a 2+ reaction when FA 31 was diluted 1/10.

c M. luteus

Table 12. Agglutination titer of antiserum 31 following dialysis against buffers of varying ionic strength.

Dialysis buffer	Whole-cell agglutinating titer[a]
0.02M Tris + 0.028 M NaCl, pH 8.2	40
0.02M Phosphate buffer, pH 6.8	40
0.02M Tris + 0.15 M NaCl, pH 8.2	320
0.15M Bicarbonate/carbonate, pH 9.0	320
Pre-dialysis	320

[a] Represents the reciprocal of the highest antiserum dilution which showed a positive reaction.

Table 13. Population densities of R. meliloti and R. meliloti serogroup 31 in soils with different cropping histories.

Site cropping history	Population density	
	R. meliloti[a]	serogroup 31[b]
	x 10^6 cells $(gsoil)^{-1}$	
alfalfa	1.5 ± 0.2	0.2 ± 0.1
	0.9 ± 0.4	0.3 ± 0.1
	1.1 ± 0.5	0.1 ± 0.1
wheat/potatoes	0.06 ± 0.02	$\leq 10^3$
rangeland	$\leq 10^3$ [c]	$\leq 10^3$

[a] Determined by the species specific fluorescent conjugate.

[b] Determined by indirect immunofluorescence using the biotinylated immunoglobulin.

[c] Below the lower limit of accurate detection.

CONCLUSIONS

The experiments presented in this thesis were designed with the simple intent of enhancing both the symbiotic and ecological understanding of indigenous R. meliloti. From an applied standpoint, the results in chapter 2 emphasize the importance of the approach taken in the evaluation of symbiotic effectiveness of R. meliloti strains on the perennial legume, alfalfa. The differences revealed in regrowth characteristics of inoculated Vernal alfalfa following the second harvest relative to regrowth of other cultivars imply that conclusions drawn from short-term effectiveness tests might be misleading. Of concern was the inability of strain 31 to perform comparatively on all cultivars. New cultivars of alfalfa evaluated in yield trials might not produce to their yield potential if the dominant nodule occupying rhizobia show inferior effectiveness. An intriguing question was also raised regarding the extent of host control over the established symbiosis. Vernal alfalfa showed the ability to control the level of N_2 fixation regardless of R. meliloti strain. Furthermore, there was evidence to suggest that Vernal selectively nodulated with the more symbiotically effective subpopulation of serogroup 31. The research findings re-emphasize the need for a strategy whereby improvements in N_2 fixation by forage legumes will depend

on co-ordinated research input from plant breeders, microbial geneticists, and soil microbiologists.

The results from chapters III and IV provide basic information of importance to developing an understanding of indigenous R. meliloti and soil microorganisms in general. Substantial variation was found between members of the species in their ability to adapt to water stress, and between their responses to permeable versus impermeable solute stress. Initially, it was of surprise that the competitive organism, strain 31, had greater difficulty in responding to low water potential than the non-competitive, strain 17. Improved growth and nodulation responses following a water potential increase by strain 31, however, support previous suggestions in literature that the ability of soil organisms to adapt to a water potential increase is of tremendous advantage. Whether the improved nodulation kinetics of strain 31 in response to the upshock has direct impact on competitive nodulation under field conditions can only be speculated upon. In addition, the results indicate that more attention should be focussed on the role of calcium during water stress, with particular regard to its potential role in maintaining cell wall integrity.

The major frustration during the course of my thesis research was the failure to produce a serogroup specific fluorescent antibody by the standard procedures utilized over the past twenty years. Success in elucidating the

causes of the problems and the consideration of alternative approaches resulted in the development of a satisfactory technique. I believe this methodology should revive interest in the study of a species about which past literature has provided inconsistent ecological information. Soil microbiologists will need to increase their understanding of immunological concepts beyond what has been traditionally expected in order to develop sophisticated procedures for studying the ecology of soil microorganisms.

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