PHYSIOLOGICAL, SEROLOGICAL, AND PLASMID CHARACTERIZATION OF FAST-GROWING RHIZOBIA THAT NODULATE SOYBEANS

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By

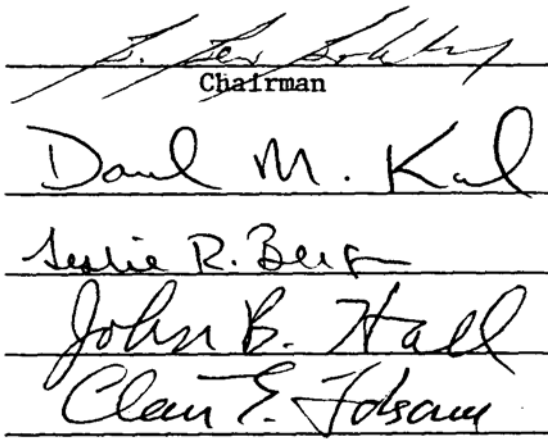
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ABSTRACT

The newly described fast-growing, acid-producing soybean rhizobia from The People's Republic of China (PRC) were examined to determine their degree of physiological and serological relatedness to each other, to the "typical" slow-growing R. japonicum, and to other fast-growing species of Rhizobium. The PRC strains were also investigated to determine: 1) whether they contained high molecular weight plasmids; 2) if there were structural relationships between plasmids from different strains; 3) if plasmids from these strains are involved in the nodulation of soybeans; and 4) whether the fast-growing soybean strains were capable of accepting, maintaining, and expressing symbiotic plasmids from other fast-growing species of Rhizobium.

Results of these investigations have indicated that: (1) While the fast-growing soybean rhizobia share symbiotic host-specificity with the typical slow-growing R. japonicum (they both nodulate the same host legume), they appear more closely related, on a microbiological, biochemical, and physiological basis, to other fast-growing species of Rhizobium than to the slow-growing species; (2) Although the fast-growing PRC strains shared several microbiological and physiological characteristics in common with other fast-growing species of Rhizobium, they possessed some unique characteristics, such as the ability to utilize ethanol as the sole source of carbon and energy and to hydrolyze gelatin; (3) The taxonomic position of the fast-growing soybean rhizobia must logically be in the new genus Rhizobium. Based on their symbiotic characteristics, may warrant seperate species status; (4) All of the fast-growing PRC soybean strains which were examined could be separated into at least three somatic serological groups based on immunofluorescence and immunodiffusion reactions. Immunodiffusion

analysis of heat-labile whole-cell antigens indicated that all the strains shared at least one heatlabile common antigen; (5) All of the fast-growing PRC strains contained 1 - 4 high molecular weight plasmids (M.W. > 100 Mdal). While most of the strains shared plasmids with similar size, restriction endonuclease profiles of plasmids from three of the strains were vastly different; (6) In one of the strains, growth in the presence of subbacteriostatic levels of acridine orange was effective in producing mutants cured of their largest plasmid. In these mutants the loss of the large plasmid led to the loss of nodulating ability indicating that nodulation genes might be plasmid borne in this group of organisms. High-temperature curing of a smaller plasmid in another strain did not lead to the loss of modulating ability or alteration of symbiotic effectiveness on soybean cultivars; (7) Although one of the fast-growing soybean strains was capable of receiving and maintaining the R. leguminosarum pea host-range (Sym) plasmid, the resulting transconjugants were unable to express the pea nodulation genes located on the plasmid. On the otherhand, transfer of pJB5JI to two R. trifolii strains resulted in pea-nodulatiog transconjugants indicating that the expression of genes on the Sym plasmid depends on the genetic backround it resides in; (8) Plasmid pJB5JI was differentially expressed in R. trifolii transconjugants depending on whether transconjugants were made from Nod(#2) or Nod^+ (0403) R. trifolii strains. That is, R. trifolii #2 transconjugants were Nod^+, Fix^+ on peas, while R. trifolii 0403 transconjugants were Nod^+, Fix^- on peas; (9) In some R. trifolii 0403 transconjugants, there was variable expression of plasmid pJB5JI depending on whether the transconjugants were isolated from culture or subsequently reisolated from pea or clover nodules. Culture isolated transconjugants were Nod+, Fix^- on peas and Nod^+, Fix^+ on clover, while nodule reisolates were Nod^+, Fix^+; Nod^+, Fix^-;

or Nod$^-$ on peas; and (10) Transfer of plasmid pJB5JI to R. trifolii 0403 resulted in the construction of unique transconjugants which had the ability to nodulate two legume hosts.

The fast-growing soybean strains from China may provide an effective tool for a better understanding of the genetics of the soybean-Rhizobium symbiosis since they appear more amenable to genetic manipulations than the typical slow-growing soybean symbiosis.

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PREFACE

Some material presented in this dissertation was obtained with the help and collaboration of several investigators. I would specifically like to acknowledge Dr. Harold Keyser for his collaboration on several biochemical tests presented in Chapter III. I would also like to acknowledge Stephen Dowdle's contributions to growth rate data presented in Chapter III. Lastly, I wish to acknowledge Heidii Fugii for her contributions to R. trifolii plasmid transfer experiments presented in Chapter IV and Ben Bohlool for the preparation of antibodies and immunofluorescence results.

CHAPTER I

INTRODUCTION AND OBJECTIVES

Recent technological advances in molecular genetics have prompted renewed interest in the genetics of nitrogen fixation. Although many organisms are capable of fixing atmospheric dinitrogen into plant-useable nitrogen sources, a large number of investigators have focused their attention on members of the genus Rhizobium. This group of microorganisms is unique in that the fixation of nitrogen only occurs when the rhizobia are in a symbiotic state with the plant host.

The "typical" slow-growing rhizobia that form nodules on the roots of soybeans, Glycine max, have now been reclassified in the new genus Bradyrhizobium (79). Recently, Keyser et al. (84) reported the isolation of fast-growing soybean-rhizobia from nodules and soil collected in the People's Republic of China (PRC). All of the strains they examined (isolated in the Honan, Shantung, and Sanshi Provinces of China) had mean generation times (in yeast extract mannitol medium) between 2 and 5 hours and lowered the pH of the culture medium (final pH's ranged from 4.3-6.7). All formed effective symbioses with wild soybeans (Glycine soja) and an unbred cultivar from China (Glycine Max, cultivar Peking), but formed ineffective symbioses with most commercial soybean cultivars. It should be noted that Glycine soja is reported to be the putative wild ancestor of todays cultivated soybeans (55). In addition, the isolates were reported (84) to ineffectively nodulate Macroptilium atropurpurum, Macroptilium laythyroides and Sesbania cannabina, all of which are promiscuous legumes which are nodulated by a wide variety of rhizobia. However, the PRC isolates did not nodulate, Leucaena leucocephala (Hale Koa),

Medicago sativa (alfalfa), Trifolium repens (clover) or Astragalus sinicus.

The genetics of the root nodule bacteria of such an important crop as soybeans has been neglected, perhaps due to difficulties in consistently demonstrating plasmids in all the slow-growing B. japonicum strains. Furthermore, the location of symbiosis-related genes in slow-growing rhizobia is largely unknown. Although several investigators (83,95,113) have transferred plasmids to slow-growing rhizobia, in all instances, the plasmids used were the P1 incompatibility group plasmids (originally from Pseudomonas aeruginosa). Also, not all of the plasmids used could be transferred to all of the strains investigated.

The objectives of this dissertation were to determine: 1, the degree of relatedness of the fast-growing soybean rhizobia to each other, to the "typical" slow-growing B. japonicum, and to other fast-growing rhizobia. Relationships between the PRC rhizobia and other fast- and slow-growing rhizobia were assessed at the biochemical and physiological levels in order to ascertain the relative taxonomic position of these newly described isolates; 2, the serological relationships of the fast-growing soybean rhizobia to other fast- and slow-growing rhizobia; 3, whether the fast-growing soybean rhizobia contain large molecular weight plasmids and if plasmids were present, whether there are structural relationships between plasmids from different strains; 4, whether symbiosis-related genes are plasmid-borne in these isolates; and 5, whether the fast-growing soybean rhizobia were capable of accepting, maintaining, and expressing plasmids from other fast-growing rhizobia.

CHAPTER II

LITERATURE SURVEY

Members of the genus Rhizobium can best be described as a heterogeneous group of Gram-negative, aerobic, heterotrophic, non-sporeforming rods which have the ability to invade and form nodules on the roots of leguminous plants (80). Within these root-nodules, a symbiotic state exists between the rhizobial partner (microsymbiont) and the plant partner (macrosymbiont). One result of this symbiosis is the fixation of atmospheric dinitrogen into ammonia by the rhizobial partner, in exchange for protection and a source of photosynthetically fixed carbon, provided by the host plant. Although the process of nitrogen fixation (via the enzymecomplex nitrogenase) is not solely restricted to members of the genus Rhizobium, it is the root-nodule-symbiosis with plants of the family Leguminosae, which is almost exclusively a property of this group of microorganisms (133).

The family Rhizobiaceae is currently listed (80) along with Pseudomonadaceae, Azotobacteriaceae Methylmonadaceae, and Halobacteriaceae in part 7 of the 8th edition of Bergey's Manual of Determinative Bacteriology (See Figure I-1 for relationships between families and genera). The only major feature shared by all these families is their aerobic metabolism and negative Gram-reaction, however, members of the Azotobacteriaceae have the ability to fix atmospheric dinitrogen in the free-living state. The Rhizobiaceae are divided into two genera, Rhizobium and Agrobacterium, with the major distinguishing characteristic between the two groups being the ability of Agrobacterium (with the exception of A. radiobacter) to form cortical hypertrophies (Crown Galls) on the roots and stems of numerous dicotyledonous plants. The current classification scheme bases species

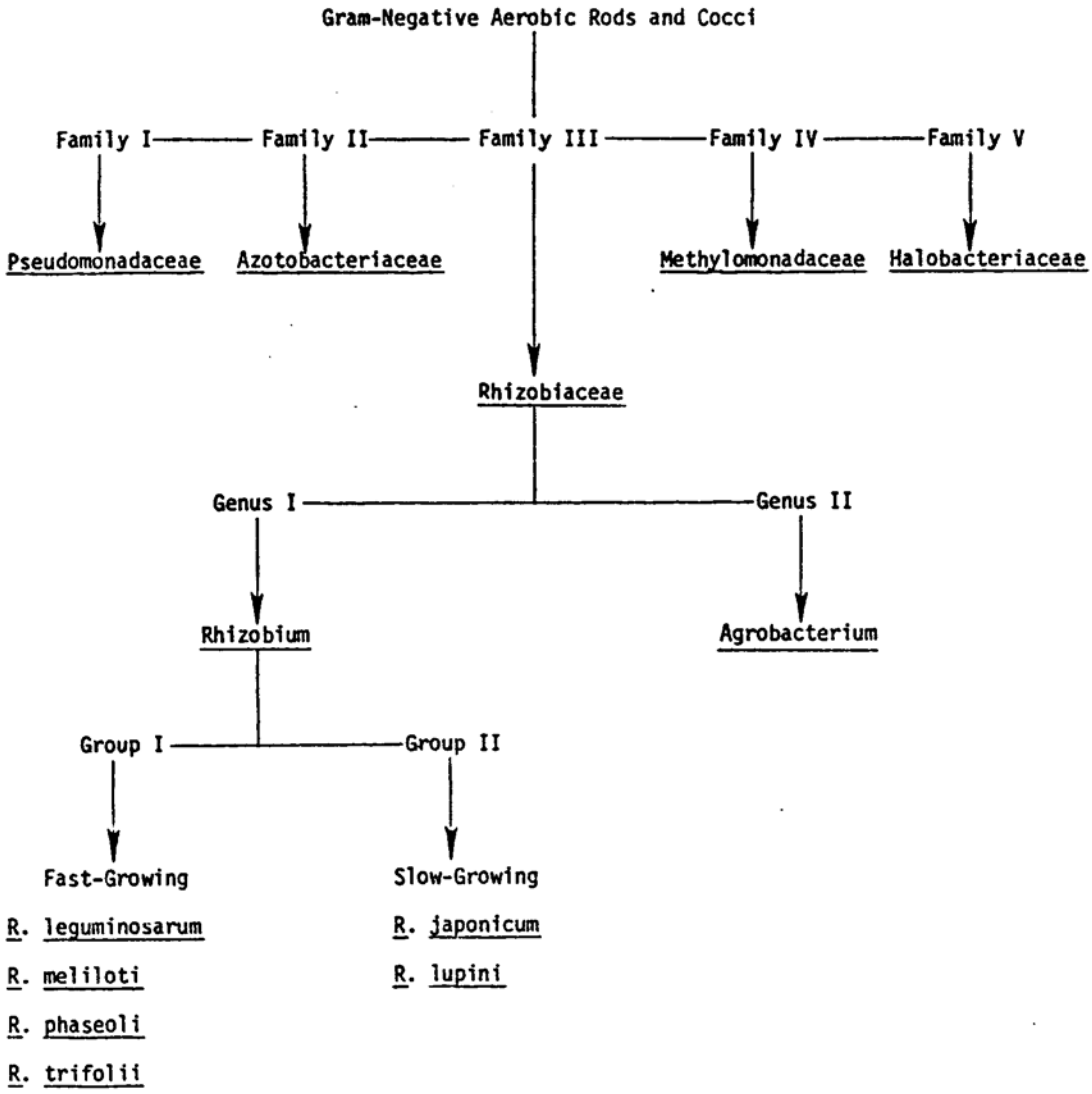


Figure I-1. Taxonomic relationships between members of Part7 of Bergey's Manual of Determinative Bacteriology (8th ed.).

designation within the genus Rhizobium exclusively on the ability of a specific bacterium to nodulate a given legume host. This approach divides rhizobia into several "plant-infection" groups and results in the designation of six species: Rhizobium japonicum (Soybean), R. leguminosarum (Pea), R. lupini (Lupen), R. meliloti (Alfalfa), R. phaseoli (Bean), and R. trifolii (Clover).

Although the division of rhizobia into "plant-infection" groups does have certain practical applications, there are also some major problems with this scheme (133,138). While some rhizobial species groups may be correctly defined by the relatively small numbers of legumes they are able to nodulate, others can nodulate a great number of different legumes. For example, R. meliloti isolates which can nodulate most species of Medicago (Alfalfa) and Melilotus (Sweet Clover) but not Phaseolus (Beans), Trifolium (Clover), or Viccia (Vetch), are said to have cross-inoculation group specificity. On the other hand, some isolates of R. leguminosarum, in addition to forming nodules on Pisum (Peas), are able to nodulate Viecia, Lathyrus (Roughpea, Grasspea), and Lens (Lentils) (138). It should be noted however, that the ability to form nodules on the roots of leguminous plants is a property contributed to by both the micro- and macrosymbiont. In addition to the above mentioned shortcomings, there exists additional problems in using schemes based on plant-infection groupings. The first problem is that there are many rhizobia that do not fit into the present scheme (they have other plant host requirements) and are thus placed into separate "catch-all" groups. One such example is the so-called "cowpea-miscellany" group. Members of this group have the ability to nodulate a wide variety of host plants, such as, Vigna (Cowpea), Glycine (Soybeans), and Macroptilium (Siratro). Another example of a "catch-all" group are the lotus rhizobia (73) which have the ability to form nodules

on legumes of the genera Lupinus (Lupine), Ornithopus (Serradella), Anthyllis (Kidney vetch), and Astragalus (Tragacanth). Another problem with the plant-infection scheme involves those organisms which are no longer capable of effectively modulating their designated host plant. For example, by definition, those organisms able to nodulate clover are referred to as R. trifolii, however, R. trifolii mutants (both spontaneous and induced) are available which have been rendered non-nodulating. By the above definition, they should no longer be referred to as rhizobia.

A. Fast- and slow-growing rhizobia

The genus Rhizobium can also be divided into two major groups depending upon growth rates and effects of growth on the pH of yeast extract mannitol (YEM) culture medium (1,41,53,73.79,80,97,106,133,138). The "fast-growing" rhizobia have mean generation times between two and four hours and produce a net decrease in the pH of YEM culture medium. On the other hand, those rhizobia referred to as "slow-growing" have mean generation times of six hours and longer and do not lower the pH of the medium (133). Generally speaking, R. leguminosarum, R. meliloti, R. phaseoli, R. trifolii, and Rhizobium spp. capable of nodulating Leucaena and Sesbania are characterized as fast-growing and acid-producing, while R. japonicum, R. lupini, and the "cowpea group" are characterized as slow-growing and alkaline-producing. As was pointed out by Vincent (133), the lotus rhizobia present some taxonomic difficulties, since there are both fast- and slow-growing strains which have similar host requirements. Similarly, among the Rhizobium lupini, both fast- and slow-growing strains can be isolated. More recently, Keyser et al. (84) reported that in addition to the slow-growers, there are also fast-growing rhizobia able to nodulate soybeans.

B. Biochemical attributes of rhizobia

Consistent with the division of rhizobia into fast- and slow-growing groups based on their growth rate and effect on the pH of YEM culture medium, is their division into two broad groups on the basis of several other characteristics (Table I-1). Graham and Parker (53) have indicated that the fast-growing rhizobia may be differentiated from the slow-growing ones by the presence of methylene blue-staining cytoplasmic granules and by colony size on YEM agar. The former have such granules and produce much larger colonies than the latter. As was pointed out by Fred et al. (44), Graham and Parker (53), and Vincent (133), fast-growing rhizobia tend to utilize a wider variety of carbohydrates than do the slow-growers. Although the utilization of a specific sugar is in itself not a useful tool for differentiating rhizobia, a clear difference does exist in the pattern of utilization of a large number of carbohydrates by fast- and slow-growing strains. The fast-growers also generally have the ability to utilize disaccharides as a sole source of carbon and energy for growth. Glenn and Dilworth (48), Graham (50), and Martinez-De Drets and Arias (101) pointed out that slow-growing rhizobia are generally unable to utilize disaccharides. Glenn and Dilworth (48) indicated that the slow-growers apparently lack both uptake systems and catabolic enzymes for disaccharide utilization. For example, fast-growing rhizobia have B-galactosidase activity, while the slow-growers lack this enzyme. Martinez- De Drets and Arias (101,102) have also shown that although both fast- and slow-growing rhizobia have NAD-linked 6-phosphogluconate activity (6-PGA), only the fast-growers have NADP-linked 6PGA. Thus, rhizobia can also be separated into fast- and slow-growing groups based on the presence or absence of enzymes of the pentose phosphate pathway.

Fast- and slow-growing rhizobia can also be separated on the basis

Table I-1. General characteristics of fast- and slow-growing rhizobia[a].

Characteristic	Rhizobia	
	Fast-Growing	Slow-Growing
Mean Generation Time (h)	2-4	6-8
pH Reaction on YEM medium	Acid	Alkaline
Utilization of Disaccharides	+	-
Utilization of Carbohydrates	Many	Few
Resistance to Antibiotics	Few	Many
NADP-Linked 6-phosphogluconate dehydrogenase	+	-
Flagellar Arrangement	Peritrichous	Polar or Subpolar
Serum Zone in Litmus Milk	+	-
Growth at pH 4.5	-	+
Growth at pH 9.5	+	-

[a]Compiled from (133) and (134).

of their relative tolerance to pH and NaCl. Previous studies of rhizobia (20,43,53,106) showed that fast-growers were relatively more alkali-tolerant and acid-sensitive, than the slow-growers. Also, Graham and Parker (53) showed that among rhizobia, tolerance to 2% NaCl was restricted to the fast-growing R. meliloti. The responses of rhizobia in litmus milk (44,53) and their relative resistance to antibiotics (128) have also been used to separate rhizobia into two growth-rate groups. Fast-growers tend to produce acid and peptonization reactions in litmus milk and are generally sensitive to antibiotics. The slow-growers on the other hand, do not possess these characteristics.

C. Deoxyribonucleic acid base ratios and homology

Rhizobia can also be separated into several groups based on DNA base ratios and nucleic acid hybridizations. While DNA base ratios, mole percent G+C, for Rhizobium are relatively broad ranged [values ranging from 59-66% (32)], they do indicate relationships between strains. De Ley and Rassel (32) indicated that the peritrichously flagellated fast-growing rhizobia, R. leguminosarum, R. phaseoli, and R. trifolii, tend to have low %G+C values, ranging from 59-63%, while the polar to subpolarly flagellated slow-growers, R. japonicum, R. lupini, and some of the cowpea miscellany, have relatively high %G+C values, 63-65%. Heberlein et al. (57) reported that with the exception of Agrobacterium pseudotsugae and R. japonicum, the agrobacteria and rhizobia which they examined had about the same %G+C content, about 59-63%. Rhizobium japonicum on the other hand, had G+C values of about 64-65%. However, Elkan (40) examined 25 strains of R. japonicum and found their mole percentage G+C to be relatively homogeneous, 61-64%. Elkan (40) also indicated that although %G+C values are significantly different between some strains of R.

japonicum, these values fall within the range of other fast- and slow-growing rhizobia and thus, are of limited taxonomic value.

DNA-DNA homologies have been used by several investigators (57,47,64) to study the degree of genetic relatedness between strains and isolates of Rhizobium. The homology studies of Gibbons and Gregory (47) and Heberlein et al. (57) have indicated that there is a sharp line of demarcation between the fast-growing species (R. leguminosarum, R. trifolii, R. phaseoli, and R. meliloti) and the slow-growing ones (R. japonicum and R. lupini). Hollis et al. (64) have recently shown that the slow-growing strains of R. japonicum which they examined could be separated into at least three distinct DNA homology groups. These authors also indicated that there was little homology between DNA from several R. japonicum strains and R. leguminosarum, R. meliloti, R. phaseoli, R. trifolii, and Agrobacterium tumefaciens. However, there was substantial homology between the slow-growing R. lupini and R. japonicum. Similarly, Crowe et al. (29) showed that the 113 fast-growing and 9 slow-growing strains of rhizobia which they examined could be placed into 4 DNA homology groups, and that the fast-growers were clearly separated from the slow-growers. Interestingly, these authors also indicated that there was little homology between fast- and slow-growing lotus rhizobia. However, the two groups were more closely related to each other than to the other fast- and slow-growing rhizobia which were examined. Thus, fast- and slow-growing rhizobia can also be separated into two groups on the basis of DNA-DNA homologies.

In addition to the above mentioned characteristics, fast- and slow-growing rhizobia have also been separated into fast- and slow-growing groups on the basis of polyacrylamide gel electrophoresis of cell proteins (119) and nodule-bacteroid inclusion bodies (28).

D. Serological relationships

Serological techniques have been used routinely in the study of rhizobia: 1, to obtain information about their antigenic composition (52,68); 2, for strain identification (13,78,104,123); 3, to investigate the serological relatedness of strains and species of Rhizobium (36,37,46,69,71,136,137) and 4, for ecological studies (13,14,15,16,104,85,121,122,123,). Three techniques in particular have found wide acceptance in serological investigations of the Rhizobiaceae: agglutination, immunodiffusion (Ouchterlony Gel Diffusion), and immunofluorescence.

Agglutination techniques were the first of the serological methods applied to the study of rhizobia (38). In general, early serological studies were concerned mainly with the relationships between serological groupings and host-specificity. Results of most studies indicated that rhizobia are a serologically heterogeneous group of organisms. Stevens (127) and Wright (140) found that different strains isolated from the same host-plant species could be serologically unrelated. Hughes and Vincent (67) found that even strains isolated from different nodules on the same plant could be unrelated serologically. However, in their study, Bushnell and Sarles (23) found that some strains from different cross-inoculation groups were serologically related. Kleczkowski and Thornton (86) indicated that the ability of a rhizobial strain to nodulate a particular host-plant is not necessarily related to it serological characteristics as detected by agglutinations. Bushnell and Sarles (23) examined rhizobia from the soybean, cowpea, and lupin cross-inoculation groups and found no correlation between the ability of certain strains in one group to cross-inoculate another, and their ability to cross-agglutinate. In addition, Stevens (127) and Bushnell and Sarles (23) indicated that due to their serologic heterogeneity, all

strains within the same Rhizobium species cannot be identified by agglutination reactions with a limited number of antisera.

Despite the inability to show correlations between host-specificity and serological groupings, agglutination reactions have been used to assess the serological relatedness of strains and species of Rhizobium. Kleczkowski and Thornton (86), using whole-cell antisera against 4 strains of R. trifolii and 2 strains of R. leguminosarum, examined the agglutination cross-reactions of antigens from 161 strains of R. trifolii, 29 R. leguminosarum, 5 strains of R. meliloti and R. lupini, and 13 non-Rhizobium soil isolates. Results of their study indicated that while no cross-reactions occurred outside of the clover and pea groups and with the 13 soil isolates, some cross-reactions occurred between the two groups. And, while some antisera were quite specific, others were relatively non-specific.

In their studies on the serological relationships of 25 strains of the slow-growing R. japonicum, Koontz and Faber (88) identified 6 somatic serogroups using agglutination adsorption reactions. Wright et al. (140) similarly found 6 serogroups among the R. japonicum they examined, however, these authors did not differentiate between somatic and flagellar antigens. Date and Decker (30) analyzed 28 strains of R. japonicum and found 17 somatic serogroups on the basis of cross-reactions and agglutination cross-adsorptions.

Graham (49) tested 113 strains of Rhizobium for agglutination by whole cell antisera produced against 58 strains of Rhizobium and 16 Agrobacterium strains. The results of his study of whole- and somatic-cell antigens indicated that the rhizobia could be separated into three serologically distinct groups: 1, R. trifolii, R. leguminosarum, and R. phaseoli; 2, R. japonicum, R. lupini, and Rhizobium spp. of the cowpea miscellany; and 3, R. meliloti. While there were no cross-reactions

between the groups, there were cross-reactions within the groups. Graham (49) also indicated that strains of R. meliloti showed some serological relatedness to Agrobacterium tumefaciens and A. radiobacter and that agglutination cross-reactions were greater with whole-cell antigens than with somatic-cell antigens.

Immunodiffusion techniques, specifically Ouchterlony double-diffusion, have also been used extensively to investigate the serological relationships between strains and species of Rhizobium (36,37,51,69,70,71,125). The technique relies on the separation of soluble, diffusible antigens through an agar-gel matrix. Relationships between various antigens and antisera are determined by examining the nature of the interaction at the junction of precipitin bands from various wells. Gel diffusion methods have been used in the study of rhizobia because they permit the rapid enumeration of soluble antigens, the techniques are relatively simple, and they can be used to study serological relationships of strains at the single antigen level. Dudman (37) has indicated that while agglutination reactions can be used to separate rhizobia into serological groups, agglutination techniques lack the resolving power of immunodiffusion in distinguishing between antigenically identical and closely related, but not identical strains.

Dudman (36) was the first investigator to use immunodiffusion techniques to study the serological relatedness of strains and species of Rhizobium. In his study of the extracellular soluble antigens of 2 strains of R. meliloti, Dudman (36) found that the two strains examined shared all antigens accept several fast-moving ones. He proposed that since the strains did not cross-agglutinate, that these strain-specific antigens could be used for identification purposes.

Using gel immunodiffusion, Skrdleta (125) divided the 11 slow-growing R. japonicum which he examined into two basic somatic serogroups.

While he detected the same serogroups using agglutination reactions, he found that immunodiffusion allowed him to show serological relationships between strains that were not agglutinated by the same antisera. Skrdleta (125) also indicated that the somatic antigens were more specific than flagellar ones in differentiating individual strains.

Dudman (37) in his study of seven strains of R. japonicum found that pretreatment of antigens (by boiling or ultrasonic disruption) was required for the proper immunodiffusion analysis of these slow-growing strains. Gibbins (46) found that while ultrasonic disruption prevented the formation of precipitin bands in immunodiffusion reactions, band formation could be restored by heating the sonicated antigen preparations.

While the use of somatic antigens (heat-stable antigens) have been more specific than flagellar ones (heat-labile) in differentiating individual strains of Rhizobium, internal antigens have also been reported (135) to provide some insight into the serological relatedness of fast- and slow-growing rhizobia. Using whole-cell antisera against three strains of R. japonicum, Vincent et al. (137) studied the internal antigens of sixty-nine strains of Rhizobium and 5 Agrobacterium strains.

Immunodiffusion reactions indicated that at least one common antigen was present in 13 strains of R. japonicum, 4 strains of R. lupini, and 4 strains of the slow-growing cowpea and lotus rhizobia. Their results also indicated that the forty-six fast-growing rhizobia examined were readily distinguished from the slow-growing strains and that the 5 strains of agrobacteria grouped with the fast-growing rhizobia. More recently, Pankhurst (110) studied the immunodiffusion cross-reactions of somatic and interal antigens from 62 fast- and slow-growing strains of lotus rhizobia. Results of his study indicated that while the fast- and slow-growers shared no common somatic antigens, internal antigens were shared

by all of the fastgrowing strains, and with seven exceptions, by all of the slow-growing strains.

The fluorescent antibody technique is the method of choice for the direct examination and identification of strains of rhizobia in culture and nodules (13,14,123) and for the enumeration of specific strains directly in soi1 (14,85,122). The major advantages of immunofluorescence over other techniques is that only small amounts of antigen and antibody are needed (121), the procedures are relatively rapid, and its the only technique readily applicable to the study of rhizobia in situ (16).

Vincent (135) in his recent review of the literature has pointed out that when serological (137) and other taxonomic evidence [see (73) and (79)] are taken together, clear relationships among rhizobia can be recognized. That is; 1, there is a closer relationship between the fast-growing species of Rhizobium and Agrobacterium than there is between the fast- and slow-growing groups of rhizobia; 2, R. trifolii and R. phaseoli should be made separate biovars of the species R. leguminosarum; 3, R. meliloti is so different from other species of Rhizobium, that it requires its own species status; 4, among the slow-growers, the slow-growing soybean rhizobia should remain as a separate species group and be included in the new genus Bradyrhizobium; and 5, the fast- and slow-growing lotus rhizobia fall within the genera Rhizobium and Bradyrhizobium, respectively.

E. Current Rhizobium taxonomy

Due to the large number of differences existing between the fast- and slow-growing rhizobia, and to inadequacies in the cross-inoculation-plant-infection group scheme, a new classification scheme for rhizobia has been proposed [see Jordan (79) and Jarvis (73)]. This scheme (since adopted by the International Subcommittee on Agrobacterium and Rhizobium)

divides rhizobia taxonomically into fast- and slow-growing groups. The first group consists of the fast-growing rhizobia. Those organisms previously designated as R. leguminosarum, R. trifolii, and R. phaseoli will be combined as one species, R. leguminosarum (Table I-2), comprising three biovars (trifolii, phaseoli, and viceae). Rhizobium meliloti [which differs significantly from other rhizobia (134)], is kept as a separate species group. The slow-growing rhizobia, R. japonicum, were transferred to a separate genus, Bradyrhizobium. Only one species is present in this genus, R. japonicum. Other slow-growing rhizobia are to be referred to as Bradyrhizobium spp., with the name of the designated-nodulated plant following in parentheses. In the remainder of this dissertation, those slow-growing organisms previously referred to as Rhizobium japonicum will be referred to as Bradyrhizobium japonicum and R. japonicum will be used only to refer to the fast-growing soybean-rhizobia from China. It should be noted that the slow-growing rhizobia formerly referred to as R. lupini are not included as a separate species in the new genus Bradyrhizobium, since their only major distinguishing characteristic is their nodulation affinity for Ornithopus and Lupinus. Subsequent to the adoption of this new scheme (which will be appearing in the forthcoming edition of Bergey's Manual of Determinative Bacteriology), Jarvis et al. (73) have proposed that another species group be included in the new genus Rhizobium. This species, Rhizobium loti refers to the heterogeneous group of fast-growing rhizobia which effectively nodulate Lotus corniculatus (birds-foot treefoil), Lupinus densiflorus (lupines) and Anthyllis vulneraria (kidney vetch). It should be noted however, that some strains of the lotus rhizobia ineffectively nodulate a great variety of platns. In addition, the slow-growing lotus rhizobia will be included in the new genus Bradyrhizobium.

Table I-2. Current and proposed classification of the root-nodule bacteria[a]

Current Classification
Genus *Rhizobium*:
Fast-Growing Species
Rhizobium leguminosarum
R. meliloti
R. phaseoli
R. trifolii
Slow-Growing Species
R. japonicum
R. lupini
Rhizobium spp. ("cowpea miscellany")
New Classification
Genus *Rhizobium*: Fast-Growing
Rhizobium leguminosarum
Biovar: *phaseoli*
trifolii
viceae
R. meliloti
R. loti
Genus *Bradyrhizobium*: Slow-Growing
Bradyrhizobium japonicum
Bradyrhizobium spp. (designated nodulated plant)

[a]Compiled and modified from (73), (79), and (80).

F. Genetics of the Rhizobium-legume symbiosis

The study of the genetics of the root-nodule bacteria had its beginning in the 1960's (91). It has been the general interest of many investigators to study those genes necessary for nodulation and nitrogen fixation. Techniques used to study the genetics of the Rhizobium symbiosis have included mutagenesis, transduction, transformation, and conjugation. More recently, the genetic aspects of the Rhizobium-legume symbiosis have been investigated using molecular genetic techniques.

Although Beringer (8) has indicated that transformation studies with Rhizobium may have begun in the 1940's by Krasilnikov (92,93), he has also pointed out that this work cannot adequately be evaluated. Initially, transduction, transformation, and conjugation were explored in order to facilitate gene transfer between rhizobia. Although transduction has been used by Kowalski (91) and Buchanon-Wollaston (21) to transfer DNA between strains of R. meliloti and between R. leguminosarum and R. trifolii, its general application in the study of rhizobia has been limited. This may be due to the fact that only small segments of DNA can be transferred and that the available phages only mediated generalized transduction (8). While transformation systems have been developed for several fast and slow-growing rhizobia (2,115,116,117), the selected markers in most cases have been streptomycin resistance, gelatinase activity, or the conversion of amino acid-auxotrophs to prototrophs. However, in 1978, Page (109) transformed a strain of the free-living nitrogen fixing organism, Azotobacter vinelandii, which was unable to fix nitrogen (Nif^-), to a Nif^+ phenotype, using DNA isolated from several rhizobia.

Conjugation within and between Rhizobium species has been the most promising system for gene transfer. Initial studies centered around the construction of chromosomal linkage maps (4,6,7,59,87). In most

instances, the P1 group plasmids from Pseudomonas aeruginosa (RP4, R68, and R68.45) were used to mobilize chromosomal DNA.

Mutants defective in symbiosis-related function are essential for most genetic studies. Three approaches were taken to construct nodulation (Nod) and nitrogen fixation (Nif) deficient strains. The first approach, used by Maier and Brill (99) and Beringer (5), utilized "conventional" chemical mutagenesis (usually N-methyl-N'-nitro-N-nitrosoguanidine). The second was that used by Beringer et al. (6) in which the mutagenic agents were transposable genetic elements (transposons). Transposons (TN) can integrate into many sites within the chromosomes of R. leguminosarum, R. trifolii, and R. phaseoli (6) and into the indigenous plasmids of R. leguminosarum (76) and in doing so, result in the production of Nif^- and Nod^- mutants. In the last approach, used by Zurkowski and Lorkiewicz (142), Casse, (25), and Higashi (60), nodulation deficient R. trifolii and R. leguminosarum mutants were obtained following acridine orange and heat curing of indigenous plasmids.

G. Rhizobium Plasmids

As was indicated above, mutagenic agents, in addition to causing chromosomal mutations, can also cause mutations in plasmid DNA. At the present time, most of the fast-growing rhizobia that have been examined have been shown to contain large [M.W. > 100 megadaltons (Mdal)] plasmids (17,18,25,54,66,94,107,114,120). Initially, these large plasmids were only infrequently detected in rhizobia. This was most likely due to the use of techniques (such as the cleared-lysate method) designed for the isolation of low M.W. plasmids. In the past few years, several plasmid-borne functions have been identified in some of the fast-growing Rhizobium species. These include; medium bacteriocin pro-

duction (61), melanin production (10), hydrogen uptake (HUP) (19), host-range specificity (10,22,60,65,66,76,142) and nitrogenase components (39,108,120).

As was indicated by Beringer (8), future genetic studies of Rhizobium will be dominated by studies of plasmids. One group of plasmids which have captured considerable interest among investigators are referred to as the symbiotic (SYM) plasmids. One such example is the R. leguminosarum SYM plasmid, pRL1JI. This plasmid, modified by the addition of the transposon TN5, (pRL1JI::TN5) (76) has been used by several investigators (10,18,35,62,77) to transfer pea nodulation ability into R. leguminosarum, R. phaseoli, and R. trifolii. This 130 Mdal plasmid carries some of the nitrogen fixation (Nif) genes as well as genes for the nodulation of peas. Plasmid pRL1JI, originally shown by Hirsch (61) to contain genes for medium bacteriocin production is a self-transmissible (due to a segment of DNA referred to as Tra), conjugal plasmid. However, the addition of TN5 to the plasmid has resulted in the loss of its expression of medium bacteriocins (presumably due to transposition into the bacteriocin genes). The addition of TN5 into the plasmid (now referred to as pJB5JI) has facilitated its use in further studies, since the kanamycin resistance phenotype (donated by TN5) of pJB5JI can be used to select for transconjugants on agar plates containing the antibiotic. Due to its transferability and selectability, the Rhizobium leguminosarum symbiotic (SYM) plasmid, pJB5JI, allows for the examination of the functioning of symbiosisrelated genes in various genetic backgrounds.

CHAPTER III

BIOCHEMICAL AND PHYSIOLOGICAL RELATEDNESS OF FAST-GROWING SOYBEAN RHIZOBIA TO OTHER FAST- AND SLOW-GROWING RHIZOBIA

Abstract

Fast-growing, acid-producing soybean-rhizobia were examined to determine their degree of biochemical and physiological relatedness to each other, to "typical" slow-growing B. japonicum, and to other fast-growing species of Rhizobium. While both the fast- and slow-growing rhizobia were positive for catalase, urease, oxidase, nitrate reductase, and penicillinase, the fast-growing R. japonicum grouped with other fast-growing species of Rhizobium in that they were tolerant to 2% NaCl, sensitive to a larger number of antibiotics, capable of growth at pH 9.5, utilized a large variety of carbohydrates (notably disaccharides), and produced serum zones in litmus milk. In addition, these fastgrowing strains were similar to other fast-growing species of Rhizobium in that they had appreciable levels of B-galactosidase, NADP-linked 6-phosphogluconate dehydrogenase, and had mean generation times much less than the typical slow-growers when growing in culture media and in sterile soil. While the fast-growing soybean-rhizobia were capable of substantial growth in ethanol, the typical fast-growers failed to grow in ethanol and the slow-growers grew only poorly. Although the fast-growing soybean-rhizobia share symbiotic host-specificity with the typical slowgrowers, they appear biochemically and physiologically more closely related to the other fast-growing species than to the "typical" slow-growing B. japonicum.

Introduction

Species within the genus Rhizobium have been divided into two groups (80,133) depending on their growth rate and effect on the pH of yeast extract-mannitol (YEM) medium under standard laboratory conditions. The fast-growing rhizobia have mean generation times of between two and four hours and produce a net decrease in the pH of YEM culture medium, while those referred to as slow-growing have mean generation times of six hours and longer and do not lower the pH of the medium (133).

The typical slow-growers that form nodules on the roots of soybeans (Glycine max) have in the past been referred to as Rhizobium japonicum (80). Recently, these rhizobia have been re-classified in a new genus, Bradyrhizobium, on the basis of their slow growth rate and other characteristics (79) to distinguish them from fast-growing, acid-producing root-nodule bacteria which now comprise the genus Rhizobium [see (73)].

Recently, Keyser et al (84) reported the isolation of fast-growing soybean-rhizobia from root nodules and soil collected in the provinces of Shansi, Honan, Shandong, and Shanghai in the People's Republic of China (PRC). The isolates have been reported (84) to have mean doubling times between 2 and 4 hours and lower the pH of YEM culture medium (final pH ranging from 4.7 to 6.7). All strains were reported to form effective nitrogen-fixing nodules on wild perennial soybeans (Glycine soja) and on an unbred soybean cultivar from China (cv Peking), but formed ineffective symbioses with most commercial cultivars (84).

This report describes the taxonomic investigation of the fast-growing, acid-producing PRC isolates. Several diagnostic microbiological, physiological, and biochemical tests were performed in order to determine the degree of relatedness of the fast-growing soybean

rhizobia to each other, to the "typical" slow-growing B. Japonicum, and to other fast-growing rhizobia. It was assumed, that in order to make any meaningful assessment of this newly described group of organisms, that their relative taxonomic relationship to other rhizobia needed to be determined.

Materials and Methods

A. Bacterial Strains and Growth Conditions

The fast-growing soybean-rhizobia, USDA 191, 192, 193, 194, 201, 205, 206, 208, 214, 217, and 257 were isolated from soil or nodules collected in the People's Republic of China [Keyser, et al. (84)]. The slow-growing Bradyrhizobium Japonicum, Chinese strains PRC-005, 74, 113-2, 121-6, 2031, and B15 were obtained from T. S. Hu, Institute of Soils and Fertilizers, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China. Bradyrhizobium japonicum strains USDA 6, 31, 34, 74, 94, 110, 122, 123, 136, 138, 142, and Y1, Yla, Y2, Y2a, Y3, K2, K2a, S1, S1a, were from the USDA Culture Collection, Beltsville, Maryland. Rhizobium lupini CC814s, NZP 2021, NZP 2238, SU 343, and NZP 2037, R. leguminosarum Nitragin 92A3, R. phaseoli NZP 5097, NZP 5253, and NZP 5260, R. trifolii NZP 560, and WU 95, and R. meliloti NZP 4013 were obtained from R. M. Greenwood, Department of Scientific and Industrial Research, Palmerston North, New Zealand. Rhizobium leguminosarum HI 5-0 was isolated in Hawaii [May and Bohlool (104)]. Rhizobium leguminosarum 6015(pJB5JI) was obtained from P. Hirsch, Max Planck Institute, Koln, FRG; Rhizobium phaseoli Bel 7.1 from E. L. Schmidt, University of Minnesota, Minneapolis; Rhizobium sp. (Leucaena) Tal-82, from the Niftal Project, Paia, Hawaii; and Rhizobium sp. (Leucaena) UMKL 19 and R. leguminosarum PRE from W. J. Broughton, Max Planck Institute, Koln, FRG.

Agrobacterium tumefaciens 79 and 101 and Rhizobium sp. (Sesbania) 3F4a4 were from USDA Beltsville, Maryland. Rhizobium meliloti L530 was obtained from B. Rolfe, Australia National University, Canberra City, Australia. All Rhizobium cultures were maintained on yeast extract-mannitol (YEM) agar slants of the following composition in g/1: yeast extract, 1.0; mannitol, 10.0; $K_2HPO_4 \cdot 3H_20$, 0.65; $MgSO_4 \cdot 7H_20$, 0.2; NaCl, 0.1; pH 6.9 (132). Agar slants used for the maintenance of fast-growing rhizobia contained 0.05% $CaCO_3$. Agrobacterium cultures were maintained on Nutrient agar (Difco) slants. All cultures were incubated at 28-30^{0}C, subcultured at least once every month and stored at 4^{0}C.

B. Staining, Morphology, and Cultural Characteristics

Cultures were examined for cell morphology and Gram reaction after 3 d of growth in YEM liquid medium. Colony morphology was examined on cultures grown for 6 d on YEM agar containing brom-thymol-blue, BTB,(0.25 mg/1). Motility was estimated from both YEM liquid and agar cultures and on B5 agar medium (45). Cell size determinations were performed using a calibrated ocular micrometer. Fast- and slow-growing PRC isolates were identified using strain specific fluorescent antibodies prepared according to Schmidt et al. (123).

C. Biochemical Tests

Tolerance to pH extremes was determined by inoculating 10^7 cells/ml from exponentially growing YEM liquid cultures into tubes containing 10 ml of YEM liquid medium which were adjusted to pH 4.5, 9.0, and 9.5. Tubes were incubated at 30^{0}C for 14 d and scored for growth. Tests were performed in triplicate.

Tolerance to sodium chloride was determined on YEM agar plates containing 2.0% NaCl. Plates were spread with 10^8 cells, and growth was scored after 14 d of incubation at 30^0C. Tests were done in triplicate.

For growth reactions in litmus milk (Difco), tubes (10 ml/tube) were incubated in quadruplicate for 6 weeks at 30^0C and were examined for pH changes, reduction of litmus, and peptonization (serum zone formation).

Production of 3-ketolactose was determined according to Bernaerts and De Ley (9). Agrobacterium tumefaciens was used as a positive control for this test.

For gelatinase activity, exponential phase cultures from YEM liquid medium were swabbed onto the surface of tryptone yeast extract (TY) agar plates (62) containing 0.4% (w/v) gelatin (Difco). Plates were incubated at 28^0C for 7 d. A positive reaction was indicated by a clearing zone surrounding the growth of the organism. If no clearing zone was detected, the plates were flooded with a 10% solution of trichloroacetic acid and re-examined.

The pH reactions of isolates on agar plates were determined using YEM medium containing 0.25 mg/l bromthymol blue.

Urease activity was determined on urea agar slants (26) incubated for 7 d at 28^0C.

Citrate utilization was determined on the solid medium of Koser (89). Plates were spread with 10^8 cells, incubated at 30^0C for 14 d, and examined for growth.

Penicillinase (B-lactamase) was detected by the method of Foley and Perret (42), oxidase by the method of Kovaks (90), and catalase by the method of Graham and Parker (53).

Hydrogen sulfide production was determined on agar slants [Hunter and Crecelius (72)]. Slants were inoculated and examined for H_2S after 14 d at 28^0C.

Nitrate reduction was tested as described in the Manual of Methods for General Bacteriology (126), in the same medium used by Graham and Parker (53) and in Difco nitrate broth.

For carbohydrate utilization, the basal medium used (Bis) was that of Bishop et al. (11) with different carbohydrates substituted for mannitol and 0.6 g/l KNO_3 used as the nitrogen source. The medium was solidified with purified agar (Difco). All carbohydrates, with the exception of dextrin and starch, were filter sterilized (0.4 um Nuclepore) before addition to gooled, molten, agar medium. Dextrin and starch were added to the medium before autoclaving at 100^0C for 15 min. Each carbohydrate was added to a final concentration of 1.0% (w/v). Inocula were prepared by removing cells from YEM agar slants (with a cotton swab) and suspending the cells to approximately $1X10^7$ cells/ml in sterile distilled water. A ten-fold dilution of each cell suspension was added to the wells of a multiple inoculator plate [Josey, et al. (81)] and inoculated onto the surface of carbohydrate containing agar plates. Bishop's agar plates without carbohydrate served as controls. Duplicate plates of each carbohydrate were incubated at 28^0C for 7 d and scored for growth.

D. Generation times in culture media and sterile soil

Growth and pH responses were determined in TY, Bis, YEM and PA (62) liquid media. All media were adjusted to pH 6.9 prior to autoclaving. Fast-growing isolates were pre-grown (in the medium into which they would be subsequently inoculated) for 3 d, while the slow-growers were pre-grown for 7 d. Inocula were added to an initial density of 10^6 cells/ml

into 50 ml of the respective medium in 125 ml "side-arm" Erlenmeyer flasks. Flasks were agitated at 150 revolutions per minute (RPM) at 28^{0}C in a water-bath shaker. Cell growth was monitored using a Klett-Summerson Photoelectric-Colorimeter (equipped with a # 66 red filter) and pH determined after four days using an Orion Research (model 501) pH meter and a glass combination-electrode.

Generation times in sterile soil were determined using 10 g samples of air-dried Kula loam soil (Typic Eutrandept, pH 6.5) in 70 ml screw-cap test tubes. Soil tubes were autoclaved for 45 min at 121^{0}C, on two successive days, and inoculated with stationary phase cultures of B. japonicum strains USDA 110 and 136, or fast-growing PRC isolates USDA 193 and 205. Fifteen tubes of each organism were inoculated to obtain initial cell numbers of about 5x10^5 cells/gm and a soil moisture tension of about 60% of water-holding capacity. Cell growth was monitored by plate counts on YEM agar using destructive samplings.

E. Intrinsic antibiotic resistances

Resistance to low levels of antibiotics was determined using the method of Josey et al. (81). Inocula were prepared as outlined above for carbohydrates. Freshly prepared, filter sterilized (0.4 um Nuclepore) solutions of antibiotics were added to cooled, molten TY agar to give the following concentrations (ug/ml): chloramphenicol 12.0, 25.0; kanamycin sulfate 10.0; naladixic acid 10.0; neomycin 2.5, polymyxin B sulfate 20.0, rifampicin 1.0, 6.0, streptomycin sulfate 2.5, 10.0; tetracycline-HCl 4.0; and vancomycin 1.5, 5.0. Controls consisted of TY agar plates without antibiotics. Isolates showing growth were scored as positive. Duplicate plates of each antibiotic were incubated (in the dark) at 28^{0}C for 7 d and scored for growth.

F. Enzyme Assays

6-phosphogluconate dehydrogenase (EC 1.1.1.43) activity was determined in cultures grown for 72 h at 26^{0}C in yeast extract-glucose medium (82). Cells were centrifuged at 6,000 X g for 10 min at 4^{o}C and washed twice in 0.05 M sodium phosphate buffer, pH 7.4. Cell pellets were resuspended in the same buffer, containing $2X10^{-4}$ M 2-mercaptoethanol (100) to a final concentration of 1.0 g wet-weight cells/1.5 ml buffer and disrupted by two passages through a French pressure cell at 15,000 pounds per square inch (PSI) and cell debris removed by centrifugation at 14,000 X g for 30 min at 4^{o}C. The clear supernate was stored at -20^{0}C until use. The activity of NADP-linked 6-phosphogluconate dehydrogenase was measured by following the reduction of NADP according to the method of Martinez-De Drets and Arias (101). Apparent endogenous enzyme activity was subtracted from the results. Specific activities were expressed in nanomoles of NADPH formed / min / mg of protein at 25^{0}C. Protein was determined by the method of Lowry, et al. (98) using bovine serum albumin as the standard.

B-galactosidase (EC 3.2.1.23) activity was determined in cultures grown for 3 d at 26^{0}C in TY medium containing 0.5% (w/v) lactose. Cells were centrifuged and washed as above and resuspended in 0.05 M sodium phosphate buffer, pH 7.2, to a final concentration of 1.0 g wet-weight / 3.0 ml buffer and broken by passage through a French pressure cell at 15,000 PSI. Cell debris was removed as before and supernatant fractions stored at 20^{0}C until use. Enzyme activity was measured by following the appearance of a colored product (o-nitrophenol, ONP) at 420 nm (126). The incubation mixture (4.8 ml) contained: 2.7 ml enzyme reaction buffer [0.1 M sodium phosphate buffer (pH 7.0), $1X10^{-3}$ M $MgSO_4 \cdot 7H_20$, $2X10^{-4}$ M $MnSO_4$, and 0.05 M 2- mercaptoethanol]; 1.8 ml ONPG solution [0.1 M sodium phosphate buffer (pH 7.0) and $1.3X10^{-2}$ M o-nitrophenyl-B-D-

galactopyranoside (Sigma Chemical Co. St. Louis, MO.)]; and 0.3 ml of cell-free extract. Enzyme assays were done at 37^0C and the reactions stopped by the addition of 1.3 ml of reaction stop buffer (8.0 M urea and 1.0 M Na_2CO_3, pH 12.0). Enzyme activities were expressed in micromoles of ONP produced per min per mg of protein at 37^0C. Corrections were made for absorbance values obtained in controls without substrate.

G. Ethanol utilization

The basal medium of Bishop (Bis) (11), with or without added mannitol, and with 0.6 g/l KNO_3 as the nitrogen source, was used in all ethanol experiments. Inocula for all studies were prepared by gently washing the cells from YEM agar slants into 50 ml of Bis medium without any carbon source. All cultures were "starved" by incubation overnight at 28°C prior to inoculation. To determine whether strains could utilize ethanol as the sole source of carbon and energy, starved cells were inoculated into 50 ml of Bis (initial concentration approximately 10^6 cells/ml) supplemented with 0.1, 0.25, 1.0, 2.0, 3.0, or 4.0% (v/v) ethanol. Cultures were examined after 7 d of incubation at 28^0C and scored for growth. Bishop's medium without any carbon source served as control.

For growth yield and mean generation time determinations when growing on ethanol, starved USDA 191 cells were inoculated to approximately 10^6 cells/ml into 50 ml of Bis supplemented with 0.1, 0.2, 0.4, or 1.0% ethanol and incubated at 28°C. Final, total cell numbers were determined using a Petroff-Hausser counting chamber. Mean generation times were calculated from the linear portion of growth curves constructed by following absorbance at 600 nm. Growth yield values were determined after 7 d for 0.1 and 0.2% ethanol and after 13 d for 0.4 and 1.0% ethanol. Substrate conversion values were determined by dividing

the final dry weight of cells in grams by the number of moles of substrate utilized. The disappearence of ethanol from the growth medium was determined by gas chromatography using a Porpak Q column at 185^{0}C with N_2 as the carrier gas.

For growth and survival studies, 50 ml aliquots of Bis containing 0.2% ethanol were inoculated to about 1X10^6 cells/ml and incubated at 25°C. Samples from each culture flask were removed at 0, 9, 14, and 18 days and viable cell numbers determined by plate counts on YEM agar.

In experiments designed to determine whether the lag phase of cells growing on limiting concentrations of ethanol were affected by a subsequent addition of ethanol (at the same or at a greater concentration), a starved USDA 191 culture was inoculated to a final concentration of about 10^6 cells/ml into three, 500 ml side-arm flasks each containing 100 ml of Bis medium. Ethanol was added to two of the flasks to a final concentration of 0.1% (a limiting concentration), while the remaining flask received 1.0% ethanol. Cultures were incubated at 25°C and growth was monitored using a Klett-Summerson Electric Colorimeter equipped with a # 66 red filter. One of the two flasks which initially contained 0.1% ethanol, received an additional 0.1% ethanol at the beginning of each stationary phase of growth, while to the other flask 1.0% ethanol was added only once. The flask which originally contained 1.0% ethanol, received no further ethanol additions.

To determine if the fast-growing soybean rhizobia were capable of showing diauxy when growing under limiting concentrations of ethanol and mannitol, a starved culture of USDA 191 was inoculated into 75 ml of Bis supplemented with either 0.2% ethanol alone, 0.02% mannitol alone, or 0.02% mannitol plus 0.2% ethanol. Flasks were inoculated, in triplicate, with approximately 10^6 cells/ml and two ml aliquots removed at different times to monitor growth (absorbance at 600 nm), ethanol utilization by

quantitative gas chromatography (see above), and mannitol utilization by the periodate-3-methyl-2-benzothiazolinone hydrazone method of Johnson and Sieburth (75). For mannitol determinations, periodate-digested samples and controls (samples without prior periodate digestion), were analyzed in triplicate. All values are the averages of three replicates.

In experiments designed to determine whether the inoculum size affected the length of the observed lag phase, 125 ml side-arm flasks, containing 50 ml of Bis supplemented with 0.4% ethanol, were inoculated, in triplicate, with USDA 191 to an initial cell concentration of $1.6X10^5$, $3.2X10^5$, $6.4X10^5$, $1.2X10^6$, or $2.5X10^6$ cells/ml. Initial cell numbers were determined by viable counts on YEM agar and growth monitored spectrophotometrically using as Klett-Summerson PhotoElectric Colorimeter equipped with a # 66 red filter.

Results

Morphological and cultural characteristics. Both the fast- and slow-growing soybean-rhizobia were Gram negative, non-sporeforming rods. The fast-growing soybean isolates tended to be slightly larger than the slow-growers, with their average dimensions being 2 to 4 um by 0.5 to 1 um. Cells from late log phase to stationary phase YEM cultures of fast-growers tended to become enlarged and exhibited marked pleomorphism. Only a few cells (1-5%) of the fast-growing rhizobia were motile in young (1-2 d) YEM cultures whereas the majority of cells (80-90%) of the slow-growing rhizobia were motile. However, when growing on the surface of moist B5 agar medium, a larger percentage (up to 25%) of the fast-growing soybean rhizobia were found to be motile. On YEM agar plates containing brom thymol blue, both fastand slow-growing rhizobia formed circular, convex, entire colonies. After 6-7 d of growth, the fast-growers formed

colonies with sizes between 1.0 and 5.0 mm in diameter and produced an acid-reaction, while the slow-growers had colony sizes of approximately 0.5 to 1.0 mm in diameter and produced an alkaline-reaction. Several of the fast-growing soybean strains had a dry-crusty (calcified) appearance when grown on YEM agar containing $CaCO_3$. In contrast to a large number of other fast-growing species of Rhizobium, the fast-growing soybean rhizobia do not produce much extracellular polysaccharides on YEM agar. Two of the isolates, USDA 191 and 192 produced "watery" colonies. Upon repeated restreaking, some of the fast-growers produced a second colony-type that was somewhat smaller than colonies produced by the parent cultures. These colonial variants were not always stable. Several of the variants that did appear stable were isolated from seven cultures of fast-growers (USDA 191, 192, 193, 208, 214, 217, and 257) and tested for serological affinity (using strain-specific fluorescent antibodies) and for effectiveness on soybeans (Glycine max) cultivar Peking. They were found to be identical to the parental cultures.

Generation times in culture medium and sterile soil

All of the fast-growing soybean rhizobia examined, had mean generation times much less than the "typical" slowgrowers, in the four culture media examined (Table III-1). For the fast- and slow-growers, the most rapid growth was attained in YEM medium, with an average generation time (MGT) for the fast- and slow-growers of 3.6 and 9.3 h, respectively. Results in Table III-1 also indicate that while TY medium is acceptable for the growth of fast-growing rhizobia (MGT ranging from 3.1-4.2 h), most of the slow-growers did not grow very well in this medium. The fast-growing PRC rhizobia acidified only the YEM culture medium (average pH 5.6). In the other three growth media, the pH either remained the same or increased substantially. The slow-growers on the other hand, raised the pH of all four culture media.

Table III-1. Mean generation times and final pH of the medium of several fast- and slow-growing rhizobia when grown in various media.

Isolate	Medium							
	TY		BIS		PA		YEM	
	MGT[a]	pH[b]	MGT	pH	MGT	pH	MGT	pH
Slow-Growing								
PRC-005	8.4	8.38	12.0	6.92	18.4	7.95	9.9	7.27
PRC-113-2	26.0	8.05	16.6	6.94	22.4	7.26	8.2	7.14
PRC-121-6	30.0	8.18	10.6	8.03	18.2	7.98	6.9	7.55
PRC-2031	22.0	8.28	27.0	7.31	14.0	8.18	13.0	7.24
PRC-B15	10.0	8.57	14.0	6.97	10.2	8.23	8.2	7.07
USDA 110	13.0	8.23	13.4	7.83	12.0	8.07	9.6	7.30
USDA 136	14.4	7.90	20.0	7.14	10.6	8.11	-	-
Average	17.6	8.22	16.2	7.30	15.1	7.96	9.3	7.26
Fast-Growing								
PRC-0B2	4.0	8.16	5.4	7.21	5.9	8.30	4.1	4.90
PRC-0B3	3.1	8.35	4.0	6.02	6.5	8.24	3.8	6.03
PRC-194	4.6	8.23	4.7	7.34	8.2	8.20	4.8	5.88
PRC-201	-	-	-	-	-	-	3.5	6.49
PRC-205	3.7	8.33	4.9	6.95	9.0	8.13	2.9	5.99
PRC-206	-	-	-	-	-	-	4.1	5.91
PRC-208	-	-	-	-	-	-	3.5	5.44
PRC-214	4.2	8.21	6.6	6.30	9.2	8.28	3.5	5.31
PRC-217	-	-	-	-	-	-	2.9	5.05
Bel 7.1	3.7	8.38	5.5	7.38	7.7	8.00	-	-
Average	3.9	8.27	5.2	6.86	7.7	8.19	3.7	5.66

[a]MGT = Mean Generation Time (h)

[b]pH = Final pH of the medium after 4 days

In sterile Kula loam soil, the fast- and slow-growing soybean rhizobia had generation times (Table III-2) consistent with those found in culture medium. The two PRC isolates examined, USDA 193 and 205, had generation times only slightly longer than those found in YEM culture medium, while the generation time of B. japonicum strain USDA 110 was almost identical to that found in YEM.

Growth responses to pH and Nacl. Both fast- and slow-growing soybean-rhizobia were examined for their ability to grow in the presence of 2.0% NaCl and at pH extremes of 4.5 and 9.5. As is shown in Table III-3, the fast-growers were uniformly sensitive to the low pH and tolerant of the high pH, while the inverse was found for the slow-growers. The slow-growers grew poorly at pH 9.0 and not at pH 9.5, while the fast-growers grew well at pH 9.5. Conversely, the slow-growers grew well at pH 4.5, while the fast-growers did not grow at this pH. The fast- and slow-growing rhizobia also differed with respect to their NaCl tolerance. While all of the fast-growing isolates examined were able to grow in the presence of the salt, there was variation among them in the extent of growth. A few isolates (USDA 193, 194, 201 and 257) produced a confluent "lawn" of growth on the medium, while others (USDA 191, 192, 205, 206, 208, 214, and 217) yielded individually tolerant colonies. In contrast, none of the slow-growing B. japonicum grew in 2% NaCl.

Biochemical characteristics. The results shown in Table III-3 indicate that both fast- and slow-growing rhizobia were catalase, oxidase, and urease positive. All of the strains examined, with the exception of the slow-growing PRC strains 2031 and B15, produced penicillinase and all isolates reduced nitrate. None of the isolates produced 3-ketolactose from lactose, hydrogen sulfide from $Fe(NH_4)_2(SO_4)_2$, or utilized citrate as the sole source of carbon.

Table III-2. Mean generation times of fast- and slow-growing soybean rhizobia in sterile soil

Organism	Mean Generation Time (h)[a]
Fast-Growing:	
USDA 193	5.4
USDA 205	4.9
Slow-Growing:	
USDA 110	9.0
USDA 136	7.6

[a]Values determined by viable counts on YEM agar.

Table III-3. Biochemical characteristics of fast- and slow-growing soybean rhizobia.

Characteristic	Percent Isolates Giving Positive Reaction[a]	
	Slow-Growing	Fast-Growing
Catalase production	100	100
Oxidase production	100	100
Urease production	100	100
Penicillinase production	71	100
Nitrate reduction	100	100
3-Ketolactose production[b]	0	0
H_2S production	0	0
Citrate utilization[b]	0	0
Gelatinase production[b]	0	100
2% NaCl Tolerance[c]	0	100
pH 4.5 Tolerance[c]	100	0
pH 9.0 Tolerance[c]	0	100
pH 9.5 Tolerance[c]	0	100

[a] Unless otherwise indicated, 7 slow-growing (Chinese strains: PRC 005, 113-2, 121-6, 2031, B15, and USDA strains: 110 and 136) and 8 fast-growing (Chinese strains: USDA 192, 193, 194, 201, 205, 206, 208, and 214) strains of soybean rhizobia were examined.

[b] Also includes fast-growing soybean rhizobia; USDA 191, 217, and 257.

[c] Fast-growing soybean rhizobia; USDA 191, 192, 193, 194, 201, 205, 206, 208, 214, 217, and 257, and slow-growing soybean rhizobia USDA 34, 110, 122, and 138 were examined.

The two groups exhibited a marked difference in gelatinase activity. The fast-growing soybean-isolates produced a clearing zone on TY-gelatin agar, while the slow-growers did not. No gelatinase activity was detected in R. leguminosarum HI 5-0 and 92A3, R. phaseoli Bel 7.1, R. trifolii NZP 560, or Rhizobium spp. USDA 3F4a4 and UMKL 19. However, a Leucaena isolate, Tal 82, was positive.

The litmus milk reactions of fast- and slow-growing rhizobia are shown in Table III-4. The slow-growing soybean rhizobia, with the exception of PRC 121-6, exhibited no peptonization (serum zone formation) but an alkaline pH change, while the fast-growers gave a variety of litmus milk reactions. These included acid and alkaline pH changes accompanied by peptonization, an alkaline pH change with a lack of peptonization, and no pH change with peptonization. The fast-growing rhizobia tended to reduce litmus, while the slow-growers did not. However, two fast-growing PRC isolates, USDA 193 and 206, peptonized litmus milk weakly, while one fast-growing isolate, USDA 208, failed to peptonize at all. The other fast-growing rhizobia examined, R. phaseoli Bel 7.1, R. phaseoli NZP 5097, R. leguminosarum 92A3, and 6015(pJB5JI), all peptonized litmus milk, had neutral pH reactions, and variable litmus reductions (data not shown).

Carbohydrate utilization. The results of carbohydrate utilization presented in Table III-5 show that the fastgrowers utilized a greater variety of carbohydrates than the slow-growers. All of the organisms examined could utilize L-arabinose, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, L-rhamnose and D-xylose. The fast-growing soybean rhizobia, although capable of utilizing arabinose, grew more slowly on this carbon source than the slow-growers. Only the fast-growing soybean-rhizobia utilized D-cellobiose, i-inositol, lactose, maltose, raffinose, D-glucitol, sucrose, and D-trehalose. None of the

Table III-4. Litmus milk reactions of fast- and slow-growing soybean rhizobia.

Organism	Litmus Reaction: Serum zone	Litmus Reaction: pH reaction[a]	Litmus Reaction: Litmus reduction
Fast-Growing:			
USDA 191,192,214,257	+	Acd	+
USDA 205,206,217	+	Neu	-
USDA 194,201	+	Alk	+
USDA 193	+	Alk	-
USDA 208	-	Alk	-
Slow-growing:			
22 strains[b]	-	Alk	-
1 strain[c]	+	Alk	-

[a] pH reactions: Acd = acid, Alk = alkaline, Neu = neutral.

[b] See materials and methods for slow-growing strains.

[c] PRC isolate: 121-6.

organisms examined could utilize dextrin, galactitol, inulin, or starch as a sole source of carbon for growth.

Antibiotic resistance patterns. The growth responses of fast- and slow-growing soybean rhizobia to various anti-biotics are presented in Table III-6. In general, the fastgrowing rhizobia were sensitive to more antibiotics than the slow-growers. Two other fast-growing species examined, R. leguminosarum 92A3 and R. phaseoli Bel 7.1, had growth responses similar to the fast-growing soybean-rhizobia (data not shown).

6-phosnhogluconate dehydrogenase (NADP-6PGD) activity. The results, shown in Table III-7, indicate that NADP-6PGD activity was only detected in the fast-growing strains. The fast-growing soybean rhizobia had NADP-6PGD activities ranging from 50 to 181 nmoles of NADP reduced per min per mg of protein. No activity was detected in the nine "typical" slow-growing B. japonicum examined. The five other fast-growing Rhizobium species tested (R. leguminosarum 92A3, R. lupins NZP 2037, R. trifolii NZP 560, Rhizobium sp. Tal-82 and Rhizobium sp. 3F4a4) had high specific activities for this enzyme, ranging from 26 to 156 nmoles NADP reduced per min per mg of protein.

B-Galactosidase activity. The results in Table III-7 indicate that only the fast-growing strains had appreciable levels of B-galactosidase activity. In general, they exhibited a 55-fold increase in enzyme activity over the slow-growers. The enzyme activity was comparable to that found with the other fast-growing rhizobia (R. leguminosarum 92A3, R. phaseoli Bel 7.1, R. trifolii NZP 560, and Rhizobium sp. Tal-82) examined (data not shown).

Table III-5. Carbohydrate utilization by fast- and slow-growing soybean-rhizobia.[a]

Carbohydrate	Percent of Isolates Showing Growth	
	Slow-Growing	Fast-Growing
L-Arabinose, D-Fructose, D-Galactose, D-Glucose, D-Mannitol, D-Mannose, L-Rhamnose, D-Xylose	100	100
D-Cellobiose, i-Inositol, Lactose, Maltose, Raffinose, D-Glucitol, Sucrose, D-Trehalose	0	100
Dextrin, Galactitol, Inulin, Starch	0	0

[a]Eight slow-growing (USDA 110, 123, 136, PRC 005, 113-2, 121-6, 2031, and B15) and ten fast-growing (USDA 191, 192, 194, 201, 205, 206, 208, 214, and 217) soybean-rhizobia were examined.

Table III-6. Growth responses of fast- and slow-growing soybean-rhizobia to various antibiotics

Antibiotic	Percent Isolates Giving Growth[a]	
	Slow-Growing	Fast-Growing
Chloramphenicol(12)[b]	100	57.2
Chloramphenicol(25)	100	0
Kanamycin(10)	57.2	28.6
Naladixic acid(10)	85.7	85.7
Neomycin(2.5)	100	0
Polymyxin(20)	100	0
Rifampicin(1)	85.7	0
Rifampicin(6)	57.2	0
Streptomycin(2.5)	100	0
Streptomycin(10)	14.3	0
Tetracycline(4)	100	0
Vancomycin(1.5)	100	100
Vancomycin(5)	100	100

[a]Seven slow-growing (PRC 005, 113-2,121-6, 2031, B15, and USDA 110, and USDA 136) and seven fast-growing (USDA 192, 193, 194, 201, 205, 206, and 208) soybean rhizobia were examined.

[b]Numbers in parentheses designate concentrations (ug/ml).

Table III-7. Enzyme activities of fast- and slow-growing soybean-rhizobia.

Strain	Enzyme	
	6-Phosphogluconate dehydrogenase[a]	B-Galacto-sidase[b]
Slow-Growing:		
USDA 31	<0.5	7.1
USDA 110	<0.5	3.8
USDA 123	<0.5	3.0
USDA 136	<0.5	7.6
USDA 138	<0.5	3.4
PRC 005	<0.5	4.2
PRC 113-2	<0.5	3.3
PRC 121-6	<0.5	4.8
PRC B15	<0.5	4.8
Fast-Growing:		
USDA 191	97	n.t.[c]
USDA 192	181	459
USDA 193	140	183
USDA 194	126	101
USDA 201	50	345
USDA 205	159	97
USDA 206	59	261
USDA 208	147	383
USDA 214	160	245

[a]nmoles NADPH produced/min/mg protein.

[b]umoles ONP produced/min/mg protein.

[c]Not tested.

Growth in ethanol. Of the 30 strains from six species of Rhizobium examined, only the fast-growing soybean rhizobia (USDA 191, 192, 193, 194, 201, 205, 206, 208, 214, and 217) were capable of substantial growth in ethanol (Table III-8). One fast-growing isolate, USDA 191, grew in ethanol up to a concentration of 3.0%. Of the slow-growing B. japonicum tested (USDA 110, 123, 136, PRC 005, 113-2, 121-6, 2031, and B15) all, except USDA 123, grew in 0.1% ethanol. However, the slow-growers grew poorly and did not produce much growth. The two slow-growing R. lupini, CC814s and NZP 2021, were able to grow in 0.2% ethanol, while the fast-growing R. lupini, NZP 2238 and SU 343, did not grow in ethanol at all. Two strains from each of the other species of Rhizobium which were tested (R. legumiaosarum PRE and 92A3, R. meliloti L530 and NZP 4013, R. phaseoli NZP 5253 and NZP 5260, and R. trifolii WU 95 and NZP 560) failed to grow with ethanol as the sole source of carbon and energy.

While the slow-growers were capable of growing in 0.1% ethanol, final cell numbers never exceeded about $4X10^7$ cells/ml (Table III-9). On the other hand, all of the fast-growing PRC rhizobia were capable of substantial growth in 0.2% ethanol, with final cell numbers reaching between 10^8 10^9/ml. The slow-growing soybean-isolates survived better in 0.2% ethanol than did the fast-growers. After 18 d of incubation, cell numbers for the fast-growers decreased by factors of 10-100 from their 9 d values, while cell numbers for the slow-growers remained about the same.

Fast-growing soybean strains USDA 191 and 205 and slow-growing B. japonicum USDA 110, were isolated from culture medium after growing in 1.0 and 0.25% ethanol, respectively, and tested for nodulating ability. All of the isolates still were able to nodulate soybean cultivar Peking (data not shown).

Table III-8. Growth of fast- and slow-growing rhizobia in ethanol.[a]

Organism[b]	Percent Ethanol					
	0.1	0.25	1.0	2.0	3.0	4.0
	Number growing/Total tested					
Rhizobium japonicum (Fast):	10/10	10/10	3/3	1/1	1/1	0/1
Bradyrhizobium japonicum (Slow):	7/8	6/8				
Rhizobium lupini:						
Fast-Growing	0/2	0/2				
Slow-Growing	2/2	0/2[c]				
Rhizobium leguminosarum:	0/2					
Rhizobium meliloti:	0/2					
Rhizobium phaseoli:	0/2					
Rhizobium trifolii:	0/2					

[a]Growth determined in Bishop's minimal medium with KNO_3 as the nitrogen source and supplimented with various concentrations of ethanol.

[b]Rhizobia examined: Fast-growing soybean strains USDA 191, 192, 193, 194, 201, 205, 206, 208, 214, and 217; *B. japonicum* strains USDA 110, 123, 136, and PRC 005, 113-2, 121-6, 2031, and B15; *R. leguminosarum* strains PRE and 92A3; *R. lupini* slow-growing strainsCC814s and NZP 2021 and fast-growing strains NZP 2238 and SU 343; *R. meliloti* strains L5-30 and NZP 4013; *R. phaseoli* strains NZP 5253 and NZP 5260; and *R. trifolii* strains NZP 560 and WU 95.

[c]Growth detected up to 0.2%.

Table III-9. Growth and survival of fast- and slow-growing soybean-rhizobia in ethanol[a]

Culture	Viable Cell Number at Day			
	0	9	14	18
Fast-Growing:				
USDA 191	$7.3X10^5$	$1.1X10^9$	$7.1X10^8$	$4.0X10^7$
USDA 192	$3.9X10^5$	$1.5X10^8$	$1.5X10^8$	$2.4X10^7$
USDA 194	$2.6X10^5$	n.t.[b]	$7.5X10^8$	n.t.
USDA 205	$2.9X10^5$	$3.1X10^8$	$3.1X10^8$	$2.6X10^7$
Slow-Growing:				
USDA 110	$7.0X10^5$	n.t.	$3.9X10^7$	n.t.
USDA 123	$5.4X10^5$	n.t.	$5.8X10^7$	n.t.
USDA 136	$6.2X10^5$	$3.5X10^7$	$4.9X10^7$	$4.2X10^7$
PRC 2031	$7.6X10^5$	n.t.	$9.1X10^6$	n.t.

[a]Ethanol concentration = 0.2% .

[b]Not tested.

To determine whether ethanol concentration affected the growth of the fast-growing soybean rhizobia, USDA 191 cells were inoculated into Bishop's medium containing 0.1, 0.2, 0.4, or 1.0% ethanol. The results presented in Table III-10 and Figure III-1 show that although ethanol can be used as a sole source of carbon and energy by the the fast-growing soybean-rhizobia, it may also be toxic to the cells at higher concentrations. Results presented in Table III-10 and Figure III-1 show that growth is substrate limited with ethanol concentrations less than 0.4%.

In previous experiments, it was observed that the higher the ethanol concentration, the longer the lag phase (data not shown). To determine whether the length of the lag phase was correlated with the concentration of ethanol and if preconditioning the cells in one ethanol concentration decreased the lag phase in the same or a different concentration of ethanol, flasks containing 0.1 or 1.0% ethanol were inoculated with USDA 191 (Figure III-2). Cells in the two flasks containing 0.1% ethanol showed logarithmic growth between 3-5 d, while cells growing in 1.O% ethanol did not emerge from lag phase until about 8 d of growth. Results in Figure III-2 show that when 0.1% ethanol was added to cells previously growing on 0.1% ethanol, there was a reduction in the length of the lag phase. Although, the addition of 1.0% ethanol reduced the growth rate of cells previously growing in 0.1%, a lag phase following this addition was not observed.

The results shown in Figure III-3 indicate that the length of the lag phase is also related to the intial inoculum size. With the exception of one initial cell density, the greater the initial inoculum size, the shorter the lag phase. Those flasks receiving $2.5X10^6$ cells/ml had a lag phase of about 2.7 d, while the flasks receiving $1.6X10^5$ cells/ml had a lag phase of about 5 d. Also, the

Table III-10. Growth parameters of fast-growing strain USDA 191 in several concentrations of ethanol

Ethanol Concentration (%)	Growth Yield[a]		Mean Doubling (h)	Ys[c]
	Final Numbers[b]	Dry Weight (mg)		
0.1	$1.42X10^9$	32.62	10.08	19.13
0.2	$2.35X10^9$	64.64	13.44	19.01
0.4	$2.43X10^9$	72.27	14.44	13.80
1.0	$5.70X10^9$	129.40	21.60	14.10

[a]Values determined after 7 days for 0.1 and 0.2% ethanol and after 13 days for 0.4 and 1.0% ethanol.

[b]Total cell numbers.

[c]Ys = gram dry weight cells/mole substrate utilized.

Figure III-1. Growth and ethanol utilization of USDA 191 in Bishop's basal minimal medium with various concentrations of ethanol. Figure: III-1A Growth; III-1B Ethanol utilization. Numbers on curves represent initial ethanol concentrations in percent.

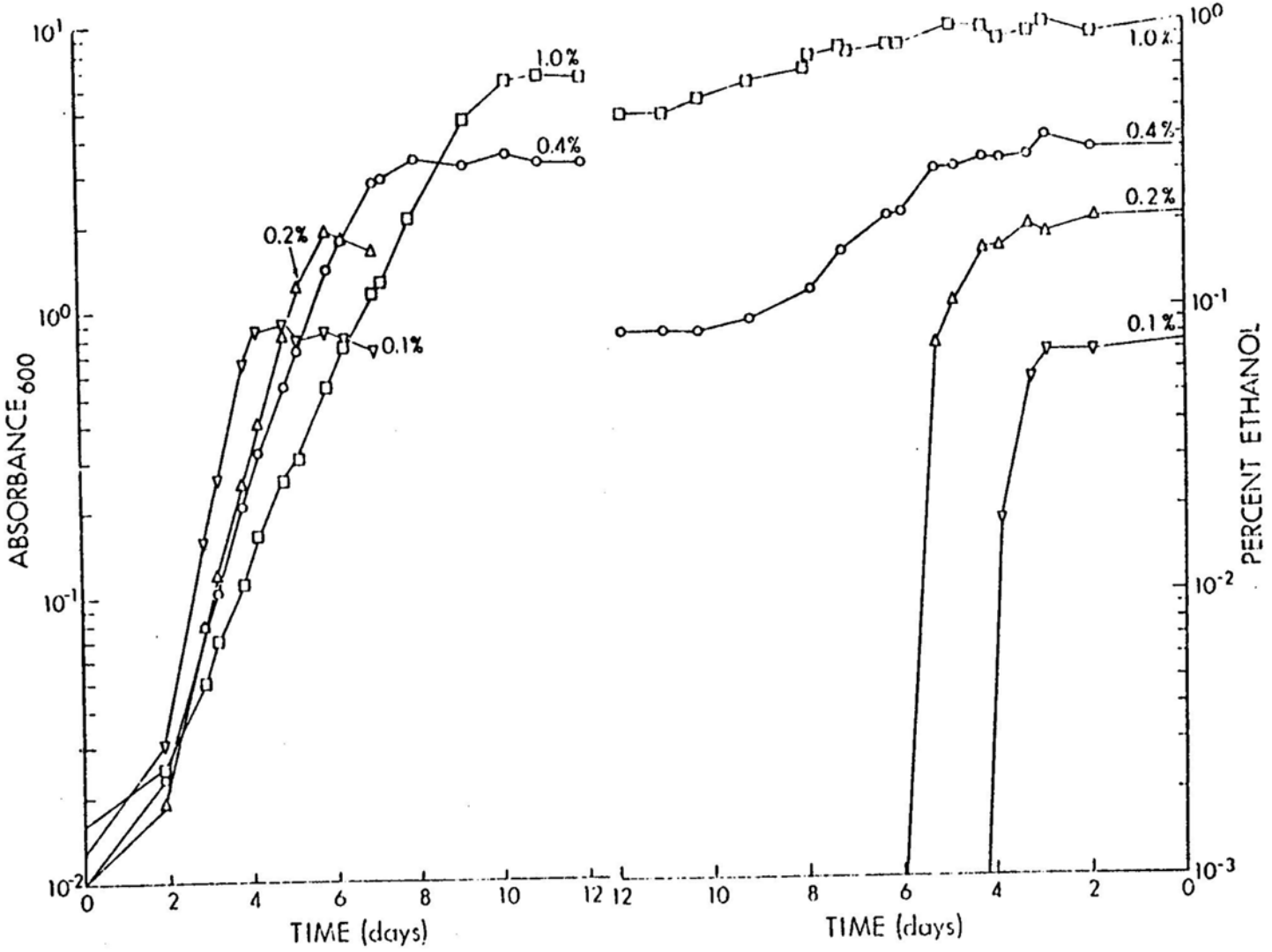

ABSORBANCE 600
TIME (days)
1.0%
0.4%
0.2%
0.1%
PERCENT ETHANOL
TIME (days)
1.0%
0.4%
0.2%
0.1%

Figure III-2. Ethanol concentration step-up of USDA 191 while growing in two concentrations of ethanol. Cultures started in 0.1% ethanol, ○ and △, received an additional 0.1% or 1.0% ethanol, respectively, at arrows. Culture started in 1.0% ethanol, □, received no extra ethanol additions.

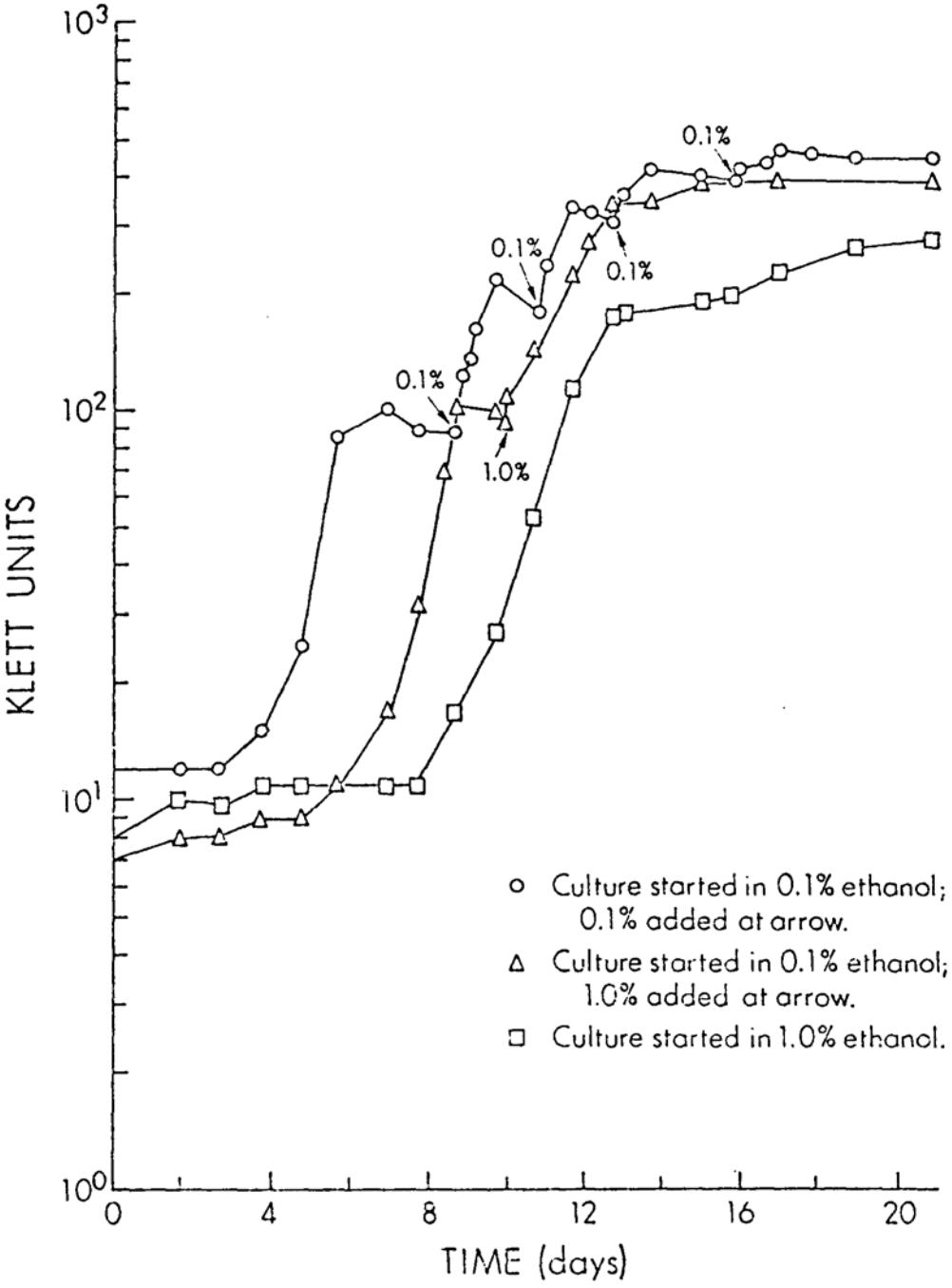

10^3
10^2
10^1
10^0
KLETT UNITS
0
4
8
12
16
20
TIME (days)
0.1%
0.1%
0.1%
0.1%
1.0%
Culture started in 0.1% ethanol; 0.1% added at arrow.
Culture started in 0.1% ethanol; 1.0% added at arrow.
Culture started in 1.0% ethanol.

Figure III-3. Relationships between inoculum size and Lag phase of USDA 191 when growing in 0.4% ethanol. Flasks were inoculated with: $1.6X10^5$, □; $3.2X10^5$, ○; $6.4X10^5$, Δ; $1.3X10^6$, X; or $2.6X10^6$ *;cells/ml.

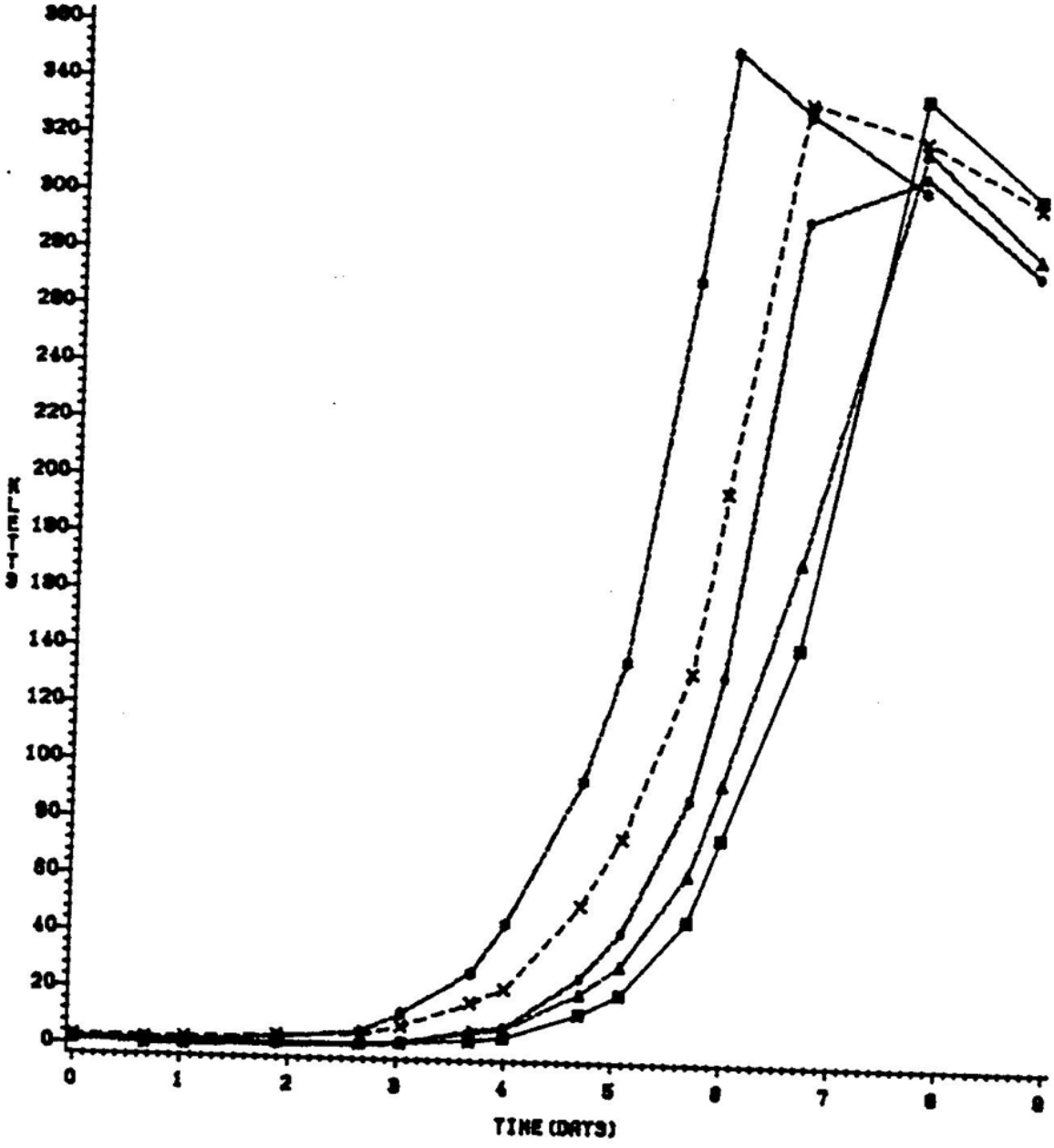

360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
TIME (DAYS)

Figure III-4. Regression line of time to reach 15 Klett units and Log number of inoculum size (cells/ml).

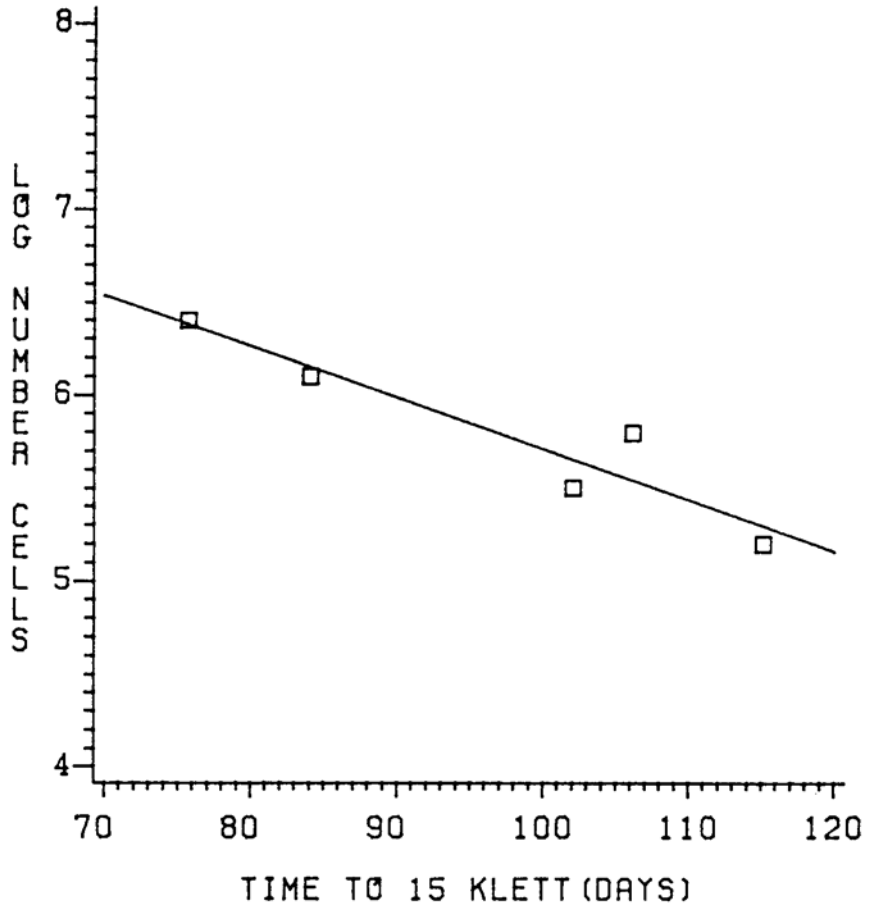
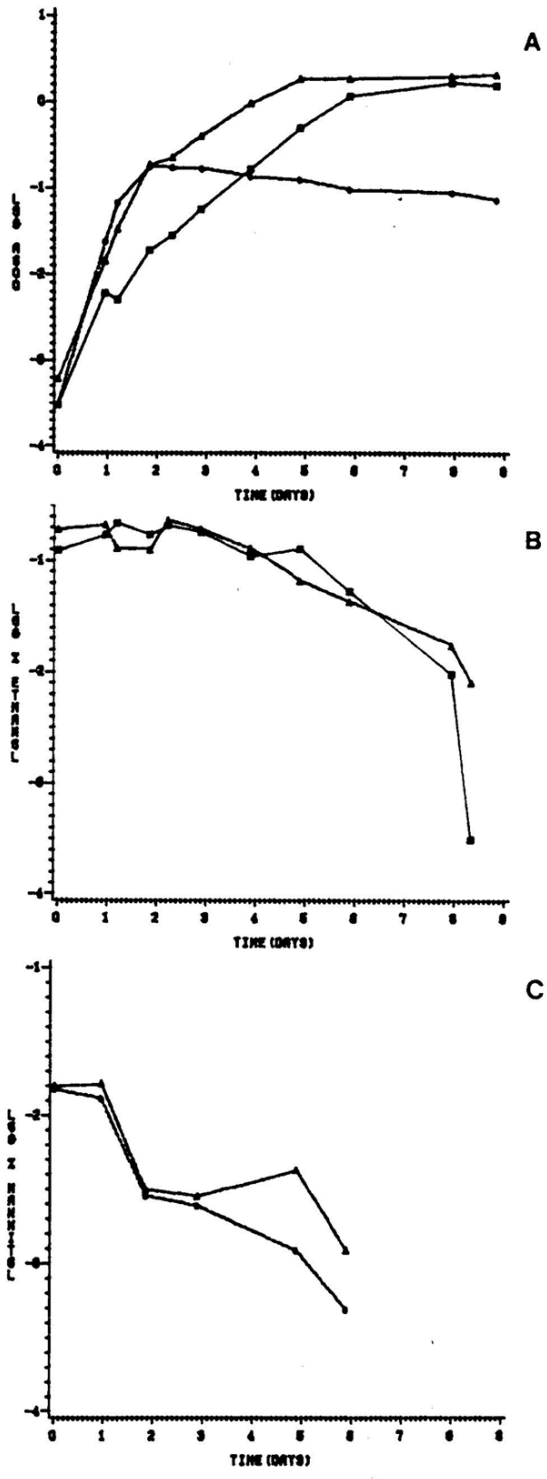

LOG NUMBER CELLS
8
7
6
5
4
70
80
90
100
110
120
TIME TO 15 KLETT (DAYS)

Figure III-5. Growth and mannitol and ethanol utilization of USDA 191. Figure III-5A: Growth in 0.2% ethanol, □; 0.02% mannitol, ○; and 0.02% mannitol plus 0.2% ethanol, Δ. Figure III-5B: Removal of ethanol from the growth medium when growing in 0.2% ethanol, □ or in 0.2% ethanol plus 0.02% mannitol, Δ. Figure III-5C: Removal of mannitol from the growth medium when growing in 0.02% mannitol, ○, or in 0.2% ethanol plus 0.02% mannitol, Δ.


A
B
C
TIME (DAYS)
TIME (DAYS)
TIME (DAYS)

graph of the time to reach 15 Klett units vs Log inoculum size is linear (Figure III-4) with an R^2 = 0.885.

To determine whether the fast-growing soybean rhizobia would show diauxic growth when grown under limiting concentrations of ethanol and mannitol, USDA 191 was inoculated into 0.2 % ethanol, 0.02% mannitol, and 0.2% ethanol plus 0.02% mannitol. Results in Figure III-5A show that when cells were grown in limiting concentrations of mannitol alone, there was no appreciable lag phase and cells reached stationary phase about 2 d after inoculation. However, when cells were grown in ethanol alone (also at a limiting concentration), the growth rate was slower than that seen in mannitol, and it took about 5 d to reach stationary phase. Cells grown under limiting conditions of both mannitol and ethanol together, initially had a growth rate similar to cells growing in mannitol alone, however, when mannitol became limited, after about 2 d, the growth rate declined to about that seen for cells growing on ethanol alone. The results in Figure III-5B indicate that ethanol did not begin to disappear appreciably from the growth medium untill after 3 d of growth and that ethanol utilization was about the same for cells grown in ethanol alone or in ethanol plus mannitol. Similarly, mannitol utilization was about the same for cells grown in mannitol only, or cells grown in mannitol plus ethanol (Figure III-5C).

Discussion

Several diagnostic tests were performed to compare fast-and slow-growing rhizobia that nodulate soybeans. The results indicate that the fast-growers are quite different biochemically from the slow-growers. While the fast-growers share host specificity with the slow-growers [both nodulate soybeans as well as other hosts (84)], they appear more similar

to other fast-growing Rhizobium species in their microbiological, physiological and biochemical characteristics. Some characteristics, however, are shared between the two groups of organisms.

All of the fast- and slow-growing soybean-rhizobia examined were typically positive for catalase, oxidase, urease, penicillinase, and nitrate reductase. Both groups were unable to utilize citrate as a sole source of carbon, produce H_2S, or produce 3-ketolactose. Graham and Parker (53) also found that most Rhizobium japonicum strains they tested possessed several of these attributes. They reported that the production of H_2S and utilization of citrate was restricted to some isolates of R. meliloti. Bernaerts and De Ley (9) found that the production of 3-ketolactose from lactose is limited to Agrobacterium species, a genus closely related to Rhizobium. Graham and Parker (53) found that the production of penicillinase seemed to be restricted mainly to the slow-growing rhizobia, R. japonicum, R. lupini and Rhizobium spp. of the "cowpea miscellany" group. However, these authors indicated that some of the R. leguminosarum strains also possess this attribute. Microbiologically, the fast and slow-growers appear to be related in that they produce similar colony types (although they do differ in size) and tend to produce morphological variants. However, as with other bacteria, the production of morphological variants is not uncommon in Rhizobium (58,134)

The PRC isolates were truly fast-growing in sterile soil and in all of the laboratory culture media examined. In most instances, when growing in laboratory media, the mean generation times of the fast-growers were 2 to 3 times that of the slow-growers. While the slow-growers grew from 1.5 to 2 times faster in sterile soil than in media, growth rates of the fast-growers in sterile soil was in the same range as that found in laboratory media. It is interesting to note that the fast-growing

soybean isolates only reduced the pH of YEM medium, while in all other media, the pH was raised. This pH reduction-property can not only be due to the presence of mannitol, since Bis also contained the same mannitol concentration. The reason(s) why Bis medium became alkaline following growth of the fast-growers, is not understood. However, it is important to remember, that for taxonomic considerations, determinations as to whether rhizobia are alkaline producing or acid-producing should only be ascertained in YEM medium.

The results of the pH and salt tolerance tests place the fast-growing soybean-rhizobia with other fast-growing rhizobia. Previous studies of rhizobia (20,43,53), in pure culture and in soil, showed that the fast-growers were relatively more alkali-tolerant and acid-sensitive, than the slow-growers. Also, Graham and Parker (53) showed that among rhizobia, tolerance to 2% NaCl was restricted to the fast-growing R. meliloti. In addition, the responses of the fast-growing soybean-rhizobia in litmus milk were more typical of fast-growing Rhizobium, e.g. R. trifolii, R. leguminosarum, and R. meliloti (44,53).

Fast- and slow-growers also appeared to be separated on the basis of their utilization of carbohydrates and sensitivities to antibiotics. Graham and Parker (53) and Fred et al. (44) showed that fast-growing rhizobia tend to use a wider variety of carbohydrates than the slow-growers. This is clearly evident in Table III-5, with the fast-growers using the majority of the carbohydrates examined and the slow-growers relatively few. It should be noted that the types of carbohydrates utilized also varies amongst the soybean-rhizobia. As was pointed out by Glenn and Dilworth (48), slow-growing rhizobia tend to lack both uptake systems and catabolic enzymes for disaccharides. My results are in agreement with these authors in that the disaccharides cellobiose, lactose, maltose, trehalose, and sucrose and the trisaccharide raffinose

clearly separated the fast- from slow-growing rhizobia. The results in Table III-7 show that only the fast-growing soybean rhizobia have appreciable B-galactosidase activities, similar to that of the other fastgrowers examined (data not shown). These results agree with those of Glenn and Dilworth (48) who found that among the rhizobia they examined, the inability of slow-growers to utilize several disaccharides was due to the lack of disaccharide uptake systems and the hydrolytic enzymes to cleave the disaccharides. The results in Table III-6 show that the fast-growing soybean rhizobia were sensitive to more antibiotics than the slow-growers. It should be noted that the fast- and slow-growing rhizobia could not be placed into any resistance groupings using the antibiotics and concentrations employed. Vincent (134) has indicated that while strains within a species of Rhizobium show differential sensitivity to antibiotics, the slow-growing species of Rhizobium are generally more resistant than the fast-growing ones.

Consistent with the division of rhizobia into fast- and slow-growing groups on the basis of carbohydrates utilized, is their division based on the presence and absence of enzymes of the pentose phosphate pathway. As was pointed out by Martinez-De Drets and Arias (101,102), although both fast- and slow-growing rhizobia have NAD-linked 6PGD activity, only the fast-growers have NADP-linked 6PGD activity. The results in Table III-7 demonstrate that the fast-growing soybean-rhizobia have levels of NADP-6PGD activity comparable to those of the other fast-growing rhizobia. These results are consistent with those of Martinez-De Drets and Arias (100,101) and Keele et al. (82) who found high levels of NADP-6PGD activity for only fast-growing species of Rhizobium.

Although mannitol and sucrose are the preferred carbon sources for fast-growing rhizobia (44) and arabinose for the slow-growers (1), both groups are capable of utilizing many different types of carbon compounds.

While it has been shown that in B. japonicum bacteroids (the morphologic state of rhizobia within soybean root-nodules), aldehydes and alcohols can support acetylene reduction and oxygen consumption (112), and that soybean nodules contain acetaldehyde and ethanol (129), there have been no reports of ethanol utilization by free-living rhizobia. Results presented in this study indicate that both the fast- and slow-growing soybean rhizobia are capable of utilizing ethanol as the sole source of carbon and energy.

Although fast- and slow-growing soybean rhizobia could not be separated on the basis of ethanol utilization, there were some clear differences in the extent in which they used ethanol. While the fast-growers routinely grew to final cell densities of $1X10^8$-$1X10^9$ on 0.2% ethanol, the slow-growers never reached densities greater than about $1X10^7$ cells/ml on any concentration of ethanol. It is apparent from results (Table III-10 and Figure III-1) showing decreased growth rates in higher ethanol concentrations, that ethanol most likely is toxic to the cells. The mean generation time of USDA 191 increased nearly 2-fold, when the ethanol concentration was raised from 0.1% to 1.0%. It is interesting to note (Figure III-4) that whether the cells were originally started in 1.0% ethanol or had their ethanol concentration raised from 0.1 to 1.0%, that their growth rates remained about the same. In the flask receiving multiple additions of 0.1% ethanol, growth rates also remained relatively constant following each ethanol addition. The results presented in Table III-10 and Figure III-1 indicate that growth is substrate limited with ethanol concentrations less than 0.4%. At ethanol concentrations of 0.1-0.2%, final cell numbers and dry weight are proportional to ethanol concentration. However, at an ethanol concentration of 0.4% and greater, growth did not appear substrate limited and growth yield values were not proportional to ethanol concentration. Results in Figure III-1 show that the maximum rate of ethanol utilization (when 191 was growing in 0.1 and

0.2% ethanol) occurred after 3 to 4 d of growth. In 0.4 or 1.0% ethanol, the rate of utilization was considerably less than that seen in lower concentrations.

Three of the fast-growing soybean rhizobia (USDA 192, 194, and 205) were also examined for their ability to use other low carbon-number compounds. While all three of the isolates utilized acetate as a sole source of carbon, only USDA 192 could use 1-propanol (data not presented). None of the organisms examined could utilize methanol, formate, or 1-butanol as a sole source of carbon (data not shown). While all of the fast-growing soybean rhizobia utilized ethanol as a sole source of carbon, I could not detect any NAD- or NADP- linked alcohol dehydrogenase (ADH) activity in cellfree extracts of the organisms (data not shown). Rigaud and Trinchant (118) have demonstrated a soluble alcohol dehydrogenase in R. meliloti. However, they did not examine whether cells were capable of growth in ethanol. Similarly, De Vries et al. (34) and Tajima and La Rue (129) demonstrated the presence of an NAD-linked ADH in pea and soybean root nodule tissue, respectively. The inabilty to detect NAD or NADP-linked ADH in cell-free extracts of the fast-growing soybean rhizobia may indicate that either the ADH of these organisms requires a different coenzyme, or that the enzyme may not be present in cell-free extracts (i.e., is membrane bound), or that the enzyme was present at undetectable levels in the cells. Since malate synthetase was detected in cell-free extracts of USDA 205 (data not shown), it is assumed that they at least have some enzymes of the glyoxylate cycle. With this cycle present, the organisms have a means for replenishing C-4 intermediates of the TCA cycle from acetyl CoA derived from acetate, or ethanol. While Johnson et al. (74) detected malate synthetase activity in culture-grown B.

japonicum cells, these authors failed to detect any isocitrate lyase activity in these isolates. However, isocitrate lyase was detected in culture-grown R. meliloti, R. leguminosarum, R. trifolii, and R. phaseoli cells (64) and in bacteroids from senescent soybean nodules (131). Interestingly, the organisms Johnson et al. (74) examined could not utilize acetate as a sole source of carbon. Thus, it is apparent that more research is needed on the mechanism(s) by which fast- and slow-growing rhizobia are able to utilize ethanol as a source of carbon.

In summary, while the fast-growing soybean-rhizobia share symbiotic host-specificity with the typical slow-growers, they appear more closely related, on a biochemical basis, to other fast-growing species of Rhizobium. The typical soybean-rhizobia are now classified as Bradyrhizobium japonicum (79). Most of the evidence presented in this thesis indicates that the newly isolated fast-growers do not fit into this species. Therefore, the taxonomic position of these new isolates must logically be in the new genus Rhizobium.

CHAPTER IV

PLASMIDS OF FAST-GROWING SOYBEAN RHIZOBIA: PROFILES RESTRICTION ENDONUCLEASE PATTERNS, AND ROLE IN MODULATION

Abstract

Several isolates from a newly-described group of fast-growing acid-producing soybean rhizobia were analyzed for plasmid content. All contained from 1 to 4 plasmids with molecular weights of 100×10^6 or larger. While most of the isolates shared plasmids of similar size, the restriction endonuclease (BamHI, EcoRI and HindIII) patterns of the plasmids from three of the isolates were vastly different.

Growth in the presence of acridine orange was effective in producing mutants cured of the largest plasmid in one of the strains. These mutants had also lost the ability to form nodules on soybeans. High-temperature curing of a smaller plasmid in another strain did not lead to loss of nodulating ability or alteration of symbiotic effectiveness on soybean cultivars.

The Rhizobium leguminosarum host-range (Sym) plasmid, pJB5JI, is a 130 Mdal self-transmissible plasmid which carries the genes for host-specificity (peas) and some of the genes for nitrogen-fixation. Plasmid pJB5JI was transferred into different species of Rhizobium and the symbiotic properties of the resulting transconjugants were examined. While, transconjugants made with the fast-growing soybean-rhizobia were unable to nodulate peas, they still retained the ability to nodulate soybeans. Transconjugants made with a Nod^+ strain of R. trifolii, 0403, could nodulate clover (effectively) and peas (ineffectively). However,

the transconjugants of a cured Nod$^-$ R. trifolii, #2, were highly effective on peas, but still did not nodulate clover. After reisolation from pea and clover nodules, #2 and 0403 transconjugants had a variety of symbiotic responses when reinoculated onto peas and clover plants. Evidence for successful transfer of the plasmid to all transconjugants included: appearance of kanamycin resistance (due to TN5 in pJB5JI) in the recipients, physical presence in the transconjugants of a new plasmid corresponding to pJB5JI, and their ability to back-cross to a non-nodulating strain of R. leguminosarum and make it infective. While plasmid pJB5JI is readily transferable to many species of Rhizobium, its symbiotic performance varies depending on the genetic background in which it resides.

Introduction

Recent advances in our understanding of the genetics of the legume-*Rhizobium* symbiosis have, for the most part, been achieved with the fast-growing species of *Rhizobium*: *R. leguminosarum* (peas, lentils, and vetch) *R. meliloti* (alfalfa), *R. phaseoli* (beans), and *R. trifolii* (clover) (10,17,18,35,76). This is in part due to the fact that symbiosis-related genes are plasmid-borne in most of these rhizobia (10,62,65,66,108). In 1967, Higashi (60) provided the first suggestive evidence that plasmid genes are involved in nodulation and the host-range specificity of *Rhizobium trifolii*. He found that *R. trifolii* and *R. phaseoli* lost their ability to nodulate their respective hosts, following treatment with acridine orange. Acridine dyes were known (27) to cause elimination of plasmids in other gram-negative bacteria and thus, it was assumed that the loss of nodulating ability was due to the loss of resident plasmid(s). Similarly, Pariiskaya (111), and Zurkowski *et al.* (141) demonstrated that growth in media containing acridine orange resulted in the development of non-nodulating mutants. It was subsequently shown (105,142) that *Rhizobium* cultures could be cured of their nodulating ability by incubation at elevated temperatures (35-37^{0}C). However, it was not until the work of Nuti *et al.* (107), that physical evidence for the presence of plasmids in *Rhizobium* was presented.

Rhizobia differ from most other Gram-negative bacteria in that they possess very large plasmids (megaplasmids). It is not unusual to isolate *Rhizobium* plasmids with molecular weights (M.W.) between 100 and 300 megadaltons (Mdal) (107,114). Two *R. meliloti* strains, T12 and T24, have been reported to carry plasmids of 190 and 460 Mdal (143). In all of the more than 60 fast-growing *Rhizobium* strains thus far investigated, large

plasmids with molecular weights > 90 X10^6 have been reported (33). Also, it is not unusual to isolate more than one size class of plasmids from a single Rhizobium strain. It is also interesting to note, that the presence of plasmids with M.W.< 85 X10^6 have rarely been reported in rhizobia. Thus, no resident rhizobial plasmids are available as molecular cloning vehicles (33).

Little information is available on the genetics of the slow-growing rhizobia, B. japonicum, R. lupini, and Rhizobium sp. of the cowpea miscellany group. This is due partly to the inability to demonstrate consistently plasmids in all infective strains. Furthermore, the location of symbiosis-related genes in the slow-growing rhizobia is still unknown. Gross et al. (54) examined plasmids in several isolates of B. japonicum (primarily from one serogroup). Although all isolates were obtained from soils with similar characteristics and were predominantly from the same serogroup, 135, they could be subdivided in 4 groups on the basis of plasmid number and size. However, these authors did not investigate the location of symbiotic genes. Recently, Masterson et al. (103) reported that in the strains of B. japonicum that they examined, the plasmids isolated did not contain genes that were hybridizable to Klebsiella pneumoniae nitrogen fixation (Nif D and H) genes. Interestingly, these authors did detect hybridization between NifDH genes and total cell DNA preparations in several slowgrowing B. japonicum strains. These results indicate that structural nitrogen fixation genes may not be plasmid borne in these slow-growers.

Genetic studies involving plasmid transfer in Rhizobium have almost exclusively been done with the fast-growing species (8). Recently, Pilicinski and Schmidt (113) demonstrated plasmid transfer within and between serologically distinct strains of B. japonicum. However, the plasmids used in their study were R68.45 and RP4

(originally from Pseudomonas), and not rhizobial plasmids. Similarly, Kennedy et al. (83) and Kuykendall (95) demonstrated the transfer of plasmids pRD1 and R68.45 from E. coli into several strains of slow-growing "cowpea" rhizobia and B. japonicum, respectively. It should be noted however, that all of these plasmids could not be transferred to all of the strains investigated.

The Rhizobium leguminosarum plasmid, pJB5JI (pRL1JI::TN5), is the pea SYM (host-range) plasmid with a TN5 (kanamycin) insertion in the genes for medium bacteriocin production (76). It has been used by several investigators (10,17,18,35,62,76,77) to transfer pea-nodulation genes into R. leguminosarum, R. phaseoli, and R. trifolii strains. Plasmid pJB5JI has a M.W. of $130X10^6$ and carries some of the nitrogen fixation (Nif) and pea-nodulation genes (Nod). This conjugal, self-transmissible plasmid also carries transfer function genes (Tra) and is transferrable to a large number of Rhizobium strains at frequencies of 10^{-1} to 10^{-3} (8). In addition, plasmid pJB5JI is in the same compatibility group as other Rhizobium plasmids, and thus is maintained intact in many recipients.

The present work describes genetic investigations of the fast-growing soybean rhizobia. These investigations were undertaken to determine: 1, whether the fast-growing PRC rhizobia contained large M.W. plasmids; 2, whether there are structural relationships between plasmids from different strains; 3, whether symbiosis-related genes are plasmid-borne in these isolates; and 4, whether the fast-growing soybean rhizobia were capable of accepting, maintaining, and expressing plasmids from other fast-growing rhizobia. In addition, this work describes investigations into the stability, maintenance, and expression of a R. leguminosarum Sym plasmid in fast-growing soybean rhizobia as well as in R. trifolii which have different genetic constitutions.

Materials and Methods

A. Bacterial strains and maintenance of cultures

The Rhizobium cultures used in this study and their sources are listed in Table IV-1. All isolates (with the exception of USDA 110) were maintained on yeast extract mannitol, YEM, (132) agar slants containing 0.05% $CaCO_3$. The fast-growing soybean rhizobia chosen for this study fall into at least four somatic serological groups (See Chapter V, This work).

B. Plasmid isolation and screening

Plasmid DNA was isolated by a modification of the method described by Hirsch et al. (62). All buffers and labware were autoclaved before use. Cultures were grown for 48 h in 200 ml of PA medium (62), supplemented with 0.20 g/1 of K_2HP0_4. Bacteria were harvested by centrifugation at 10,000 X g and washed, two times, in TEN buffer (50 mm Tris, 20 mM Na_2-EDTA, and 50 mM NaCl, pH 8.0). The cell pellet was resuspended in 16 ml of TEN buffer and lysozyme (Sigma Chemical Co., St. Louis) (5 mg/ml in sterile TEN) added to a final concentration of 0.55 mg/ml. The mixture was incubated at 37°C for 30 min and Pronase B (CalBiochem Corp., California) (5 mg/ml in sterile TEN, pre-digested at 37°C for 1 h) and sodium dodecyl sulfate (Sigma) [10% (w/v) in TEN] were added to final concentrations of 0.5 mg/ml and 0.9% (v/v), respectively. The mixture was incubated at 37°C for 1 h or until lysis was complete and the viscous lysate titrated to pH 12.4 by the addition of 3 M NaOH with gentle, but thorough stirrinig (using the back-end of a plastic knife). After incubation at room

Table IV-1. Bacteria used and their sources.

Isolate	Relevant Characteristic	Source
Rhizobium japonicum:		
USDA 192	wild-type	USDA, Beltsville
USDA 193		
USDA 194		
USDA 201		
USDA 205		
USDA 206		
USDA 208		
2051A03-R	Nod^-, Str^r, Chl^r	This study
Transconjugants		
201-8	Kan^r	This study
201-11	201(pJB5JI), Kan^r	
201-13	Kan^r	
201-14	201(pJB5JI), Kan^r	
Rhizobium leguminosarum:		
6015(pJB5JI)	Nod^+:300 phe-1, trp-12, rif-392, str-37, inf-6007, Nod^+, Kan^r	B. Rolfe
6015	Nod^-:300 phe-1, trp-12, rif-392, str-37, inf-6007, Nod^-, Kan^s	P. Hirsch
Transconjugants		
6015-11-7	6015(pJB5JI), derived from back-cross of 6015 to 201-11	This study
6015-14-8	6015(pJB5JI), derived from back-cross of 6015 to 201-14	This study
Rhizobum trifolii:		
#2	Nod^-	W. Broughton
403	Nod^+, Wild-Type	W. Broughton
Transconjugants		
2-33	2(pJB5JI) Nod^+, Fix^+	This study
2-54	2(pJB5JI) Nod^+, Fix^+	This study
403-33	403(pJB5JI) Nod^+, Fix^-	This study

temperature for 20 min, the lysate was adjusted to pH 8.5 with 2 M Tris-HCl (Sigma) and transferred to a 40 ml polycarbonate centrifuge tube. Cold (4°C) 5 M NaCl was added to a final concentration of 1 M and the contents of the tube mixed by gentle inversion. The mixture was incubated at 4°C for 4 h and the SDS/NaCl precipitate removed by centrifugation at 10,000 X g for 20 min at 4°C. The supernatant was transferred to a 40 ml centrifuge tube and polyethylene glycol (PEG) 6000 (Sigma) [50% (w/v) in TEN] was added to a final concentration of 10% (v/v). The tube was mixed by inversion and incubated overnight at 4°C and plasmid DNA collected by centrifugation at 7,000 X g for 15 min at 4°C. The supernatant was gently decanted and the pellet was allowed to dissolve in 0.5 ml of TEN buffer. Plasmid samples were stored at 4°C for up to 1 week.

For preparative plasmid extractions, all isolation steps were scaled up 10-fold and the plasmids were further purified by CsCl-ethidium bromide (EtBr) equilibrium density gradient centrifugation. Crude plasmid DNA, 4.5 ml, was added to 20 ml of CsCl-saturated TEN buffer in a 40 ml centrifuge tube, the solution gently mixed by inversion of the tube, and centrifuged at 10,000 X g for 20 min at 4°C. The resultant PEG/SDS precipitate was removed from the upper portion of the solution and ethidium bromide (Sigma) (10 mg/ml in sterile distilled water) was added to a final concentration of 0.31 mg/ml. The refractive index of the solution was adjusted to 1.3925±0.001 and transferred to 13 ml nitrocellulose ultracentrifuge tubes (Beckman). The DNA preparation was centrifuged, 2 times, at 36,000 RPM and 17°C for 48 hours, in a Type 40 (Beckman) rotor (25). Ethidium bromide and CsCl were removed from the plasmid preparation as is outlined by Hirsch et al. (62) except that CsCl-saturated n-butanol was used as the ethidium bromide extractant.

For plasmid screening, samples were mixed with tracking dye [50%(w/v) glycerol, 0.125% (w/v) bromphenol blue, and 50 mM Na_2-EDTA, pH 8.0] and plasmids were resolved by electrophoresis on horizontal 0.7% agarose (Biorad, Richmond, CA; Standard Low) gels (18 by 13 by 0.6 em) at 60 mA for 6 h at 4^0C with Tris-borate buffer (62). Gels were stained for 20 min in Tris-borate buffer containing 5 ug/ml ethidium bromide and bands were visualized by reflective, short-wave, UV light and photographed using Kodak Pan-X film and a yellow (Hoya, K2) filter.

C. Restriction endonuclease analysis

For restriction enzyme analysis, 60 ul of purified Plasmid DNA was digested with either BamHI, EcoRI, or HindIII (Bethesda Research Labs, Inc.) for 1 h at 37°C according to the suppliers directions. Following digestion, samples were heated at 65°C for 3 min to inactivate endonucleases, and 15 ul of tracking dye was added. Digested samples were electrophoresed on 0.7% agarose gels at 50 mA for 16 h at 4° with Tris-acetate buffer (65). Gels were stained for 20 min in Tris-acetate containing 5 ug/ml ethidium bromide and bands were visualized and photographed as before. For molecular weight estimations, the electrophoretic mobilities of plasmid bands were compared to the reference strain R. leguminosarum 6015 (pJB5JI) (62,114) and the BamHI, EcoRI and HindIII digests of lambda DNA (31).

D. Acridine orange and heat-curing of plasmid DNA

Plasmid curing was done by the acridine orange procedure of Zurkowski et al. (141) or by the heat-treatment curing procedure of Zurkowski and Lorkiewicz (142). For acridine orange curing, overnight tryptone yeast extract (TY) (62) cultures were diluted to approximately 10^4 cells/ml in YM medium (141) containing 1,3,7,15 or 20 ug/ml acridine

orange (Sigma Chemical Co.,St. Louis, Mo.). Cultures were incubated at 25°C in the dark for 6 d. After incubation, cultures were diluted and spread-plated onto YEM agar plates and the resulting colonies which formed were streaked for purification two consecutive times on the same medium. For heat curing, overnight TY cultures were inoculated to approximately 10^6 cells/ml into PA medium. Cultures were incubated at 37, 40, and 42°C and transferred at weekly intervals. After heat-treatment, cultures were spread-plated on YEM agar plates and isolates purified as is outlined above.

E. Bacterial matings

Crosses were done according to the membrane-filter method of Buchanan-Wollaston et al. (22) from overnight TY cultures. Aliquots from serial dilutions of the bacterial mixtures, harvested from the membrane filters (with aid of a magnetic stir bar), were spread-plated onto the appropriate selective media. To select for R. japonicum and R. trifolii transconjugants receiving plasmid pJB5JI (kan^r), and against the auxotrophic (Phe, Trp) donor 6015 (pJB5JI), the minimal Y-medium (3) supplemented with 50 or 100ug/ml kanamycin was used. In back-crosses to the Nod^- mutant, 6015 (Rif^r,Str^r), the same medium was supplemented with kanamycin (50 ug/ml), rifampiein (20 ug/ml), streptomycin (100 ug/ml), phenylalanine (50 ug/ml), and tryptophan (50 ug/ml). This medium was called Ysupp. In crosses between R. trifolii (pJB5JI) and a Nod^- USDA 205 (Str^r, $Chlr^r$) mutant, the medium used was Y-medium supplemented with kanamycin (100 ug/ ml), streptomycin (200 ug/ml), and chloramphenicol (10 ug/ml). All mixtures were also spread-plated onto nonselective TY medium to obtain total viable counts.

F. Construction of antibiotic resistant mutants

Spontaneous antibiotic resistant mutants were obtained by incubating cells, obtained from the surface of YEM agar slants, in 100 ml of TY broth medium supplemented with various concentrations and types of antibiotics.

G. Assessment of culture purity

Culture purity was ascertained for all donors, recipients, and transconjugants in the following manner: each culture was streaked two consecutive times on the appropriate selective medium and isolated colonies transferred to TY liquid medium. After 2 d of growth, cultures were restreaked on plates of selective media. Colonies were transferred to slants of TY or TY supplemented with 50 ug/ml kanamyein, as appropriate.

Each pure culture thus obtained was further identified by immunofluorescence (123) or immunodiffusion (132) using strain specific antiserum prepared according to Schmidt et al. (123).

H. Plant infection assays

Nodulation tests for soybeans and peas were performed in modified Leonard jar assemblies (96) consisting of a 250 ml wide-mouth Erlenmeyer flask with a 13 mm hole in the bottom. The flask was glued (silicone seal) onto the cover (also having a 13 mm hole) of a 500 ml short, wide-mouth screw-cap bottle, which served as the nutrient reservoir. An absorbant cotton wick was connected between the two reservoirs to facilitate transfer of nutrient solution from the lower reservoir into the plant growth vessel above. The growth vessel was filled with a Vermiculite:Perlite mixture (3:1) and the nutrient reservoir with quarter-strength Hoagland's plant nutrient solution (63). The modified flask assembly was capped with aluminum foil and autoclaved before use.

Clover nodulation was tested in screw-cap tubes (2.5 by 20 cm) containing 25 ml of quarter-strength Hoagland's solution (63) with 1.0% agar. The tubes were slanted after sterilization. Peas (Pisum sativa var Wisconsin Perfection) and soybean (Glycine max vars Peking, Chippew a 64, and Lee) seeds were surface sterilized by immersion in a 4.0% (w/v) calcium hypochlorite solution for 20 min followed by exhaustive washings in sterile distilled water. Three sterile seeds of the same variety were aseptically transferred to each plant growth vessel and allowed to germinate and grow until they reached the aluminum foil cap. Plants were selected for uniform size and two of the three seedlings were aseptically removed from each vessel. Plants were inoculated with one ml aliquots of 2-3 day-old YEM cultures and the vessels topped-off with approximately 2 cm of sterilized Perlite and 2-3 cm of sterilized paraffin-coated sand (1.0 ml of paraffin:chloroform (1:100) per 10 gm silica sand). Clover seeds (Trifolium repens var ladino) were surface sterilized by immersion in a 2.0% solution of sodium hypochlorite for 10 min followed by exhaustive washings in sterile distilled water. Seeds were aseptically transferred to Petri dishes (150 by 25 cm) containing 1.0% water-agar and germinated at 25°C in the dark. Seedlings were transferred to Hoagland's-agar slants and inoculated with 0.1 ml of 2-3 day-old YEM liquid cultures.

Plant vessels were covered with aluminum foil to exclude light from the root zone and incubated at 24±2°C in a plant growth chamber with an average light intensity of 250 uEinsteins/m^2/sec and a day length of 16 h. All plant tests were done in triplicate. After four weeks of growth (unless specified), plants were examined for the presence of nodules, for color, and for nitrogenase activity by the acetylene reduction method (56).

I. Isolation and identification of nodule occupants

For the recovery of isolates from nodules, root sections (with attached nodules) were excised from the plants and surface sterilized in a solution consisting of 75% ethanol and 8% H_20_2. After exhaustive washings in sterile distilled water, nodules were macerated in 10% glycerol (peas and clover) or in distilled water (soybeans) and streaked onto YEM agar containing 0.25 mg/1 brom thymol blue. Macerates were also spread onto slides and nodule occupants examined by direct immunofluorescence using strain specific antibodies (123).

Results

Plasmid profiles

All of the ten fast-growing PRC isolates examined contained from 1 to 4 plasmids of approximately 100 Mdal and greater. Figure IV-1 shows the relative electrophoretic mobilities of plasmids from USDA isolates 217, 208, 206, 193, 191, 201, 192, 214, 205 and 194 (Lanes: A, D, E, F, G, H, J, K, L and M, respectively). The reference plasmids from R. leguminosarum 6015 (pJB5JI) appear in Fig. IV-1, Lanes C and I. All of the fast-growing PRC isolates with the exception of USDA 194 and USDA 191 shared a plasmid of similar size (approximately $200x10^6$ Mdal). Isolates 217, 191, 201, 192, 205, and 194 each had 2 to 3 demonstratable plasmids (Fig. IV-1, Lanes A, G, H, J, L and M, respectively). One isolate, USDA 206, contained 4 plasmids (Fig. IV-1, Lane E), while the other PRC isolates (Fig. IV-1, Lanes D, F and K) contained one plasmid. Isolate USDA 191 (Fig. IV-1, Lane G) contained two plasmids, one of which exhibited the highest electrophoretic mobility (lowest molecular weight) of all the PRC isolates examined. No plasmids could be detected in the slow-growing R. japonicum isolate USDA 110 (Fig. IV-1. Lane N). However,

Figure. IV-1. Agarose gel electrophoresis of plasmid DNA from fast- and slow-growing rhizobia. Lanes A, D, E, F, G, H, J, K, L, and M fast-growing R. japonicum, PRC isolates 217, 208, 206, OB3, 440, 201, OB2, 214, 205, and 194, respectively; Lanes C and I, R. leguminosarum 6015(pJB5JI); Lanes B and N, slow-growing B. japonicum PRC isolate 121-6 and USDA 110, respectively. Numbers in margin refer to the size of the reference plasmids from isolate 6015(PJB5JI) in Mdal (57,109).

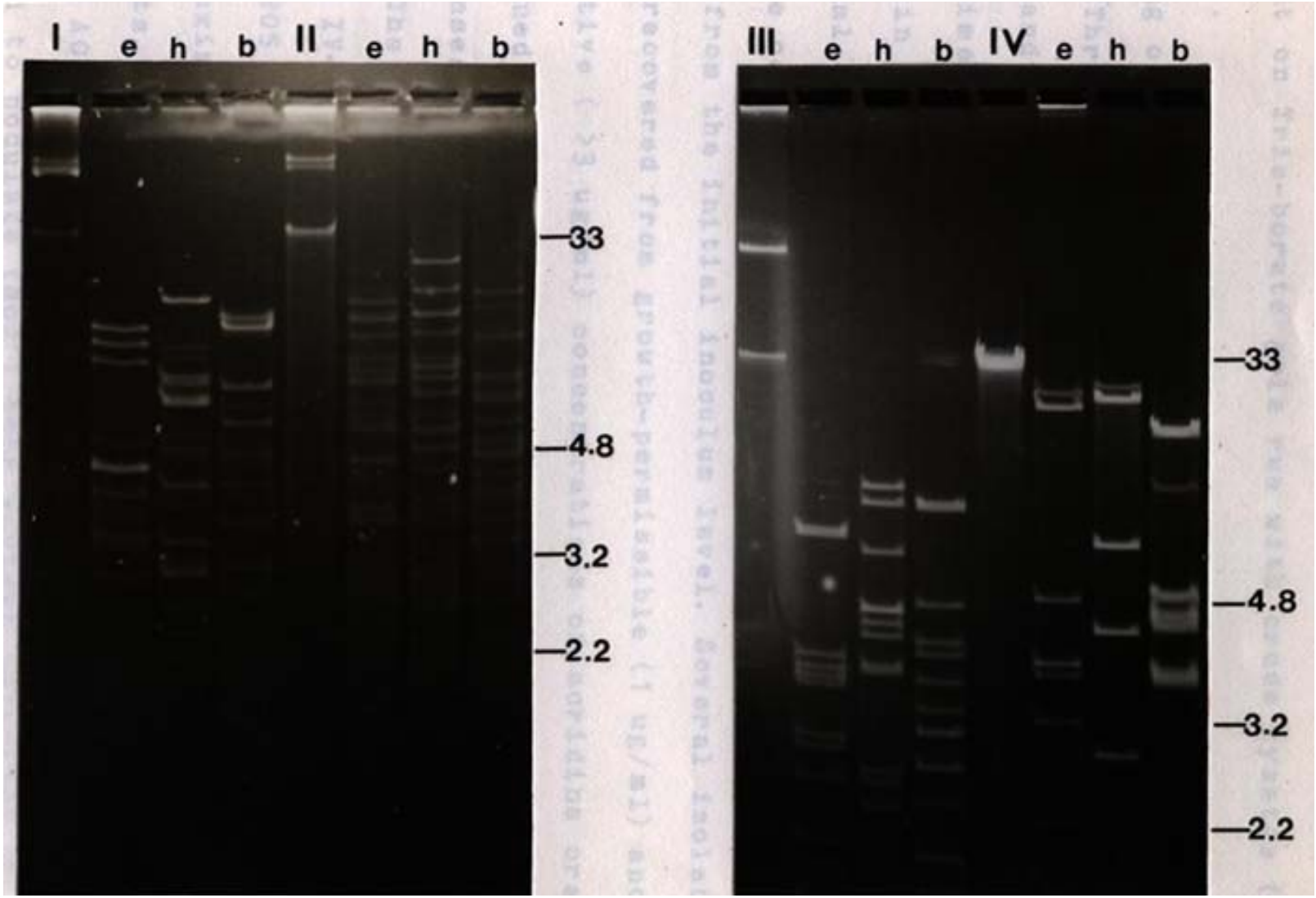

A
B
C
D
E
F
G
H
204
165
130
100
I
J
K
L
M
N
204
165
130
100

another slow-growing R. japonicum, isolate PRC 121-6 from China (Fig IV-1, Lane B), consistently exhibited one plasmid with a molecular weight of about $100x10^6$.

Restriction enzyme analysis

Purified plasmid DNA from USDA isolates 192, 194 and 205 [each a member of a separate serological group (see Chapter 5)] was used for restriction enzyme analysis, with three restriction endonucleases. Results in Figure IV-2 show that the EcoRI, HindIII and BamHI (e, h and b, respectively), restriction patterns of plasmids from the three isolates are vastly different from one another. For molecular weight comparisons, the patterns of EcoRI, HindIII and BamHI digests of lambda DNA are presented in Figure IV-2, Lanes IVe, h, and b, respectively. In all instances, restriction endonuclease digestion gave rise to fragments with molecular weights less than 33 Mdal. The BamHI digest of plasmids from USDA 205 (Fig. IV-2, Lane Ib) and the HindIII digest of plasmids from PRC 194 (Fig. IV-2, Lane IIb) gave fragments with the highest molecular weights. Although USDA isolates 205 and 194 (Fig. IV-2, Lanes I and II, respectively) contain plasmids of similar number and size on Tris-acetate gels (Figure IV-2), their restriction digest profiles clearly show them to be different. These differences are most evident by the absence of restriction fragments in several regions of the EcoRI, HindIII and BamHI digests of plasmids from PRC 205 (Fig. IV-2, Lanes Ib, e, and h). Similarly, the restriction digest profile of plasmids from USDA isolate 192 is different from either of the other two isolates. It should also be noted that after plasmid purification by CsCl centrifugation, one extra plasmid band of approximately 33 Mdal became evident on Tris-acetate gels (Fig. IV-2, Lanes I and II). This band was absent on Tris-borate gels run with crude lysates (Fig. IV-1).

Figure. IV-2. Restriction endonuclease digest of plasmids from fast-growing PRC R. japonicum. The undigested samples are given in lanes I (PRC 205), II (PRC 194), and III (PRC OB2). The three lanes following each number are digest patterns of e= Eco R1, b= Bam H1, and h= HindIII restriction endonucleases. Intact lambda DNA and the respective endonuclease digests are shown in lanes IV, e, h, and b, for comparison. Numbers in the margin refer to the size of several lambda fragments in Mdal (28).


I
e
h
b
II
e
h
b
33
4.8
3.2
2.2
III
e
h
b
IV
e
h
b
33
4.8
3.2
2.2

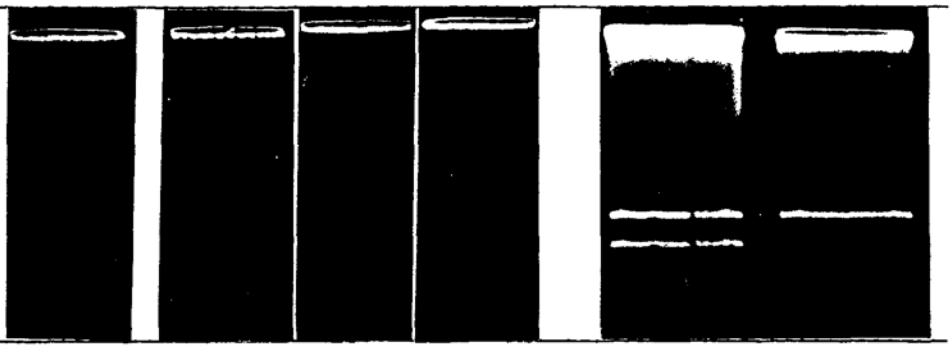
Curing of nodulation plasmids

Three of the fast-growing soybean isolates, USDA 192, 194, and 205, were used for acridine orange and heat curing experiments. All three of the isolates examined, failed to grow in acridine orange (AO) at concentrations greater than 1 ug/ml. At an AO concentration of 3 ug/ml or greater, viable cell numbers either decreased slowly or remained the same from the initial inoculum level. Several isolates which were recovered from growth-permissible (1 ug/ml) and restrictive (>3 ug/ml) concentrations of acridine orange were examined for their plasmid profiles and for their nodulation responses on soybeans.

The plasmid pattern of the "cured" mutants is shown in Table IV-2A. The results show that the AO mutants of USDA 205 (205 1AO1, 1A03, and 1A05) are devoid of the largest (approximately $200X10^6$ Mdal) of the two plasmids. These mutants, isolated from cultures grown in the presence of 1 ug/ml A0, and shown to be cured of their largest plasmid failed to nodulate (Nod^-) both soybean cultivars (Table IV-2B). However, all of the isolates recovered from AO at 3 ug/ml and greater, effectively nodulated (Nod^+,Fix^+) soybean cultivar Peking. Likewise, all of the USDA 194 and 192 isolates from 1 ug/ml AO retained their nodulating ability. The cured Nod^- mutants of 205 were also used in blocking experiments to see whether they would inhibit nodulation by Nod^+ parental strain as well as other fast-growing R. japonicum. There was no significant suppression of nodulation even when the Nod^- mutant was inoculated 48 h before the addition of the parent strains.

Immunofluorescence analysis of the somatic antigens of Nod^- mutants indicated that the mutants were serologically indentical with the parent strain. Immunodiffusion analysis using antisera produced against somatic antigens as well as whole-cell antigens also indicated that the nodulation deficient mutants were serologically identical to the parent

Table IV-2. Plasmid profiles, immunologic reactions, and symbiotic properties of acridine orange- and heat-cured isolates of *R. japonicum*.

	Cultures					
	Parental	Cured Mutants			Parental	Cured mutant
	USDA 205	205 1A01	205 1A03	205 1A05	USDA 194	194 1X 40°C
A Plasmid Profile						
B Symbiotic Properties on cultivar:						
Peking	Nod^+,Fix^+	Nod^-	Nod^-	Nod^-	Nod^+,Fix^+	Nod^+,Fix^+
Chippewa	Nod^+,Fix^-	Nod^-	Nod^-	Nod^-	Nod^+,Fix^-	Nod^+,Fix^-
C Immunologic Reactions[a]						
FA	4+	4+	4+	4+	4+	4+
ID	5 (2)	4 (2)	4 (2)	4 (2)	4 (2)	4 (2)

[a]Antiserum for ID (Immunodiffusion) was prepared against whole and somatic cell antigens and antiserum for IF (Immunofluoresence) against somatic antigens. For IF, the intensity of the immunofluoresent reaction is expressed as; 4+ = very intense, - = no reaction. For ID, numbers represent the number of precipitin bands formed with whole-cell antiserum; numbers in parentheses are number of bands formed with somatic antiserum.

(Table IV-2C). Both somatic and whole-cell antigens from the mutants show patterns of identity with the parent strain.

Incubation of USDA 192 and 205 at elevated temperatures did not alter the plasmid profiles of the recovered isolates. Organisms recovered from growth medium incubated at 37 and 40°C for one week, still nodulated both soybean cultivars and their plasmid profiles were identical to their respective parents. Similarly, incubation of USDA 194 at 37°C for 7 weeks or 42°C for 1 week did not alter the plasmid profiles of the recovered mutants. However, one USDA 194 isolate, 194 1X40 (Table IV-2A), which was incubated at 40°C for 7 d, had a plasmid profile different from the parent strain. This isolate, was missing its lowest molecular weight plasmid band (approximately $100X10^6$ Mdal). However, it retained the ability to nodulate soybeans. Immunofluorescence and immunodiffusion analysis of its somatic and whole cell antigens showed that it was serologically the same as the parental strain (Table IV-2C).

Frequency of kanamycin resistance in fast-growing soybean rhizobia

Before examining the ability of the fast-growing soybean isolates to receive plasmid pJB5JI (Tra^+, Nod^+, Fix^+, Kan^r) from R. leguminosarum strain 6015(pJB5JI), it was necessary to determine if the isolates would grow on Y minimal medium supplemented with 50 ug/ml kanamycin. Under conditions identical to those used in mating experiments, only one, USDA 201, out of the seven isolates examined failed to grow in the presence of 50 ug/ml kanamycin (Table IV-3). The remaining six isolates, however, had natural kanamycin resistance frequencies of $1.3x10^{-5}$ to $5x10^{-6}$. Three of the isolates, USDA 192, 201 and 205 (each a member of a different serological group) were also examined for their ability to grow in the presence of higher concentrations of kanamycin. One isolate, USDA 192, grew at a kanamycin concentration

Table IV-3. Frequency of natural kanamycin resistance in several fast-growing *Rhizobium japonicum* and *R. trifolii* isolates[a].

Isolate	Frequency of Resistance to Kanamycin (50 ug/ml)
R. japonicum:	
USDA 192	$1.2X10^{-5}$[b]
USDA 193	$4.7X10^{-6}$
USDA 194	$3.6X10^{-6}$
USDA 201	$< 1.0X10^{-9}$
USDA 205	$3.9X10^{-6}$[c]
USDA 206	$2.9X10^{-4}$
USDA 208	$5.0X10^{-6}$
R. trifolii:	
#2	$< 1.0X10^{-9}$
0403	$1.5X10^{-7}$[d]

[a]Frequency determined by dividing the number of resistant colonies on Y-Kan medium by the total number of colonies on non-selective medium.

[b]Resistant to 100 ug/ml with $3.0X10^{-6}$ frequency.

[c]Resistant to 100 ug/ml with $< 1.0X10^{-9}$ frequency.

[d]Resistant to 100 ug/ml with $3X10^{-8}$ frequency.

of 100 ug/ml (frequency of $3x10^{-6}$) while the other two isolates failed to grow at this concentration. None of the three isolates grew at a kanamycin concentration greater than 100 ug/ml (Table IV-3).

Plasmid transfer to fast-growing PRC isolates

Fast-growing soybean isolates, USDA 201 and 205, were examined for their ability to receive, maintain, and express the pea-nodulating host-range plasmid, pJB5JI, from R. leguminosarum 6015(pJB5JI). Transfer of the kanamycin marker to USDA 201 occurred at a frequency of $4.0X10^{-4}$ (Table IV-4) while transfer to USDA 205 occurred at a frequency of $4.0X10^{-9}$. The low frequency of transfer to USDA 205 preeluded its use in further studies.

To confirm the presence of pJB5JI in the USDA 201 transconjugants, two isolates 201-11 and 201-14, were backcrossed to the Nod^- R. leguminosarum strain, 6015. Transfer of pJB5JI from transconjugants 201-11 and 201-14 to R. leguminosarum 6015 occurred at frequencies of $2.0X10^{-3}$ and $2.7X10^{-3}$, respectively (Table IV-4). R. leguminosarum 6015 transconjugants were picked from Ysupp plates and purified as described previously.

In order to ascertain if transconjugants received pJB5JI intact, or if the kanamycin resistance phenotype was due to spontaneous mutation or transposition of TN5 from pJB5JI (PRJ5JI::TN5) into the chromosome or the resident plasmids, plasmid DNA was isolated from four USDA 201 and two 6015 transconjugants and visualized following agarose gel electrophoresis. Three classes of USDA 201 transconjugants could be detected by plasmid profiles (Figure IV-3). The first class consisted of those transconjugants, 201-8 and 201-13, which had plasmid profiles similar to the parent strain USDA 201. Each of these isolates possessed two plasmids

Table IV-4. Frequency of transfer of plasmid marker in crosses between *Rhizobium leguminosarum*, *R. japonicum*, *R. trifolii* and their transconjugants.

Donor	Recipient[a]	Transfer Frequency
1. *R. leguminosarum* 6015(pJB5JI)	*R. japonicum* USDA 201	$4X10^{-4}$
2. *R. leguminosarum* 6015(pJB5JI)	*R. japonicum* USDA 205	$4X10^{-9}$
3. *R. japonicum* 201(pJB5JI)	*R. leguminosarum* 6015	$2X10^{-3}$
4. *R. leguminosarum* 6015(pJB5JI)	*R. trifolii* #2	$4X10^{-4}$
5. *R. leguminosarum* 6015(pJB5JI)	*R. trifolii* 0403	$2X10^{-4}$
6. *R. trifolii* #2(pJB5JI)	*R. leguminosarum* 6015	$7X10^{-3}$
7. *R. trifolii* 0403(pJB5JI)	*R. leguminosarum* 6015	$1X10^{-2}$

[a]Transconjugants from crosses 1 and 4 were selected on Y minimal medium containing 50 ug/ml kanamycin, 2 and 5 on Y medium with 100 ug/ml kanamycin, 3, 6, and 7 on Y supp (Phe, Trp, Str, Rif, and Kan) medium.

p201a and p201b, of approximately 130 and 200 Mdal., respectively. The second class, represented by transconjugant 201-11, had in addition to the two plasmids present in USDA 201, a third smaller plasmid of approximately 100 Mdal. The third class of transconjugants, represented by 201-14, appears to have lost its largest plasmid, but like 201-11, has gained an additional smaller plasmid. It should be noted that the smallest plasmid present in USDA 201 has an electrophoretic mobility similar to pJB5JI (Figure IV-3). The plasmid profiles of two back-crossed USDA 6015 transconjugants, 6015-11-7 and 6015-14-8 along with their respective parents are shown in Figure IV-4. Both 6015 transconjugants have received a plasmid band corresponding to plasmid pJB5JI (130 Mdal) present in their respective parents (Figure IV-4).

Seven USDA 201 transconjugants, five 6015 transconjugants derived from back-crosses with 201-11, seven 6015 transconjugants derived from back-crosses with 201-14, and whole mating mixtures from both 6015 crosses, along with USDA 201, 6015(pJB5JI), and 6015, were inoculated onto individual plants of peas and soybeans (Glycine max var Peking). All seven of the USDA 201 transconjugants (Table IV-5) effectively nodulated (Nod^+, Fix^+) soybeans, but failed to nodulate peas. All of the 6015 back-crossed transconjugants examined effectively nodulated (Nod^+, Fix^+) peas, whereas none of the transconjugants nodulated soybeans (Table IV-5). Several 6015 transconjugants were reisolated from nodules, and after purification, were reinoculated onto pea plants. All nodule-isolated transconjugants retained the ability to nodulate peas. All plants inoculated with whole mating mixtures also possessed effective nodules. Plants inoculated with the control mating mixtures of R. japonicum USDA 201 (parental) and R. leguminosarum 6015 were not nodulated.

Two USDA 201 transconjugants, 201-11 and 201-14, were inoculated onto the commercial soybean cultivars Chippewa and Lee to determine

Figure IV-3. Plasmid profiles of R. japonicum USDA 201 and its transconjugants. Lane: 1, R. leguminosarum 6015(pJB5JI); 2, R. japonicum 201; 3, R. japnonicum 201-11(pJB5JI); 4, R. japonicum 201-8 kan^r ; 5, R. japonicum 201-14(pJB5JI); 6, R. japonicum 201-13 kan^r. Arrow indicates plasmid pJB5JI.

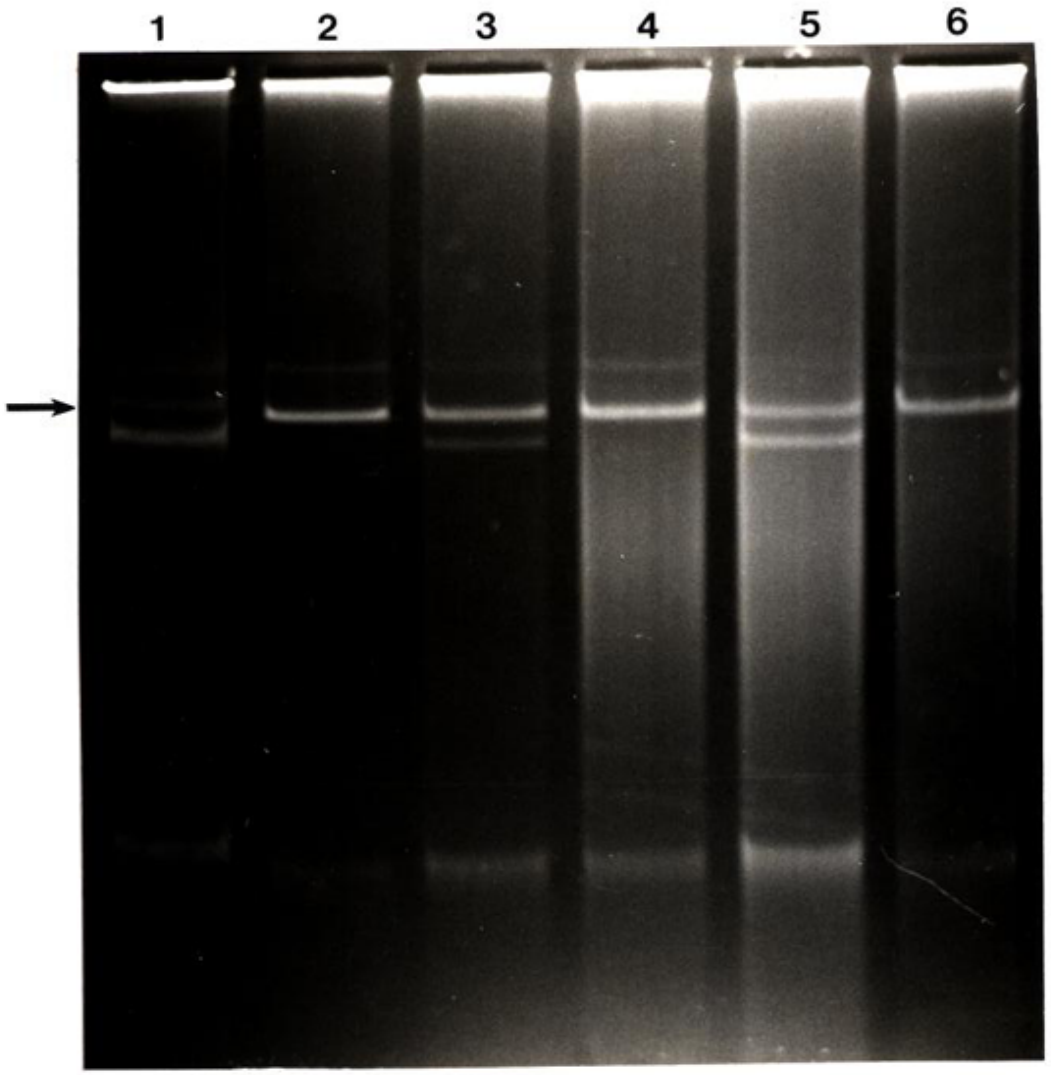

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4
5
6

Figure IV-4. Plasmid profiles of R. leguminosarum 6015 and its transconjugants. Lane 1: R. leguminosarum 6015(pJB5JI); 2, R. japonicum USDA 201; 3, R. japonicum 201-11(pJB5JI); 4, R. japonicum 201-14(pJB5JI); 5, R. leguminosarum 6015; 6, R. leguminosarum 6015-14-8(pJB5JI); 7, R. leguminosarum 6015-11-7 (pJB5JI). Arrow indicates plasmid pJB5JI.

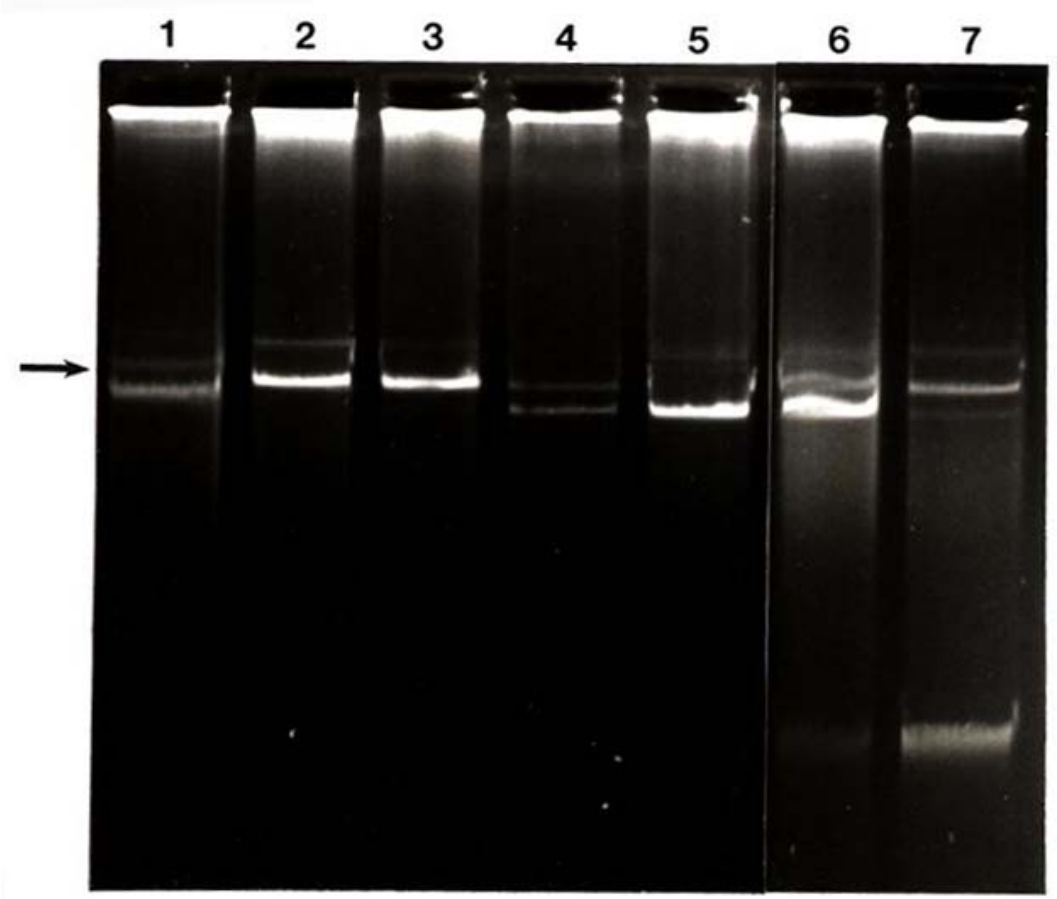

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Table IV-5. Symbiotic properties of *R. japonicum*, *R. leguminosarum*, *R. trifolii*, and their transconjugants.

Culture	Symbiotic Properties On:				
	Soybean Variety			Pea	Clover
	Peking	Chippewa	Lee		
Rhizobium japonicum					
USDA 201	Nod^+,Fix^+	Nod^+,Fix^-	Nod^+,Fix^-	Nod^-	
USDA 201 Trans-conjugants	Nod^+,Fix^{+a}	Nod^+,Fix^{-b}	Nod^+,Fix^{-b}	Nod^{-a}	
Rhizobium leguminosarum					
6015(pJB5JI)	Nod^-			Nod^+,Fix^+	Nod^-
6015	Nod^-			Nod^-	
6015 Transconjugants[c]	Nod^-			Nod^+,Fix^+	
Rhizobium trifolii					
#2				Nod^-	Nod^-
403				Nod^-	Nod^+
#2 Transconjugants[d]				Nod^+,Fix^+	Nod^-
0403 Transconjugants[d,e]				Nod^+,Fix^-	Nod^+

[a] Seven isolates from conjugation with 6015(pJB5JI) examined.

[b] USDA 201 trasnconjugants, 201-11 and 201-14 were examined.

[c] Twelve isolates from conjugation with 201-11 and 201-14, three isolates from conjugation with #2 transconjugants, and three isolates from conjugation with 0403 transconjugants were examined.

[d] Six isolates from conjugation with 6015(pJB5JI) were examined.

[e] One 403 transconjugant had one Nod^+,Fix^+ nodule on peas.

whether the presence of plasmid pJB5JI would now enable them to nodulate commercial varieties of soybeans effectively. As is indicated in Table IV-5, both transconjugants remained unable to produce an effective symbiosis (Nod^+, Fix^-) with these cultivars.

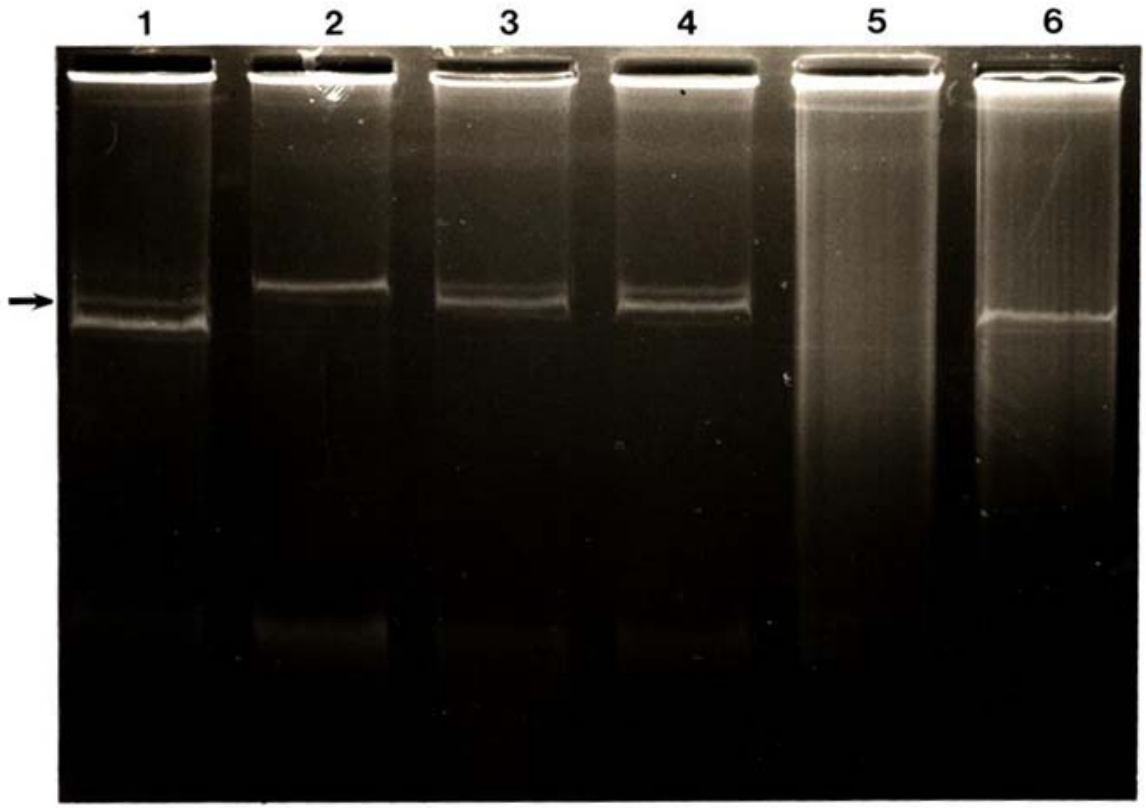
Plasmid transfer to R. trifolii

Rhizobium trifolii isolates, 0403, and #2, were examined for their ability to receive, maintain, and express the pea-nodulating, host-range plasmid, pJB5JI, from R. leguminosarum 6015(pJB5JI). R. trifolii 0403 is a "wildtype" clover-nodulating strain, while R. trifolii #2 is a clover nodulatinn-deficient (Nod^-) strain. As before, both strains were examined for natural resistance to kanamycin. While strain #2 was unable to grow on Y medium with 50 ug/ml kanamycin, strain 0403 was resistant to kanamycin at this concentration. However, at a kanamycin concentration of 100 ug/ml, strain 0403 was resistant only at a frequency of $3.4X10^{-8}$ (Table IV-3).

Transfer of plasmid pJB5JI from R. leguminosarum to R. trifolii 0403 and #2 occurred at frequencies of $2.1X10^{-4}$ and $4.4X10^{-4}$, respectively (Table IV-4). Both of the #2 transconjugants (2-33 and 2-54) and the one 0403 transeonJugant (403-33) examined received a plasmid band corresponding in size to pJB5JI (Figure IV-5). Interestingly, the "wild-type" parent strain 0403, was not found to contain any plasmids despite repeated attempts at plasmid isolation and visualization.

Six 0403 and #2 transconjugants, along with parental strains 0403, #2, and 6015(pJB5JI), were inoculated onto peas and clover. All six of the #2 transconjugants (Table IV-5) effectively nodulated (Nod^+, Fix^+) peas, but still remained unable to nodulate clover. The six 0403 transconjugants examined formed nodules on both peas and clover. While

Figure IV-5. Plasmid profiles of R. trifolii strains #2 and 0403 and their transconjugants. Lane: 1, R. leguminosarum 6015(pJB5JI); 2, R. trifolii #2; R. trifolii 2-33(pJB5JI); 4, R. trifolii 2-54(pJB5JI); 5, R. trifolii 0403; 6, R. trifolii 403-33(pJB5JI). Arrow indicates plasmid pJB5JI.


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clover nodules were effective, all the nodules on peas, with the exception of one, were completely ineffective. One of the three plants inoculated with 0403 transconjugant 403-22, had one Fix^+ nodule. All of the 0403 transconjugants retained the ability to effectively nodulate clover (Nod+, Fix^+) (Table IV-5).

Two 0403 transconjugants (403-42 and 403-33) and two #2 transconjugants (2-54 and 2-33) were backcrossed to R. leguminosarum 6015. Plasmid pJB5JI transferred from R. trifolii transconjugants 403-42, 403-33, 2-54, and 2-33 to R. leguminosarum strain 6015 at frequencies of $1X10^{-2}$, $1.4X10^{-2}$, $6X10^{-3}$, and $7.6X10^{-3}$, respectively (Table IV-4).

Several R. leguminosarum 6015 transeonjugants derived from backcrosses to R. trifolii transconjugants 403-42, 403-33, 2-54, and 2-33, were inoculated onto individual plants of peas and clover. All of the 6015 transeonjugants effectively nodulated (Nod^+, Fix^+) peas, whereas none of the transconjugants nodulated (Nod^-) clover (Table IV-5).

To determine the stability of plasmid pJB5JI in transconjugants after passage through plant nodules, several 0403 and #2 transconjugants were reisolated from clover and pea nodules and after purification and identification (using immunofluorescence), were reinoculated onto individual plants of clover and peas. The results shown in Table IV-6 indicate that the nodule-reisolated #2 and 0403 transconjugants produced a variety of modulation and nitrogen fixation patterns on peas and clover plants. After reisolation from pea and clover nodules, some of the 0403 transconjugants (which originally formed an ineffective symbiosis with peas and an effective symbiosis with clover) lost their ability to modulate-peas, still produced an ineffective symbiosis, or produced an effective symbiosis with peas. All of the 0403 nodule reisolates (with the exception of 5 plants) still retained the ability to effectively modulate clover. Interestingly, two of the #2 transconjugants gained the

Table IV-6. Symbiotic properties of *Rhizobium trifolii* transconjugants reisolated from nodules.

Organism	Isolated From:	Symbiotic Properties On: Peas	Clover
		(Nodulation/N_2-Fixation)	
R. trifolii			
#2 transconjugants			
(2)[a]	Pea Nodules	+/+	+/-
R. trifolii			
0403 transconjugants			
(8)	Pea Nodules	+/-	+/+ & -[c]
(9)	Pea Nodules	+/- & +[b]	+/+ & -[c]
(4)	Clover Nodules	-/-	+/+
(7)	Clover Nodules	+/-	+/+
(4)	Clover Nodules	+/- & +[b]	+/+ & -[c]

[a]Numbers in parentheses refer to the number of plants.

[b]Approximately 150-250 small ineffective nodules and 5-15 large effective nodules on each plant.

[c]One to two plants were ineffectively nodulated.

ability to nodulate clover after they were subsequently isolated from pea nodules. However, these transconjugants only produced an ineffective symbiosis on clover.

Restoration of nodulating ability

To determine whether pea nodulating ability could be transferred to a fast-growing PRC isolate which has lost its own nodulation plasmid, an acridine-orange-cured, nodulation-deficient USDA 205 mutant [2051A03 (see above)] was used in crosses with R. leguminosarum 6015(pJB5JI) and R. trifolii 403-42 [0403 Chl^r, Str^r(pJB5JI)]. Despite repeated conjugation attempts, with either donor, kanamycin resistant 2051A03 transconjugants could not be isolated (data not shown).

Discussion

In this report, I have demonstrated the presence of from 1 to 4 plasmids in ten fast-growing strains of soybean-rhizobia. The recent results of Masterson et al. (103) have indicated that several of the fast-growing isolates contain plasmids which hybridize to the structural nifK and D genes of Klebsiella pneumoniae. However, these authors did not show the location of nodulation genes in these isolates. My results suggest that in at least one of these isolates, a large plasmid may also be the location of some of the nodulation genes. All of the acridine orange-generated, nodulation-deficient mutants of USDA 205 had lost their largest plasmid. Zurkowski and Lorkiewicz (143) showed a correlation between the loss of nodulation character and the elimination of a plasmid in Rhizobium trifolii T12 and 24. Morrison, et al. (105) have recently demonstrated that a

Lablab purpureus isolate NGR234, which can nodulate some legumes and the nonlegume Parasponia, could be cured of its nodulating ability by treatment at elevated temperatures. In this isolate, the loss of nodulating ability was found to result from the elimination of its largest resident plasmid. In my study the heat-treated USDA 194 mutant, missing a smaller plasmid, was still able to effectively nodulate soybeans. Masterson et al. (103) indicated that in USDA 194, the structural nif genes, were not located on any of the plasmids.

Zurkowski and Lorkiewicz (142) have shown that R. trifolii strains can easily lose their nodulation plasmid at elevated temperatures. The fast-growing soybean rhizobia that I examined did not lose their nodulating ability at restrictive temperatures of 37°C for up to 7 weeks, or at 40 and 42°C for 7 d. These isolates seem to be resilient to the heat-curing-treatments used effectively for other species of Rhizobium. However, at levels of acridine orange that allowed slight growth, one isolate was cured of its nodulating plasmid, while in bacteriostatic concentrations, no curing of any plasmids could be demonstrated These results are in agreement with those of Pariiskaya (111) who found that R. meliloti L-1 was only significantly cured of modulating ability in subbaeteriostatic levels of acridine orange. However, Higashi (60) and Zurkowski et al. (141) cured different R. trifolii isolates of their nodulating ability in bacteriocidal concentrations of acridine orange.

The results of this study indicate that plasmid pJB5JI, the pea-modulation, host-range plasmid from R. leguminosarum 6015(pJB5JI) could be transferred to the fastgrowing soybean rhizobia and R. trifolii but was variably expressed in different genetic environments. When the plasmid was transferred to a

modulation-deficient isolate of R. trifolii, #2, nitrogen-fixation and modulation genes were expressed, since the resulting transconjugants effectively nodulated peas. Initially, it was thought that plasmid pJB5JI did not restore the clover modulation deficiency present in the #2 transconjugants. However, after recovery from pea nodules, two of the transconjugants gained the ability to nodulate clover. When pJB5JI was transferred to a modulation-eom petent R. trifolii isolate, 0403, the resulting transconjugants were capable of nodulating both clover and peas. However, peas (with the exception of one plant) were only nodulated ineffectively (Nod^+, Fix^-). It should be noted however, that after isolation from pea and clover nodules, there was a differential expression of nodulation and nitrogen fixation genes among some of the 403 transconjugants. While one out of the six transconjugants from clover nodules had lost the ability to nodulate peas (most likely indicating a lost or deleted plasmid) three gained the ability to effectively nodulate peas. While none of the five 403 transconjugants reisolated from pea nodules lost the ability to nodulate peas or clover, two of the reisolates could now nodulate peas effectively. Thus, those plasmid-borne genes required for an effective symbiosis, were differentially expressed in some of the transconjugants after passage through host plants. The mechanism for this phenomenon is unknown. Beynon et al. (10) noted that while some plasmids controlling host-range specificity may be able to coexist (and behave one way) in culture, they may not be able to do so within the nodules of a given host legume. That is, there may be some “physiological incompatibility” or functional interference between the resident nitrogen fixation and nodulation genes and those specified on plasmid pJB5JI. Likewise, there may be functional

interference between the clover nitrogen fixation genes present in R. trifolii 0403, and the pea genes on pJB5JI, which accounts for their lack of expression in 403 transconjugants. Passage through the appropriate host plant may in some way modify this incompatability and allow for the expression of both sets of genes.

Transfer of plasmid pJB5JI to the fast-growing soybean isolate, USDA 201, did not bestow upon the transconjugants the ability to nodulate peas. The presence of the plasmid in the transconjugants did not interfere with their ability to nodulate their own host soybeans. However, in some transconjugants, a loss of, or a deletion in, a resident plasmid was evident on agarose gels. This did not, however, affect the nodulating ability of the transconjugants, implying that the lost or deleted fragments may not be involved in modulation. While plasmid pJB5JI was transferable to fast-growing soybean strain USDA 201, other isolates failed to accept the plasmid. One strain, USDA 205 was not able to receive plasmid pJB5JI from R. leguminosarum 6015(pJB5JI) or R. trifolii 0403(pJB5JI) even when cured of its largest plasmid.

Plasmid pJB5JI has been used by Brewin et al. (17, 18) and Hirsch et al. (62) to transfer pea-nodulating ability to non-nodulating mutants of R. leguminosarum. Similarly, Beynon et al. (10) used pJB5JI to transfer pea-nodulating ability to R. phaseoli and Djordjevet et al. (35) used the same plasmid to transfer pea-nodulating ability to R. trifolii. Even though the fast-growing USDA 201 transconJugants that received pJB5JI do not themselves nodulate peas, they are, nevertheless, capable of transferring this property to a non-nodulating strain of R. leguminosarum, 6015. This strain is a non-nodulating derivative of R. leguminosarum strain 300 (77), similar to 6015(pJB5JI), but it is non-nodulating because of a deletion in its

largest Plasmid (62). The smallest plasmid which appeared in 201-11 and 201-14 transconjugants, may be due to either the co-transfer of another plasmid from R. leguminosarum 6015(pJB5JI), or a deletion in an incoming or resident plasmid. Since USDA 201 contains a plasmid which has a similar electrophoretic mobility as pJB5JI, deletions in, or recombinational events between, this and other plasmids and the major replicon are very probable. This may explain why transconjugants 201-8 and 201-13 did not show any additional plasm id bands despite their kanamycin resistance. However, the 6015 back-crosses, 6015-11-7 and 6015-14-8, appear to have received pJB5JI from 201-11 and 201-14 intact as judged by the appearence of a new plasmid (Figure IV-3) with an electrophoretic mobility similar to pJB5JI. Thus, the plasmid may have "recombined-out" of 201-11 and 201-14 during conjugation to R. leguminosarum 6015. As was indicated by Keyser et al. (84), the fast-growing soybean rhizobia only form effective symbiosis with Glycine Max var Peking and with Glycine soja, but form ineffective nodules on most New World commercial varieties. The results of this study show that despite the presence of the extra nitrogen fixation genes (present in pJB5JI), USDA 201 transconjugants still form ineffective nodules on the two commercial cultivars of soybeans examined.

In conclusion, the results of this study indicate that: 1, the loss of a large plasmid from the fast-growing R. japonicum leads to loss of nodulating ability. These nodulation-deficient Rhizobium japonicum mutants may prove useful in genetic manipulations of the soybean/Rhizobium symbiotic system; 2, although the pea SYH plasmid, pJB5JI, is transferrable to many different species of Rhizobium, the plasmid is differentially expressed in different genetic backgrounds. Despite the fact that the R. leguminosarum

pea host-range plasmid, pJB5JI, can effectively function in closely-related rhizobia, my results suggest that it does not by itself carry all the genetic information necessary for the nodulation of peas in a genetically dissimilar environment. However, the plasmid is maintained intact in the different genetic backgrounds despite its lack of expression; and 3, the host may play some role in modifying the genetic make-up of the Rhizobium in the nodules, since some of the nodule-reisolates had a different pattern of nodulation than the parent cultures.

CHAPTER V

SEROLOGICAL RELATEDNESS OF FAST-GROWING SOYBEAN RHIZOBIA TO OTHER FAST- AND SLOW-GROWING RHIZOBIA

ABSTRACT

Immunofluorescenee and immunodiffusion reactions have been used extensively for the characterization of strains within different species of Rhizobium. The fast-growing soybean rhizobia from China (PRC) were examined to determine their serological relatedness to each other, to the "typical" slow-growing B. japonicum, and to other fast- and slow-growing rhizobia. Somatic antigens of thirty-nine strains of Rhizobium were examined for immunofluorescence cross-reactions with fluorescent antibodies (FAs) produced against the somatic antigens of five fast-growing soybean-rhizobia. Immunofluorescence (IF) analysis of seven of the fast-growing soybean strains indicated that the isolates could be separated into at least three somatic serogroups; 192, 194, and 205. One out of the eleven typical slowgrowing B. japonicum (which were from different serogroups) cross-reacted with one of the PRC FAs (194), while members of the other serogroups failed to react with any of the FAs. The two slow-growing cowpea rhizobia examined did not cross-react with any of the PRC FAs. Of the 19 other fast-growing rhizobia examined, one strain of Rhizobium sp. for Leucaena, Tal 82, and three strains of Rhizobium spp. for Sesbania, Tal 1117, Allen 770, and PL 146, fell within the 194 IF serogroup. One strain of Rhizobium sp. for Lablab, NGR 234, cross-reacted with all of the PRC FAs, however, iF cross-reactivity was greatest with FAs prepared against USDA 192 and 205.

Somatic and whole cell antigens of 33 fast- and slow-growing Rhizobium strains were tested for immunodiffusion (ID) cross-reactions with antisera

from three fast-growing soybean rhizobia. While the six fast-growing soybean strains could be separated into at least three somatic serogroups, they could not be grouped on the basis of whole-cell reactions. The eight typical B. japonicum (belonging to 8 different serogroups) and two slow-growing cowpea strains failed to react with any of the whole- or somatic-cell antisera. While antigens from two strains of R. leguminosarum, R. phaseoli, R. trifolii, and R. lupini did not react with any of the antisera, several fast-growing rhizobia clearly exhibited ID cross-reactivity. Antigens from two R. meliloti strains and one strain of Rhizobium sp. for Lablab cross-reacted with all three whole-cell antisera. Two out of the four strains of Rhizobium sp. for Sesbania and one out of the two strains of Rhizobium sp. for Leucaena cross-reacted with only USDA 192 whole-cell antiserum.

Results of this study indicate that the fast-growing PRC soybean rhizobium are serologically related and can be separated into at least three somatic serogroups using immunodiffusion or immunofluorescence reactions. Also, that ID analysis using whole-cell antigens indicates that the PRC isolates share at-least one antigen in common.

Introduction

Immunodiffusion, agglutination, and fluorescent antibody techniques have been used widely to identify and characterize strains of B. Japonicum grown in culture (36,23,37,49,125), present in nodules (15,24,37,38,104) and growing in soil (15). One major finding of these studies is that the slow-growing soybean rhizobia exhibit a high degree of serological specificity. That is, strains of B. japonicum are serologically heterogeneous (38,49). Another is that somatic antigens are more strain specific than flagellar or internal antigens.

Koontz and Faber (88) and Wright (140) identified 6 somatic serogroups of B. japonicum using agglutination reactions. However, Date and Decker (30) analyzed 28 strains of B. Japonicum and found 17 somatic serogroups on the basis of cross-reactions and agglutination-adsorptions. Skrdleta (124) found only four somatic serogroups among the 40 strains he examined, but reported that 22 did not react with any of the 11 antisera used.

While agglutination reaction was the first serological method applied to the study of rhizobia, it lacks the resolving power to distinguish between antigenically similar and closely related, but not identical strains (38). Skrdleta (125), using gel-immunodiffusions, divided the eleven B. Japonicum he examined into two basic somatic serogroups. While he detected the same serogroups using agglutinations, he found that immunodiffusions allowed him to show serological relationships between strains which were not agglutinated by the same antisera.

Lastly, the fluorescent antibody (FA) technique has been used by others (13,14,85,122,123) for the direct examination of rhizobia in culture, nodules, and soils. The major advantages of FA over

other serological techniques is that only small amounts of antigen and antibody are needed (16,121), the procedures are relatively rapid, and it is the only technique which allows for the direct examination of rhizobia in situ (16).

This study describes a serological investigation of the fast-growing soybean rhizobia for their degree of relatedness to each other, to the "typical" slow-growing B. japonicum, and to other fast- and slow-growing species of the root nodule bacteria.

Materials and Methods

A. Bacterial strains, sources and maintenance of cultures.

The strains used in this study and their sources are listed in Table V-1. All cultures were maintained on YEM agar slants (132) (see Chapter III for composition). Agar slants used for the maintenance of fast-growing rhizobia contained 0.05% $CaCO_3$. All cultures were incubated at 28°C.

B. Preparation of antigens.

For immunodiffusion (ID) analysis, antigens were prepared from cells grown on the surface of B5 (45) medium. Cells from the fast-growers were harvested from 30 ml agar flats after 3 d of growth, while the slow-growers were harvested after 7 d. Cells were resuspended in 2-3 ml of 0.85% saline (containing Thimerosal at a final concentration of 1:10,000) and stored at 4°C until use. For immunofluorescence (IF) studies, antigens were prepared from cells grown in YEM liquid medium for 2 to 3 d. Somatic cell antigens for injections and IF studies were prepared by placing cell suspensions in a boiling water bath for 1 h.

Table V-1. Bacteria used and their sources[a]

Culture	Source	Culture	Source
B. japonicum (Slow):		*R. phaseoli*	
USDA 31	1	Bel 7.1	2
USDA 110		CC 511	5
USDA 123	2		
USDA 136	1	*Rhizobium* spp.	
USDA 138		For Cowpea	
PRC 005	3	32H1	12
PRC 113-2		CB 756	5
PRC 121-6		Webster 48	13
PRC B15			
R. japonicum (Fast):		For Leucaena	
USDA 191	4	RL 18	8
USDA 192		RL 33	
USDA 193		RL 38	
USDA 194		RL 78	
USDA 201		RCR 3817	1
USDA 205		Tal 582	9
USDA 214		Tal 1145	
		UMKL 19	6
R. leguminosarum			
Nitragin 92A3	5	For Sebania	
PF2	6	3F4a3	1
		3F4a4	
R. lupini		3F4c1	
CC 1814a	5	KH	10
NZP 2037		PL 146	11
		Tal 1113	9
R. meliloti		Tal 1114	
L5-30	7	Tal 1115	
NZP 4013	5	Tal 1116	
		Tal 1117	
R. trifolii		Tal 1119	
TA 1	5	Allen 770	
NZP 560			
#2	6	For Lablab	
		NGR 234	14

[a]Sources: 1, USDA, Beltsville, Md.; 2, E. L. Schmidt, Univer. Minnesota; 3, T.S. HU, Institute Soil and Fertlizers, Beijing, People's Republic of China; 4, H.H. Keyser, USDA, Beltsville; 5, R.M. Greenwood, DSIRO, Palmerston North, New Zealand; 6, W.J. Broughton, Max Planck, Institute, FRG; 7, B.G. Rolfe, ANU, Canberra City Australia; 8, H. Moawad, U. Hawaii; 9, Niftal Project, Paia, Hawii; 10, D. Evans, Univer. Hawaii; 11, Origin Unknown; 12, Nitragin Co., Milwaukee, Wi.;13, B.B. Bohlool, Univer. Hawaii; 14, M.J. Trinck, Australia.

C. Preparation of antisera.

Boiled cell suspensions were used to produce somatic-cell antisera according to the method of Schmidt et al. (123). For the production of whole-cell antisera, unboiled cell suspensions (whole-cell antigens) were injected into the same rabbits previously used for the production of somatic antisera. Rabbits were injected with one ml (0.5 ml in each hip) of an equal mixture of antigen and Freund's complete adjuvant (Difco). After three weeks, two ml of the same culture was injected intravenously without adjuvant. The rabbits were bled one week after the second injection.

D. Immunofluorescenee

Fluorescent antibodies (FA) were prepared against the somatic cell components of USDA 192, 193, 194, 205, and 214. Antisera were conjugated according to the procedures described previously (123).

Smears from pure cultures and nodules were stained according to the method of Schmidt, et al. (123). Gelatin-rhodamine isothiocyanate conjugate (12) was used to suppress non-specific binding of FA. Stained smears were examined with a Zeiss standard 14 microscope equipped for epifluorescence and phase contrast. Incident illumination was provided by an HBO-50 (OSRAM) mercury-vapor light source with a fluorescein isothiocyanate (FITC) filter.

The cross-reactions of FA-stained strains were assessed by subjective evaluation of the relative intensity of fluorescence, from 0 to 4+, with 4+ being the highest degree of fluorescence.

E. Immunodiffusion.

Immunodiffusion analyses (ID) of whole and somatic cell antigens were performed according to the procedures of Vincent (132). Cell suspensions, containing 10^{10}-10^{11} cells/ml were added to the outer wells (6mm dia.) of Petri dishes containing 20 ml of immunodiffusion agar [0.75% (w/v) Nobel agar (Difeo), 0.85% (w/v) NaCl, 0.01% (v/v) Thimerosal, and $8X10^{-5}$ M Na_2-EDTA, pH 6.5] and allowed to prediffuse for 4 h before the addition of antiserum to the center well. Gels were incubated at room temperature for 3 to 4 d and stained with amido black [0.1% amido black, $4.25X10^{-1}$ M acetic acid, $4.25X10^{-2}$ M sodium acetate, and 15% (w/v) glycerol]. Gels were destained with a 2.0% (w/v) solution of acetic acid untill the background was clear and the resultant precipitin bands which formed were recorded photographically.

Results

Immunofluorescence Analysis

The results of the immunofluorescence cross-reactions of 39 strains of fast- and slow-growing rhizobia with 5 fluorescent antibodies are shown in Table V-2. The results presented indicate that the 7 fast-growing PRC soybean rhizobia can be separated into at least three somatic serogroups. While the only member of the 192 serogroup is USDA 192, the 194 serogroup consisted of USDA 194 and 201. The 205 serogroup consists of USDA 191, 193, 205, and 214.

Results of this study indicate that with the fastgrowing PRC soybean-rhizobia, the intensities of the immunofluorescence reactions were greatest when young (1-2 d old) YEM cultures were

used. If older cultures were stained with FAs, iF reactions decreased in intensity and a smaller percentage of cells reacted. In addition, the FA staining of the fast-growing soybean strains differed from the other rhizobia examined in that cultures had to be boiled, prior to FA staining (data not shown). Unboiled cells either failed to react or reacted poorly with the FAs.

Results of this study also indicated while media other than YEM could be used for the growth of the fast-growing soybean rhizobia, FAs did not stain cells grown in TY (62) or PPM (see Chapter IV, this work). However, FAs would stain cells grown in modified YEM medium where galactose had been substituted for mannitol.

The five PRC FA's were also used to determine if there were cross-reactions with other fast- and slow-growing rhizobia (Table V-2). Of the 11 slow-growing B. japonicum examined (comprising members of 11 different serotypes), only B. japonicum USDA 136 reacted with one of the FAs (FA 194). Of the 19 other fast-growing rhizobia examined, one Rhizobium sp. for Leucaena, strain Tal 82 and three Rhizobium sp. for Sesbania, strains Tal 1117, Allen 770, and PL 146 fell within the 192 serogroup. One Rhizobium sp. for Lablab, NGR 234, cross-reacted with all of the fast-growing PRC FA's. However, the IF cross-reactivity of NGR 234 was greatest with FA's prepared against USDA 192 and 205.

Immunodiffusion analysis

The results of immunodiffusion cross-reactions of 33 strains of Rhizobium with somatic and whole cell antisera produced against USDA 192, 194, and 205 are summarized in Table V-3. The results show that the fast-growing soy bean-rhizobia could not be

Table V-2. Immunofluorescence cross-reactivity of somatic antigens of fast-growing soybean-rhizobia

Somatic Antigens Of:	Fluorescent Antibodies Against Somatic Antigens of Fast-Growing Isolates				
	USDA 192	USDA 193	USDA 194	USDA 205	USDA 214
R. japonicum:					
Fast-Growing					
USDA 191	-[a]	2+	-	2+	2+
USDA 192	+4	-	-	-	-
USDA 193	-	4+	-	3+	4+
USDA 194	-	-	4+	-	-
USDA 201	-	nt	4+	-	nt
USDA 205	-	3+	-	4+	3+
USDA 214	-	3+	-	3+	4+
Slow-Growing					
10 Serotypes	-	-	-	-	-
USDA 136	-	-	2+	-	-
R. leguminosarum:					
2 strains	-	-	-	-	-
R. phaseoli:					
1 strain	-	-	-	-	-
Rhizobium spp.					
For Leucaena					
6 strains	-	-	-	-	-
Tal 82	3+	-	-	-	-
For Sesbania					
5 strains	-	-	-	-	-
Allen 770	2+	nt	-	nt	-
Tal 1117	2+	nt	-	nt	-
PL 146	2+	nt	-	nt	-
For cow-pea					
2 strains	-	-	-	-	-
For Lablab					
NGR 234	3+	2+	2+	3+	2+

[a]Numbers represent the relative degree of fluorescence: - = no fluorescence 1+ = weekly fluorescent, 4+ = very fluorescent. Not tested = nt.

separated into serological groups based on their immunodiffusion reactions with the three PRC whole-cell antisera (see Figure V-1 A for example). All of the PRC strains examined shared at least one common heatlabile antigen. However, the fast-growing soybean strains could be separated into at least three distinct serogroups based on ID reactions with the three somatic antisera (see Figure V-1B for example). Generally speaking, the ID patterns obtained with whole cell antisera were quite complex and involved at least four discernable precipitin bands; while reactions with somatic antisera produced 1-2 bands.

While no precipitin bands were detected in diffusion reactions between the 8 "typical" slow-growing rhizobia and the three PRC whole or somatic cell antisera, several fastgrowing rhizobia clearly exhibited ID cross-reactivity. Two R. meliloti strains, L5-30 and NZP 4013 produced 1-2 precipitin bands with whole-cell antisera from each of the serogroups (192, 194, and 205), while the same isolates only produced one precipitin band with somatic-cell antiserum from USDA 192. Although two Rhizobium spp. for Sesbania, PL 146 and Allen 770, formed one precipitin band with whole-cell antiserum against USDA 192, only Allen 770 reacted with 192 somatic-cell antiserum. While strain NGR 234 of Rhizobium sp. for Lablab (which also nodulates Leucaena and several other legumes) formed precipitin bands with all three whole-cell antisera, it only gave one band with somatic-cell antisera. No immunodiffusion cross-reactivity was detected between the PRC antisera and any other of fast- and slow-growing rhizobia examined.

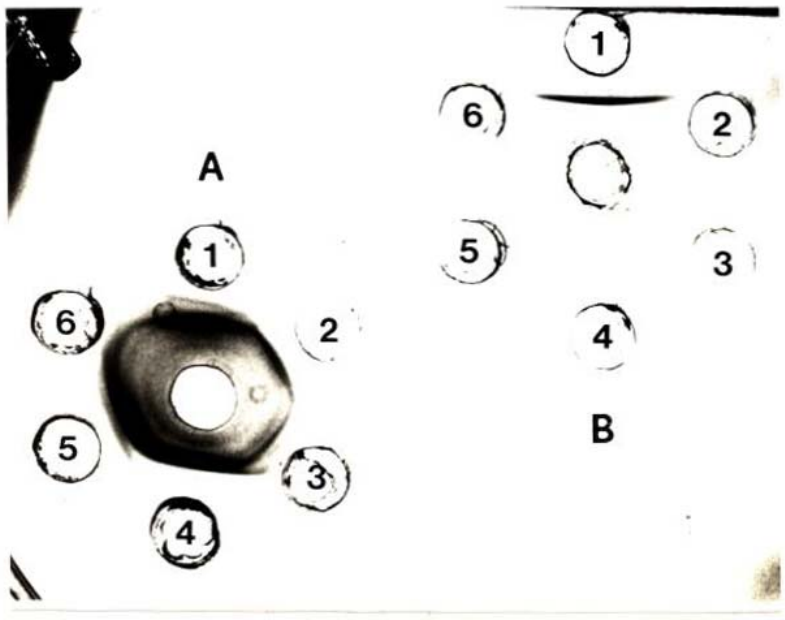


Figure V-1. Serological analysis of several fastgrowing soybean-rhizobia. Figure V-1 A. Immunodiffusion of whole-cell antigens of USDA 191, 192, 193, 194, 205, and 214 against antiserum prepared from USDA 205 whole-cell antigens. Well: 1, USDA 192; 2, USDA 193; 3, USDA 191; 4, USDA 194; 5, USDA 205; 6, USDA 214. Center well contains wholecell antiserum against USDA 192. Figure V-1B. Immunodiffusion of somatic antigens of USDA 191, 192, 193, 194, 205, and 214 against antiserum prepared against the somatic antigens of USDA 192. Well: 1, USDA 192; 2, USDA 193; 3, USDA 191; 4, USDA 194; 5, USDA 205; 6, USDA 214. Center well contains somatic-cell antiserum against USDA 192.

Table V-3. Immunodiffusion analysis of fast- and slow-growing rhizobia.

Antigen	Antiserum Prepared Against Whole-Cell Antigen of: 192	194	205	Somatic Antigen of: 192	194	205
R. japonicum (Fast):						
USDA 191	3[a]	3	1	0	0	1
USDA 192	4	3	1	1	0	0
USDA 193	1	2	2	0	0	1
USDA 194	3	4	4	0	2	0
USDA 205	2	3	4	0	0	1
USDA 214	1	1	2	0	0	1
B. japonicum (Slow):						
8 Serogroups	0	0	0	0	0	0
Rhizobium leguminosarum						
2 strains	0	0	0			
Rhizobium lupini	0	0	0			
2 strains						
Rhizobium meliloti						
L5-30	1	2	2	1	0	0
NZP 4013	1	2	1	1	0	0
Rhizobium trifolii						
2 strains	0	0	0			
Rhizobium phaseoli						
2 strains	0	0	0			
Rhizobium spp.						
For Leucaena						
1 strain	0	0	0			
RL 38	1	0	0			
For Sesbania						
2 strains	0	0	0			
PL 146	1	0	0	0	0	0
Allen 770	1	0	0	1	0	0
For Lablab						
NGR 234	2	2	3	0	0	1
For Cowpea						
2 strains	0	0	0			

[a]Numbers represent the number of precipitin bands formed.

Discussion

Although the fast-growing soybean rhizobia were isolated from geographically diverse regions of China, they were found to be serologically related. Immunodiffusion reactions with whole-cell antisera indicated that all of the PRC isolates share at least one heat-labile agar diffusible antigen in common. The heat-stable (somatic) antigens analyzed by immunofluorescence or immunodiffusion, were useful in separating the fast-growing soybean rhizobia into at least three serological groups. Other workers (49,110,125,135) have found that somatic antigens of fast- and slow-growing rhizobia are more specific than flagellar or internal antigens and that these thermostable antigens are suitable for separating strains within a species into distinct serological groups.

The results of this study indicate that while the fastgrowing soybean rhizobia share symbiotic host-specificity with the typical slow-growing B. japonicum, they are not serologically related. None of the strains from 8 serogroups of slow-growing soybean rhizobia examined, with the exception of B. japonicum USDA 136 (CB 1809), cross-reacted with the five antisera against the PRC strains. In addition, antisera against the fast-growing PRC rhizobia did not react with two slow-growing "cowpea" rhizobia or the one slowgrowing R. lupini isolates. It should be noted, that the cross-reaction of USDA 136 with PRC 194 somatic antiserum was only detected by immunofluorescence. No precipitin bands could be detected in immunodiffusion reactions between USDA 136 and PRC 194 somatic antiserum. Pankhurst (110) found that among the 62 fast- and 76 slow-growing Lotus rhizobia which he examined, there was no sharing of somatic antigens between the two groups, when analyzed by immunodiffusion reactions. The PRC rhizobia showed some serological relatedness to some of the other fast-growing rhizobia. Of the 19 fast-growing rhizobia

examined by immunodiffusion, 6 showed serological cross-reactivity with the fast-growing PRC rhizobia. Out of these six strains, the two R. meliloti strains and Rhizobium sp. Allen 770, fell within the USDA 192 somatic serogroup, while the fast-growing Lablab isolate, NGR 234 belonged to the PRC 205 somatic serogroup. Similarly, immunofluorescence analysis of 22 strains of fast-growing rhizobia revealed that one Leucaena strain, Tal 82, and three Sesbania strains, Tal 1117, Allen 770, and PL 146 were members of the USDA 192 somatic serogroup. On the other hand, the one Lablab strain, NGR 234 examined by immunofluorescence, cross-reacted with all five of the FA's used.

An interesting result of this study was the finding that the two R. meliloti isolates cross-react with USDA 192 somatic antiserum. At present, all available taxonomic and serologic evidence (see chapters 1, 2 and 5 this work) indicate that R. meliloti are so unique among the rhizobia, that they deserve separate species status. Vincent et al. (137) have indicated that agglutination patterns of internal antigens placed strains of R. meliloti with fast-growing rhizobia capable of nodulating Lotus and Leucaena. Trinick (130) also noted agglutination of R. meliloti by antisera prepared against a strain of Leucaena rhizobia.

Another interesting finding was that while the fast-growing rhizobia were capable of substantial growth in several different laboratory media, they would not react with fluorescent antibodies when the cells were grown in TY or PPM medium. The phenomenon of antigenic variation due to cell growth conditions has been reported by others. Vintikova et al. (139) indicated that the nature of the growth medium affected the antigenic properties of R. meliloti. Dudman (38) also indicated that R. meliloti antigens vary more than those of R. trifolii when grown in different media. However, in this study it was found that cells grown in TY medium would not even react with FA produced from TY antigens (data not shown).

In summary: 1, although the fast-growing soybean rhizobia were isolated from geographically different regions of China, they are serologically related to each other and can be separated into at least three distinct somatic serological groups; 2, immunodiffusion analysis of whole-cell antigens revealed that all of the fast-growing PRC strains shared at least one heat-labile antigen in common; and 3, somatic antigens were more specific than whole cell antigens in separating these rhizobia into serogroups.

CHAPTER VI

GENERAL SUMMARY

The newly described fast-growing soybean-rhizobia from China have provided a unique opportunity to study the biochemical, physiological, and genetical relationships between fast- and slow-growing rhizobia which have the ability to nodulate the same legume host. While soybeans represent a major agricultural crop in the United States, China is thought to be the center of origin and diversity of soybeans (and most likely soybean rhizobia).

The results of these investigations have indicated that: 1, while the fast-growing soybean rhizobia share symbiotic host-specificity with the typical slow-growers (they both nodulate the same legume host), they appear more closely related, on a microbiological, biochemical and physiological bases, to other fast-growing species of Rhizobium. Although the fast-growing soybean isolates share some biochemical characteristics in common with other fast-growing rhizobia, there are some biochemical properties (such as ethanol utilization and gelatinase activity) which are unique to this group; 2, all of the fast-growing soybean isolates which were examined fell within at least three somatic serogroups, based on immunofluorescence reactions. While immunodiffusion analysis of heat-labile whole-cell antigens indicated that all the strains shared at least one heat-labile common antigen, immunodiffusions performed with heat-stable somatic antigens were more specific and separated the isolates into at least three somatic serogroups; 3, the taxonomic position of the fast-growing soybean rhizobia must logically be in the new genus Rhizobium and based on their symbiotic characteristics

they may warrant separate species status; 4, all the isolates of this group which were examined contain at least one high molecular weight plasmid. In one of the isolates the loss of a large resident plasmid led to the loss of nodulating ability which indicates that nodulation genes might be plasmid borne in this group of organisms; and 5, the pea host-range (Sym) plasmid, pJB5JI, could be transferred to one of the fast-growing soybean isolates, but the resulting transconjugants were unable to form nodules on peas. On the other hand, the transfer of pJB5JI to two R. trifolii strains resulted in pea-nodulating transconjugants indicating that the expression of the Sym plasmid depends on the genetic background it resides in.

The fast-growing soybean rhizobia may provide an effective tool for a better understanding of the genetics of the soybean-Rhizobium symbiosis since they appear more amenable to genetic manipulations than the typical slow-growing soybean symbionts.

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