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Characterization of *Bradyrhizobium japonicum* serocluster 123 member isolates native to Iowa soils

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Characterization of *Bradyrhizobium japonicum* serocluster 123 member isolates native to Iowa soils

by

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GENERAL INTRODUCTION

Nitrogen is one element essential in the biosynthesis of amino acids, proteins, and DNA. Despite the abundance of N in our atmosphere, estimated at 755 g of N over every cm^2 of the earth's surface (Richards, 1987), many plants and animals suffer from N deficiency. While many plants obtain their N from the soil solution, legumes have evolved a symbiotic relationship with N_2-fixing bacteria, Rhizobium and Bradyrhizobium. This literature review will address several aspects of N_2-fixing bacteria and their interactions with bacteriophages, plants, and other soil microorganisms.

Members of the prokaryotic bacterial genera Rhizobium or Bradyrhizobium, which once may have been plant pathogens, have developed symbiotic relationships with leguminous plants. The symbiosis between these two living organisms, presumably based on C/N exchange, gives rise to many unanswered questions. This plant/microbe interaction became increasingly important when legumes were cultivated globally, having an impact on world food markets and the world economy.

In the midwestern United States (including Iowa), soybean is infected with a dominant rhizobial strain, Bradyrhizobium japonicum belonging to serocluster 123. Members of this serocluster, specifically USDA 123, have been found to be relatively ineffective in N_2 fixation when compared with other strains of B. japonicum.

Two aspects of the symbiotic relationship were evaluated in these studies: 1) a method to identify strains based on viral lysis; and 2) an assessment of the N_2-fixation efficiencies of rhizobial soybean isolates from Iowa soils. Bacteriophage typing was used to study the variations and relatedness among the native serocluster 123 member strains. Effectivity and competitiveness of the isolates were evaluated and compared with each other and with strains from the USDA culture collection. Our overall objective was to find a superior strain for use in inoculants that would replace the indigenous B. japonicum strains that normally occupy Iowa soybean nodules. We tried to exploit interstrain diversity of the indigenous population and to identify strains within the native serocluster 123 that were as effective, or nearly as effective, in fixing nitrogen as USDA 110.

Explanation of Dissertation Format

In the Literature Review, published work pertinent to our studies has been reviewed and summarized. This section will not be submitted for publication and is used only as a source of background information for the following sections.

Paper 1, "Native Bacteriophages Infective to Bradyrhizobium japonicum Serocluster 123 from Iowa Soils," reports the isolation and characterization of Bradyrhizobium-specific bacteriophages. Phages were characterized morphologically by using transmission electron microscopy and genetically by using gel electrophoresis. Host-range

sensitivity of bacterial isolates to these bacteriophages were recorded, and diversity within this serocluster is discussed.

Paper 2, "Interstrain Competition and N_2 Fixation Evaluation Among Bradyrhizobium japonicum Serocluster 123 Member Isolates," evaluates the competitiveness and effectivity of native bacterial isolates belonging to serocluster 123 compared with USDA 110 and USDA 123 in greenhouse studies.

Finally, an overall summary follows the two papers, entitled "Summary and Conclusions." References cited in the Literature Review follow the Summary and Conclusions.

LITERATURE REVIEW

Ecology of Bacteriophages Infective to Rhizobia

Bacteriophage-Induced Variation Among Rhizobial Populations

Bacteriophages belong to a group of bacteria-specific viruses of diverse morphology and characteristics. The name "bacteriophage," meaning "eater of bacteria," first was given by F. d'herelle (Adams, 1959) to a bacteriolytic substance he isolated from dysenteric patients.

Rhizobiophages--bacteriophages infective against rhizobia--are commonly found in soils cultivated with leguminous plants (Kleczkowska, 1957; Vincent, 1970; Barnet, 1972; Patel, 1976). The bacteriophages occur associated with rhizobia in the rhizosphere (Katznelson and Wilson, 1941; Barnet, 1972; Golebiowska et al., 1972) and root nodules (Kowalski et al., 1974) of legumes. Bacteriophages may affect nodulation of legume roots by influencing rhizobial populations in two ways. First, there may be a direct infection of the bacteria by bacteriophages, followed by lysis of rhizobial cells. Demolon (1951) obtained higher plant yields in a field inoculated with bacteria resistant to polyvalent bacteriophages than in a field inoculated with sensitive strains. Kowalski et al. (1974) and Evans et al. (1979a, 1979b) found that yield reductions in plants inoculated with bacteriophage-sensitive strains were related to a lower number of root nodules because of the lytic

activity of the virulent bacteriophage on the host rhizobial population.

A second influence by rhizobiophage on rhizobial populations is that the rhizobiophage might give rise to ineffective substrain mutants, which may occupy major portions of legume-root nodules (Gupta and Kleczkowska, 1962). Reduced plant yields resulted presumably from lower N_2-fixation rates. Ineffective mutants were observed by Evans et al. (1979a) when, under controlled conditions, the presence of a virulent bacteriophage in the root zone of clover caused populational reductions of susceptible Rhizobium trifolii strains. Less bacteriophage-sensitive strains, ineffective in N_2 fixation, tended to dominate. Kleczkowska (1957) showed that mutant strains varied in their N_2 fixation from ineffective to partially effective. Bacteriophages presumably have an effect on bacterial survival and variation, but Wiggins and Alexander (1985) suggested that a bacterial density of 10^4 CFU/mL in the environment is a threshold below which phages do not multiply.

Heterogeneity of R. meliloti was first suggested by Hughes and Vincent (1942) and later by Purchase et al. (1951) by using serology. Since then, serological techniques have been used extensively to differentiate heterogeneity within a particular rhizobial strain. It has become evident more recently, however, that these techniques underestimate the diversity of indigenous populations (Noel and Brill, 1980; Dughri and Bottomley, 1983; Dughri and Bottomley, 1984).

Other means have since been used to further characterize interstrain variability, e.g., protein profiling (Jenkins and Bottomley, 1985; Hickey et al., 1987) and bacteriophage typing (Lesley, 1982; Bromfield et al., 1986).

A bacteriophage sensitivity-pattern system may have advantages in replacing serology as a means of strain identification. This simple and inexpensive method was first employed by researchers as early as the 1920s (Gerretsen et al., 1923) to show the host range of a bacteriophage infecting R. meliloti strains. Today, a bacteriophage-typing system may be used to differentiate within an indigenous population of rhizobia based on rhizobial susceptibility to a number of lytic rhizobiophages. Bromfield et al. (1986) found variation in bacteriophage sensitivity among R. meliloti in the nodules of Medicago sativa cultivars at two field sites. They analyzed 1,920 nodules and found 55 and 65 different bacteriophage types, respectively, distributed over the two sites. Thurman and Bromfield (1988) also showed great diversity among the indigenous R. meliloti populations between and within the legume species of Medicago, when a sensitivity pattern was developed from typed nodules obtained from cross-inoculation groups. These authors suggested that the legume host participated in selection of particular Rhizobium-bacteriophage types. Lesley (1982), by using an enrichment technique, isolated 15 specific bacteriophages, each active on a R. meliloti strain. He then developed a

bacteriophage-sensitivity pattern based on Rhizobium susceptibility. Eighty distinct patterns were developed, which discriminated among the strains.

Thus, bacteriophage-host interactions have been examined to distinguish variability within a strain with more accuracy and greater range than available through serological techniques. Still insufficient data are available regarding how the interactions are affecting the Rhizobium-legume symbiosis, particularly in terms of efficiency and competition, and little work is available regarding the soybean-rhizobial symbiosis.

Host Specificity of Bacteriophages

The specific mechanisms by which rhizobia attach to the roots of the host leguminous plant are not fully known. There are many systems suggested, generally based on host specificity of Rhizobium species. Lectins are plant-specific proteins and are suggested to be involved in recognition of bacterial cell-surface carbohydrates.

Lectins have been shown to have high affinity for binding rhizobia (Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1975; Bhuvaneswari et al., 1977). Stacey et al. (1984) used a bacteriophage that lysed all but a non-nodulating mutant of a B. japonicum strain but did not find evidence of capsular polysaccharides (CPS) or exopolysaccharide (EPS) participation in plant-cell lectin binding systems. Binding of all mutants to plant-root lectins suggested that other factors, such as

lipopolysaccharides (LPS), may be involved in recognition and binding.

Highly specific bacteriophage receptors also have been found in various components of bacterial cell walls. Atkins and Hayes (1972) noted that changes in LPS and lipoproteins of R. trifolii mutant strains resulted in higher resistivity to bacteriophage lysis than found in wild-type strains. Barnet and Vincent (1970) suggested that certain bacteriophages recognize antigenic portions of the cell wall of Rhizobium as their receptor sites. Similarly, Zajac and Lorkiewicz (1983) presented evidence that O-acetyl groups in LPS receptors of R. trifolii played a major role in recognition of bacteriophage P1. Treatment of bacterial cells with alkali deaceytylated the LPS, thus causing the loss of receptor sites. The cleavage of O-acetyl groups of LPS by bacteriophage also has been suggested.

The evolution of less-efficient rhizobial mutants caused by rhizobiophages and loss of competitive ability of bacteriophage-susceptible strains (Schwinghamer and Brockwell, 1978; Evans et al., 1979a) has been complicated by the mutagenic effect of bacterial host cells on bacteriophages, and vice versa. Adaptive mechanisms by viruses as a whole to a continuously changing environment are known to occur by frame-shift mutations, rearrangements, and duplications at the chromosomal levels. Additionally, genetic-exchange mechanisms between the bacterial host and the infecting bacteriophage

readily occur, altering phenotypic properties of both organisms. Hashem et al. (1986) reported the isolation of a mutant bacteriophage specific for B. japonicum USDA 117 after long-term inoculation of the parental bacteriophage in soil. According to these investigators, this mutant gained leverage in its ecological competitiveness. Soybean plants grown in association with the wild-type bacteriophage showed lower nodule numbers, nodule weights, shoot weights, and acetylene reduction activities than those plants grown with mutant bacteriophage strains. This phenomenon also was reported by Schwinghamer (1965) in a study that demonstrated the occurrence of host-induced modifications of Rhizobium bacteriophages in some of the closely studied bacteriophages.

The effect of bacteriophages on bacterial hosts also has been studied. Hancock and Reeves (1976) observed that bacteriophage-resistant mutants isolated from Escherichia coli K-12 had alterations in their LPS envelopes and, additionally, had changed responses to levels of antibiotics. A study of outer-membrane protein composition by polyacrylamide gel electrophoresis indicated modifications of the major cell-wall proteins.

Another implication for the specific interaction between bacteriophage and the bacterial host was suggested based on the activity of bacteriophage-encoded carbohydrate-specific depolymerases (Atkins and Hayes, 1972; Barnet and Humphrey, 1975). These depolymerases formed halos, diffusing beyond the

normal plaque size produced by bacteriophages, during their lytic activity. The exopolysaccharide was depolymerized by the enzyme activity and short-chain carbohydrates or monosaccharides were produced and released into the environment.

Hartung et al. (1988) cloned a depolymerase-encoding bacteriophage gene specific for Erwinia amylovora surface polysaccharides into E. coli. This gene was expressed by producing abnormal halos similar to plaques formed by virulent bacteriophages carrying the depolymerase gene. The occurrence of this unique system is an example of a successful expression of genetic factors transferred between bacteriophage and its bacterial host. Exchange of genetic information also may occur between a bacteriophage and its host rhizobia, resulting in alteration of the host phenotype/genotype because bacteriophage-mediated transduction and other methods for genetic transfer in the soil environment have been reported to commonly occur (Zeph et al., 1988; Zeph and Stotzky, 1989).

Bacteriophage Taxonomy

Genomic analysis of animal viruses for classification purposes first was used by Cooper (1961). Later, in 1967, Bradley employed virion nucleic acid strandedness, a technique described earlier by Lwoff et al. (1962), in addition to morphological makeup, as a taxonomical means to classify the viruses infecting bacterial cells. Six morphological bacteriophage types (A-F) were originally introduced by

Bradley, which thereafter increased to 18 morphotypes, based mainly on differences in bacteriophage tail structures.

The discovery of bacteriophage particles in the soil environment was first reported in fields of "fatigued" lucern (N_2-deficient alfalfa) by Demolon and Dunez (1935). Rhizobiophage research began when bacteriophages, known to influence the population and ecological evolution of the bacterial symbiont, were found in the rhizosphere and nodules of economically important legumes (Kleczkowska, 1957; Barnet, 1972; Kowalski, 1974). To understand the action of rhizobiophages, methods were employed to isolate pure, single-strain bacteriophage cultures, and then to classify them based on their morphology. Patel (1975) isolated 19 virulent bacteriophages for fast- and slow-growing rhizobia. Electron microscopy showed differences in bacteriophage structures, and a bacteriophage-sensitivity pattern revealed the degree of their specificity for two rhizobial hosts.

Morphotyping also has been used to classify R. trifolii bacteriophages (Atkins, 1973), based on variations in spike attachment, base plates, and tail types of 14 isolates. A bacteriophage with colibacteriophage T4 morphology lytic to a B. japonicum strain native to Tennessee soils was isolated by Stacey et al. (1984).

Recently, new biotechnology tools have been used to characterize bacteriophages more specifically and quickly. Bacteriophages belonging to the same morphological groups,

having broad host ranges, can be differentiated by using restriction endonuclease patterns (Werquin et al., 1988). Molecular techniques such as DNA-DNA homology, restriction mapping, and molecular weights, in conjunction with serology, morphotyping, and host specificity ranges, now are commonly used to identify bacteriophages and their bacterial hosts.

Competitive Evaluation Among Rhizobial Species

Nodule Initiation Process

1. Infection Process

A successful interaction between plants and bacteria is not confined just to exploitation of the plant by the microorganism, based on its nutritional requirements, but also evolutionary adaptation of both organisms has extended to a mutualistic relationship, benefiting both. In this association, the bacteria are no longer considered pathogens because they may benefit the plant in some physical or biochemical manner.

Rhizobia are bacteria that fix atmospheric N_2 in a symbiotic relationship with legumes. The symbiosis occurs in many legumes when bacteria initiate an infection thread by colonizing a root-hair tip and then move toward cortical cells through the infection thread (Dart, 1974; Bauer, 1981; Verma and Long, 1983). The infection thread starts on a curled and deformed root hair ("Shepherd's Crook"), formed by elongation of root-hair cells (Fahraeus, 1957; Callaham and Torrey,

1981). The already-dividing bacteria move through the infection thread and enter the cytoplasm of the host meristematic cells while surrounded by a prebacteroid membrane. Differentiation of rhizobial cells to bacteroids occurs at this stage, and the nitrogenase enzyme is synthesized and N_2 fixation is initiated. The first product of N_2 fixation in the nodule of leguminous plants is ammonia (NH_3) (Bergersen, 1965). Ammonia is produced by rhizobial bacteroids--now encoding for the nitrogenase enzyme--which is excreted into the plant-cell cytoplasm (Bergersen and Turner, 1967). This product is then incorporated into organic compounds that are translocated throughout the plant. Two groups of compounds are produced by legumes as NH_3 carriers: amides and ureides. Amides are produced in legumes of temperate regions, which mainly are infected by species of Rhizobium (Kennedy, 1966). These fast-growing bacteria have colony diameters of 2-4 mm after three-to-five days of growth on Yeast Mannitol Salt Agar. They are motile by a polar or subpolar flagellum, or by two to six peritrichous flagella. Rhizobium are acid producers when mannitol or other sugars are used as the source of carbohydrate in the growth medium, with an optimum growth pH of 6-7 (Jordan, 1982).

The B. japonicum and other Bradyrhizobium spp., on the other hand, infect and colonize root nodules mainly of ureide-producing legumes native to tropical regions (Pate et al., 1980). These slow-growing bacteria are motile by only

one polar or subpolar flagellum. The diameters of the colonies do not exceed 1 mm when grown five-to-seven days on Yeast Mannitol Salt Agar, with an optimum growth pH of 6-7 (Jordan, 1982).

2. Binding

Successful infection of legumes by rhizobia is necessary for effective N_2 fixation to occur. There are a variety of models explaining mechanisms of attachment of bacteria to the legume-root surface. Some models suggest that the attachment is host specific and that binding is mediated by lectin produced by the host-legume root. These lectins react to very specific saccharide receptors on the bacterial cell surface (Dazzo and Hubbell, 1975; Bal et al. 1978; Dazzo and Brill, 1979; Stacey et al., 1980; Dazzo et al., 1984). Other studies suggest attachment of bacteria to the root surface is non-specific and that lectin does not play a major role in the binding process (Bohlool and Schmidt, 1976; Law et al., 1982; Badenoch-Jones et al., 1984; Pueppke, 1984; Mills and Bauer, 1985; Vesper and Bauer, 1985; Vesper and Bauer, 1986).

Lectin has been widely used as a tool for recognition of specific sugar molecules in biological experiments (Ridge and Rolfe, 1986). This specific recognition by lectin can be further manipulated for cell surface-carbohydrate profiling by using lectin of different affinities. This differentiates among organisms that may have only a single sugar substitution in their cell-surface structure. Ridge and Rolfe (1986)

reported that the binding of different lectins to different parts of the legume-root surface was an indication of various lectin-specific sugar molecules present on the root surface. In their 1986 study, ten labeled lectins were tested on the roots of the legume M. atropurpureum Urb. Four of the ten lectins were bound to epidermal cells on various portions of the root, suggesting the involvement of lectins as a signal compound for rhizobial gene expression.

Wong and Shantharam (1984), in an attempt to define the specificity of lectin binding to multiple sites on the bacterial cell surface, worked with pole bean (Phaseolus vulgaris L.) nodulated by certain species of Rhizobium phaseoli, R. leguminosarum, and R. trifolii, but not by B. japonicum 61A118. In all cases, pole-bean lectin reacted with all strains tested, whether the legume was nodulated or not. The authors concluded that pole-bean lectin and soybean lectin have different sugar specificities for the infecting species of rhizobia. B. japonicum 61A118 may have multiple sites for lectin binding by different legumes.

Recently, mechanisms other than lectin binding have been suggested to be the main factor in binding of B. japonicum to legume-root surfaces. Chahal and Vilkhu (1985), for example, reported that capsular polysaccharides play an important role in lectin binding. In a culture study of Rhizobium species that nodulate pea (Pisum sativum L.), they found that homologous and heterologous strains of Rhizobium were capable

of binding to pea lectin. Culture age and lectin binding were related, based on the bacterial-capsular polysaccharide production. The highest level of binding between the bacteria and the legume roots occurred during the log phase of growth. Binding decreased when the culture passed the log phase, indicating the transient nature of polysaccharide-related receptors on the bacterial cell surface.

In another report (Vesper and Bauer, 1986), pili (fimbriae) were observed to play an important role in the attachment of B. japonicum to soybean plant roots. When B. japonicum were treated with pili-specific antiserum, nodulation of the host legume was reduced by 80% and attachment reduced by 90%. Pili are reported to be proteinaceous (fibrillar structure) and are commonly observed on the surface of most gram-negative bacteria (Ottow, 1975). They are frequently used as a tool of attachment to other bacteria during conjugation; to substrates as energy sources; and, in B. japonicum, to the root surface of soybean for initiation of symbiosis (Wadstrom et al., 1980; Gaastra and DeGraaf, 1982; Korhonen et al., 1983).

The exact mechanism by which legume-root exudates stimulate rhizobial populations in the rhizosphere is not fully known. A rhizosphere:soil (R:S) population ratio of approximately 1000:1 in the legume rhizosphere, however, is commonly accepted. Halverson and Stacey (1984), in a study on the role of root exudates in bacterial binding to legume-root

surfaces, showed that alterations in nodulation of soybean roots by mutant strains of HS111 (defective soybean root nodulator) were cultivar independent. The defective trait reverted to wild type when treated with root exudates. The root exudates may have a signal compound involved in the biochemistry of nodulation.

In addition to capsular polysaccharides (CPS), bacterial cell-surface carbohydrates may play a major role in the attachment of rhizobia to the legume roots because lectins mediate binding of very specific carbohydrates in specific symbiont recognitions (Bauer, 1981). Kovalevskaya et al. (1985) examined EPSs of some related legume-nodule bacteria and observed that all had similar monosaccharide compositions, but differed in quantity and in the lipopolysaccharides present. They also noticed that major EPS components (glucose, galactose, pyruvic acid) were structurally similar in all strains tested, but differed in proportions among species.

Major Abiotic Factors Affecting Competition and Survival

Soil pH

The adaptability of soil microorganisms is mainly determined by soil physical, chemical, and biological factors. Many unknown parameters in the soil matrix dictate the suitability of a niche to a particular organism. Soil pH is readily determined in bulk soil but soil acidity affects the

symbiosis at both the bacterial and plant levels. Nodulation failure of legumes in acid soils has been attributed to limited growth and survival of rhizobia in those soils (Robson and Loneragan, 1970). Low pH also has been reported to affect the rhizobial population of introduced inoculum in acid soils of the tropics (Danso, 1988). Mulder and Van Veen (1960) studied the growth patterns of clover in acid soils and found that normal nodulation of clover roots occurred when numbers of Rhizobium in the inoculum exceeded 6×10^4 per 500 g of soil. Rhizobium and Bradyrhizobium strains behaved differently in response to the pH of their environment. Lowendorf and Alexander (1983) reported that strains of R. phaseoli were able to divide at pH 3.8. In further studies, R. meliloti did not grow below pH 5.3, and many species of Rhizobium did not grow at all in slightly acid cultures.

The effects of soil acidity on bacterial growth are complicated by soil clay contents (Bushby, 1990) and by increases in toxic metals in the soil solution; i.e., Al at toxic concentrations (Franco and Munns, 1982). High Al concentrations, however, are reported to affect the growth of the host plant to a greater extent than growth of rhizobia (Hartel et al., 1983). Bushby (1990) reported that certain strains of Bradyrhizobium could only be isolated from soils having less than 20% or greater than 40% clay. This phenomenon was accounted for by the bacterial surface-charge properties and their interactions with negatively charged

clays. The clay-surface charges were attributed to pH, soil type, and other edaphic factors.

Legumes generally are less sensitive to acidity than rhizobia and can grow well below their optimum pH range. Certain steps in the infection process during rhizobia-legume symbiosis are pH sensitive; however, the bacterial growth and legume-root development may not be affected at these pH levels. Highly alkaline soils also affect competition and survival among rhizobial species. Damirgi et al. (1967) examined soybean-nodule isolates from Iowa soils by using agglutination. They reported the prevalence of serogroup 135 in highly alkaline soils, whereas 123 was the dominant serogroup in most other soils.

In many soils, high pH levels are associated with high levels of salt that inhibit the growth of legumes. Bhardwaj (1975) studied the effect of salinity and high pH on both the legume host and the infecting rhizobial strain. He reported that bacteria are generally more salt tolerant than legumes and, in soils with high salt content, plant-host growth is inhibited, whereas bacterial growth is little affected. The adaptation of rhizobia to higher salt concentrations in alkaline soils was related to mutation of existing strains to more salt-resistance phenotypes (Steinborn and Roughley, 1975).

Soil Type

Rhizobia in the soil are not in pure culture and are accompanied by other living tissues, chemical compounds, and various soil-type matrices. A change in a specific soil component may alter the survival and competitiveness of a specific rhizobial species. Pena Cabriales and Alexander (1983) added alfalfa residues to soil and noted a reduction in the numbers of R. meliloti, R. phaseoli, B. japonicum, and cowpea rhizobia, but an increase in the number of protozoa. When the soils were amended with antibiotics, four antibiotic-resistant strains proliferated successfully.

Soil types also have been reported to influence the predominance of a particular rhizobial strain over other strains. Damirgi et al. (1967) reported that 52% of all soils in Iowa were dominated by members of serogroup 123, and that 79-100% of the soybean nodules in some of the fields tested were occupied by this serogroup. As the pH of soils increased, a higher percentage of the nodules was formed by other serogroups, especially members of serogroup 135. At pH 8.3, approximately 92% of the nodules were occupied by serogroup 135.

Another study to determine the factors affecting interstrain competition was conducted by Kosslak and Bohlool (1985). Two Minnesota soils, Waseca and Waukegan, reacted differently when soybean was inoculated with strain USDA 123. This strain occupied 69% of root nodules formed in Waseca soil

and only 24% of the nodules in Waukegan soil. This behavior was attributed to the biotic and abiotic factors differing between the two soil types.

Temperature and Moisture

For rhizobia to survive and infect respective host legumes, the ability to tolerate varied environmental conditions is essential. Temperature and moisture conditions often are found to be influential in soil rhizosphere competition and in serogroup distribution of certain strains in root nodules. Weber and Miller (1972) observed that B. japonicum strains occupying soybean root nodules changed as temperatures changed. Higher temperatures were correlated with isolation of strains that were isolated infrequently at lower temperatures. High temperatures also were reported by Favre and Eaglesham (1986) to affect soybean nodulation by different B. japonicum strains and to affect the nitrogenase activity of rhizobia (Parkhurst and Layzell, 1984). The combination of moisture and temperature stress has been found to be more deleterious to rhizobial survival than temperature stress alone (Wilkins, 1967). In a study to show the survival of cowpea rhizobia in soils affected by both temperature and moisture stresses, Boonkerd and Weaver (1982) measured the survival of two cowpea and two peanut rhizobia at two temperatures (25 and 35 °C) and under three moisture conditions--air dry, moist, and saturated. The higher temperature, in conjunction with either air dry or saturated

soil, was the most detrimental and caused a two-to-three log decrease in bacterial populations. Hartel and Alexander (1984) reported a relationship between tolerance to high temperature and tolerance to low-moisture levels. They tested 21 strains of cowpea rhizobia from cool/wet and hot/dry areas of West African savanna regions. They concluded that the strains from hot/dry regions were more temperature and desiccation tolerant than those strains from cooler and more humid regions.

Chemicals

1. Fungicides

Reduced yields of legumes have been widely attributed to seed rot and other seed-colonizing pathogens. Fungicides are one of the agents to inhibit or reduce pathogenic fungi, which are responsible for the destruction of seed and extensive yield reductions, particularly in hot and humid regions. These chemicals, however, are usually toxic to symbiotic N_2-fixing bacteria, causing a reduction of N_2 fixed for the associated legume (Afifi et al., 1969; Jones and Giddens, 1984; Ruiz-Sainz et al., 1984).

Graham et al. (1980) reported that fungicides are seed protectants necessary for bean production in Latin America. Numbers of R. phaseoli declined when Thiram, Captan, or PCNB was used as an antifungal seed-protecting agent. Jones and Giddens (1984) tested the effect of Thiram on B. japonicum USDA 110 and rhizosphere microorganisms. Rhizosphere

populations of bacteria declined when fungicide-treated seeds were used. They suggested the need for fungicide-resistant mutants of B. japonicum as inoculants. Ruiz-Sainz et al. (1984) also studied the effect of the fungicide Captafol on R. trifolii. Concentrations above 75 μg/mL of Captafol were found to be highly toxic to the Rhizobium, but, even below this level, the bacteria lost their nodulating capabilities. The resistant mutants isolated from plant roots were shown to have lost their plasmids, resulting in nodulating-defective mutants.

Use of fungicides, where affordable, is a feasible seed protectant; however, inoculation with effective N_2-fixers resistant to fungicides becomes essential when fungicide seed protectants are used.

2. Nitrogen Fertilizers

The relationship between symbiotic N_2-fixation and the amount of soil N taken up by the legume is not completely understood. Legumes take advantage of rhizobial symbiosis, and its nitrogenase activity, to acquire the much-needed N for vegetative and reproductive growth. However, N_2-fixation alone may not be adequate for best plant growth in some instances (Gibson, 1976). In addition, nodulation is inhibited when legumes are planted in highly N-rich soils. This phenomenon is particularly pronounced when high amounts of NO_3-N fertilizers are present (Herridge et al., 1984; Danso, 1987). Danso (1987) suggested breeding for N_2-fixing

legumes that produce high yields in soils having low amounts of N. It is conceivable as a consequence of such a breeding program that the costs for N fertilizers would be reduce while environmental safety increases. Most legumes in the midwestern United States are planted in previously N-rich soils.

Interstrain Diversity and Competitiveness within Rhizobial Species

Genotypic and phenotypic variations among the known serogroups/strains of rhizobia have greatly contributed to the understanding of the ecology and biochemistry of symbiotic N_2 fixation. Distinct genetic differences have been found between fast- and slow-growing rhizobia (Jordan, 1982). In fast-growing Rhizobium, genes responsible for nodulation (nod) and N_2 fixation (nif) are carried on a large plasmid, the sym plasmid (Banfalvi et al., 1981; Hombrecher et al., 1981; Hooykaas et al., 1981). Ruvkun et al. (1982) found that these two genes were closely linked on the R. meliloti sym plasmid. Differences between fast- and slow-growing rhizobia have been described in host specificity (Keyser et al., 1982), growth morphology and nodulating characteristics (Elkan, 1981), and biochemical analysis of the bacteria (Sadowdky et al., 1983).

The fast-growing Rhizobium fredii were first isolated from wild-type (Glycine soja) and unimproved soybean lines (Glycine max cv. Peking) (Keyser et al., 1982). The reports on classification of R. fredii have been controversial.

Buendia-Claveria et al. (1986) claimed that the fast-growing Rhizobium did not relate closely to the Rhizobiaceae family, whereas Sadowsky et al. (1983) reported that although members of R. fredii have a common host specificity with B. japonicum, biochemically speaking, they belong to fast-growing Rhizobium. It was further shown that these symbiotic bacteria were unable to effectively compete with most indigenous strains to form an effective symbiosis with improved North American soybean cultivars (Israel et al., 1986). This lack of competitiveness prevented the development of these bacteria commercially (McLoughlin et al., 1986). When the fast growers were tested against two slow-growing B. japonicum strains, the slow growers out-competed R. fredii in nodulating soybean (60% occupancy), even after a ten-fold higher number of fast growers was used. In a study on the competition of strains of R. fredii and indigenous populations of B. japonicum in midwestern soil, only 50% of the soybean nodules were occupied by the fast growers in the first year and, in the second year, no nodules were occupied by the fast growers (McLoughlin et al., 1986). They suggested that, except for USDA strain 191, other strains of R. fredii were unsuitable for commercial use and were effective only on the unimproved Peking cultivar. USDA 191 nodulated some of the improved soybean cultivars, but lacked competitiveness against slow growers, especially against members of serocluster 123. In general, slow-growing rhizobia have been poorly described because of low efficiency

in gene transfer and slow growth habits (Doctor and Modi, 1976; Kuykendall, 1979). In midwestern United States, the commercially important legume soybean is mainly nodulated by members of B. japonicum serocluster 123. Replacing this strain by more effective strains has been extremely difficult because of the highly competitive nature of members of this serocluster (Ham, 1980). The introduction of a commercially effective strain at one billion rhizobia per 2.5 cm of row to 22 different soils was assessed by Weaver and Frederick (1974). No increase in nodule occupancy was observed by the inoculum strain in soils having more than 1,000 rhizobia per gram of soil. They suggested that 1,000 times greater numbers of inoculum rhizobia over the indigenous populations were necessary to establish occupancy in 50% of the soybean nodules. Berg et al. (1988) reported that, among the 22 B. japonicum strains used in a competition study in Iowa soils, strains of serocluster 123 were the best competitors; they found no correlation between effectiveness in N_2 fixation and nodule occupancy by the test strains. Moawad et al. (1984) reported in an earlier study that members of serocluster 123 out-competed other indigenous strains in nodule formation even though the rhizobial population densities were similar. Members of serocluster 123 were found not to be as effective in N_2 fixation as other strains, especially USDA 110 (Caldwell and Vest, 1970).

Reports on ineffectivity in N_2 fixation and the highly competitive nature of this serocluster caused attention to be focused on environmental factors and interstrain variability. Kosslak and Bohlool (1985) studied the behavior of B. japonicum serocluster 123 in the soil environment. They reported that USDA 123 was not very competitive under defined conditions (e.g., in vermiculite) with USDA 110. Because members of serocluster 123 were reported to be heterogenous (Gibson et al., 1972; Schmidt et al., 1986), the USDA 123 type strain may not represent the diverse population of serocluster 123.

Older literature uses the word "serogroup", but Schmidt et al. (1986) proposed use of the term "serocluster" for 123 rather than serogroup. Serological relatedness among members of serogroup 123 and serogroups 122, 127, and 129 established the new terminology, presenting a more accurate description of the existing broad diversity within the serocluster.

Recent use of molecular techniques has shown higher degrees of genetic variation existing within strains of rhizobia than was previously estimated by serology (methodologies will be discussed later in this review). Hartmann and Amarger (1991) reported genomic heterogeneity among R. meliloti field isolates by using plasmid profiling, DNA fingerprinting, and insertion sequence (IS) typing. Field inversion gel electrophoresis (FIGE) was used to analyze enzymatically restricted genomes of 11 field isolates

belonging to serocluster 123 (Sobral et al., 1990). Genetic diversity was observed among the isolates examined.

The genetics and the biology of competition have been poorly understood. Because of natural growth habits of slow-growing B. japonicum, advances in defining the genes involved in competition have been slow. Haugland and Verma (1981) did not find a sym plasmid in any slow-growing B. japonicum, whereas Stanley et al. (1985) reported B. japonicum fell into two distinct categories based on conserved homologous genomic DNA sequences in their nif and nod genes. These genetic findings, combined with differences observed in physiology and symbiotic properties of B. japonicum, led to speculation that interstrain diversity may exist within the species (Fuller and Verma, 1974; Keyser et al., 1982).

Little work has studied the properties and advantages of interstrain diversity with Bradyrhizobium in terms of N_2 fixation and competitiveness within the bacterial population. A study by Berg et al. (1988) evaluated the symbiotic properties of B. japonicum belonging to serocluster 123. Phenotypic variation was observed based on their nodule occupancy. Members of serocluster 123 occupying soybean nodules were the best competitors among all the seroclusters tested. Furthermore a greenhouse study of these field isolates showed an existing variability in their N_2 fixation.

Testing of field isolates necessitates methods where isolation and purification of a particular strain is possible.

Furthermore, test strains should be checked for recurrent genomic rearrangement and reshuffling so that symbiotic properties would not be lost due to laboratory and/or environmental stresses. Currently, genomic restriction digestion patterns (DNA fingerprinting) appear to be the most stable markers to identify strains in a mixed population. If a particular isolate has a competitive advantage over other interstrain members and is a highly effective N_2 fixer, the isolate may be useful as an inoculant strain where it could readily establishment in the soil environment. It seems logical to manipulate the existing diversity among rhizobial species until the governing mechanism for competition becomes known.

Measurement of N_2-Fixation

Measurement of N_2 fixed by the rhizobia-legume symbiosis is necessary to understand bacterial contributions and thus to manipulate N_2-fixation efficiencies between symbiotic partners. Many methods have been used to estimate the rate of N_2 fixation (Havelka et al., 1985), generally classifiable as either direct or indirect. The use of an indirect and non-destructive chlorophyll assay for screening B. japonicum strains recently has been suggested (Mirza et al., 1990). A positive correlation between plant leaf chlorophyll content and total plant N was observed in both greenhouse and field studies. Other indirect methods include plant color and plant-dry-matter weight measurements.

Kjeldahl Method

The most widely used direct methods, for the most part, are destructive, e.g., total N and ^{15}N analysis. The simplest and most commonly accepted technique to determine plant total N is the Kjeldahl procedure (Bremner, 1965). This is mainly used to differentiate between symbiotic N_2 fixation of a nodulated legume variety and its uninoculated counterpart or a non-nodulating isoline. The procedure is a variation of the "difference method" in which, by employing the proper controls, the rate of N_2 fixation can be estimated in many situations. Bezdicek et al. (1978) determined the rate of N_2 fixed in field-grown soybean by using uninoculated plots as controls. Assuming the soil was uniform, the difference in total N between the inoculated and uninoculated soybean indicated the amount of N contributed to the plant by Bradyrhizobium. The Kjeldahl procedure has gained wide acceptance among many research workers but it has several disadvantages: it uses expensive reagents and instruments; it is time consuming; and it is destructive to plant tissue.

^{15}N Analysis

^{15}N analysis is based on a naturally found stable N isotope in the atmosphere. Plants grown under ^{15}N-enriched conditions are analyzed for their $^{15}N/^{14}N$ ratios by mass spectrometry and, knowing the natural abundance of the isotope (i.e., 0.4% of total atmospheric N), the amount of $^{15}N_2$ fixed by Rhizobium and Bradyrhizobium can be estimated (Burris and

Wilson, 1957; Hauck and Bremner, 1976). Hauck and Bremner (1976) reported over 1,500 published papers related to the agronomic use of this ^{15}N tracer technique. The commonly used ^{15}N analysis also is a destructive technique. Additionally, necessary instruments and the isotope gas are costly, but the major advantage of this system is the very small sample portions needed for testing.

A variation of this method was reported by Norhayati et al. (1988), where the use of enriched ^{15}N isotopic gas was eliminated. Instead, the technique depended on naturally found ^{15}N. Existing levels of the tracer were sufficiently high in the plant tissue that, through direct analysis of soil and plant tissue, the rate of N_2 fixation could be accurately estimated. This procedure eliminated the high cost of ^{15}N isotopic gas.

C_2H_2 Reduction Assay

Symbiotic N_2 fixation depends on the ability of the nitrogenase enzyme to convert N_2 to NH_4+ within the legume-root nodule. This enzyme is the only biological compound known to reduce acetylene (C_2H_2) to ethylene (C_2H_4) as an alternative pathway to N_2 reduction (LaRue et al., 1981). In this procedure, acetylene usually is injected into a closed container containing newly excised roots of a legume and incubated for 30-120 min. A sample of the gas is analyzed by gas chromatography for the amount of ethylene present (Hardy et al., 1973). The acetylene reduction assay, in general, is

simple, quick, and inexpensive to perform, but has many limitations. A major limitation is high variability among similarly handled plants. Also, acetylene may inhibit the oxidation of ethylene in the soil atmosphere by soil anaerobic microorganisms (Richards, 1987). This results in higher rates of measured ethylene. Acetylene reduction, as commonly performed, also is a destructive method.

Recently, non-destructive methods have been devised to overcome the single-point measurements in acetylene-reduction assays and also to reduce the variability among the plants tested. Use of a glass chamber to monitor nitrogenase activity throughout the legume life cycle was proposed by Habte (1983).

An in situ adaptation of a non-destructive assay also was proposed by Glenister and LaRue (1986), where acetylene was directly injected into the soybean-root zone. Ethylene present in the soil atmosphere was determined and the rate of nitrogenase activity was calculated. They observed large variability among individual plants, but no significant differences among cultivars tested. When the rates of in situ nitrogenase activity of intact soybean was compared with those taken from conventionally excised roots, it was found that the soil type had an influence on the latter assays.

Strain Identification

In many studies involving mixed cultures, it is imperative to use an identification technique of bacterial

strains such as serology, immunofluorescence, immunodiffusion, bacteriophage typing, antibiotic-resistance, plasmid profiling, or genomic restriction analysis before and/or after experiments have been performed.

Antibiotic Resistance

To study rhizobial competitiveness and effectiveness, bacteria must infect the plant and be competitive with other strains, and subsequently be recovered. Antibiotic resistance is one method that enables the recovery of the introduced strain from a sterile or non-sterile environment. The use of bacterial strains resistant to high levels of antibiotics to study rhizobial ecology was demonstrated by Danso et al. (1973), Schwinghamer and Dudman (1973), and Pankhurst (1977). The strains usually were added to soil, or other matrices, and then recovered in a selective medium containing a high dosage of antibiotics.

The performance of antibiotic-resistant mutants of rhizobia has been debated. Antoun (1982) questioned the reliability of genetically marked, resistant mutants for symbiotic properties when used to study rhizobial ecology. Schwinghamer and Dudman (1973) reported no change in the N_2-fixation effectiveness of antibiotic-resistant mutants of Rhizobium when compared with their parental strains. Several other workers have reported loss of symbiotic effectiveness of mutant antibiotic-resistant strains (Zelazna-Kowalska, 1971; Pankhurst, 1977; Bromfield and Jones, 1980).

The competitiveness of antibiotic-resistant mutants also has been evaluated. Bromfield and Jones (1979) reported that resistant mutants of R. trifolii were less competitive and formed fewer nodules than did the wild type. Turco et al. (1986), in a study of doubly-marked antibiotic-resistant mutants of B. japonicum and R. leguminosarum, found that 93% of the mutants were less competitive in nodule formation than the parental strains. Jones and Bromfield (1978) stated that large-scale selection procedures gave a better chance of isolating mutants identical to the wild type in their symbiotic properties.

The rhizobia, in a natural ecosystem, are usually resistant to low levels of antibiotics, and slow-growing rhizobia generally exhibit more resistance than the fast growers (Graham, 1962). Schwinghamer and Dudman (1973) reported that the frequency of isolation of rhizobial mutants, resistant to 250 μg/mL of streptomycin, was very low, but that this intrinsic resistance was genetically encoded and was a stable trait in four B. japonicum strains tested. Streptomycin is the most frequently used antibiotic in selective media. Rhizobia, and bradyrhizobia in particular, are resistant to high levels of this antibiotic. Streptomycin, unlike other antibiotics, can be used in ecological studies involving rhizobia in either the field or laboratory situations (Schwinghamer, 1963). Many other antibiotics are not suitable for rhizobial studies because

loss of symbiotic properties was associated with use of high dosages. This loss was a result of mutational resistance to particular antibiotics (Schwinghamer, 1963).

Serology

One of the most-used methods in identification of bacterial strains is serology. Many variations of serological techniques exist, e.g., agglutination, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA).

The agglutination method is the reaction of antibodies present in the antiserum with cell-surface antigenic determinants of the corresponding bacteria. The reaction can usually be observed as a white precipitant on a clear surface. This procedure, although less sensitive than other immunological techniques, can differentiate strains or serogroups (Read, 1953). Chan et al. (1988) used serological agglutination to differentiate 11 Bradyrhizobium species isolated from Astragalus sinicus (Chinese milkvetch) in China and Japan. Two subgroups were observed among the 11 effective isolates.

The immunofluorescence technique is a similar procedure to agglutination. The antibodies have a fluorescent dye attached, which can be seen directly with aid of an ultraviolet microscope. Isolation and enumeration of B. japonicum in the soil environment became possible by using this technique (Bohlool and Schmidt, 1968, 1970, 1973; Kosslak

et al., 1983), but cross reactions occur frequently with other strains.

The ELISA method, which is the most accurate and recent of the three procedures, is the conjugation of a soluble antigen or antibody attached to an insoluble solid phase. Asanuma et al. (1985) used ELISA to identify rhizobial species within the 'cowpea' group, and detected B. japonicum in culture and from root nodules in the field. This method also was used to evaluate the competitive ability of R. meliloti in Canadian fields (Rice et al., 1984) and to characterize inter-strain variability among R. meliloti isolates (Olsen and Rice, 1984).

Bacteriophage Typing

Even though serology has been commonly used to identify various strains of rhizobia in legume-root nodules, bacteriophages exhibit greater sensitivity in explaining diversity among the indigenous rhizobial populations (Bromfield et al., 1986). Thurman and Bromfield (1988) used bacteriophage typing and examined diversity among the indigenous R. meliloti as affected by legume host. Lesley (1982) found extensive variation among the R. meliloti field isolates when he used bacteriophage typing. He suggested that the usefulness of the system was due to good reproducibility. Kowalski et al. (1974) studied rhizobial diversity within the nodules of field-grown soybean and used bacteriophage typing to explore various occupants of root nodules and compared the

typing with serological techniques. Bacteriophage typing is quick and economical, but not all isolates show sensitivity to bacteriophages, and some may be infected by a number of bacteriophage particles, producing similar patterns.

Genetic Techniques

Genetic tools recently have been exploited to determine diversity, as well as to identify rhizobial species. Methods such as serotyping, bacteriophage typing, and antibiotic resistance were neither definite nor very sensitive for characterization of rhizobia isolated from the soil environment. Therefore, bacterial genetic techniques, which were largely developed on enterobacteria, recently have been applied to agricultural and specifically to rhizobial technology.

Extrachromosomal DNA (plasmid) profiling, less sensitive than chromosomal DNA restriction patterns (Hartmann and Amarger, 1991), has frequently been used to characterize plasmid-bearing rhizobial strains. Although plasmids had been found previously in all species in the Rhizobiaceae family, Nuti et al. (1977) were first to report the presence of megaplasmids in different Rhizobium species. Prakash et al. (1982) used restriction endonucleases to distinguish the R. leguminosarum symbiotic plasmid pRle1001a. Earlier, they had differentiated this plasmid from the homologous sym plasmid of R. trifolii (Prakash et al., 1981). Other workers have used plasmid profiling, in conjunction with intrinsic antibiotic

resistance, to identify indigenous R. meliloti strains (Shishido and Pepper, 1990). Plasmid analysis of B. japonicum serogroup 135 isolates from 15 alkaline soils in Nebraska established four different subgroupings of these extra-slow-growing strains (Gross et al., 1979). Their effectivity along with plasmid profiles were used to explore the potential of an indigenous strain as an inoculant in high-pH soils. Hartman and Amarger (1991) found 24 distinct plasmid-profile patterns when they examined 125 R. meliloti isolates from field-grown alfalfa in France. Subgroupings of isolates were based on the number (one to five) and the size (45 to >600 kb) of their plasmids.

Ecological studies of bradyrhizobia also have involved plasmid typing (Nuti et al., 1977; Gross et al., 1979). But not all Bradyrhizobium studied in general, and B. japonicum strain USDA 123 and its member isolates in particular, carry plasmid(s). The inability to detect and isolate plasmids from USDA 123 led to a more definite method of strain characterization, mainly direct chromosomal fingerprinting. Sobral et al. (1990) analyzed 11 B. japonicum serocluster 123 field isolates by field inversion gel electrophoresis to determine genotypic diversity among the isolates. Rare DNA-cutting endonucleases were used for genomic digestion, where large pieces of DNA provided the restriction bands (fingerprints) of the bacterial genome. Recently, genomic restriction digestion patterns have been used in addition to

plasmid profiling and insertion-sequence (IS) typing, to characterize R. meliloti field isolates (Hartmann and Amarger, 1991). IS typing as a new genetic method in rhizobial ecological studies was based on the description of an IS element in R. meliloti strain RCR2011 (ISRm1) (Ruvkun et al., 1982). Wheatcroft and Watson (1988) described the usefulness of this method based on the number and the locations of IS elements existing in the genomes of R. meliloti isolates. Hartmann and Amarger (1991) showed that IS typing was more distinctive than plasmid profiling among R. meliloti field isolates. Widespread application of this new technique depends on the detection of these elements in genomes of various species of rhizobia and the engineering of corresponding probes suitable for hybridization. The sites and the frequency of transposition may provide yet other means to study interstrain diversity.

PAPER 1.

NATIVE BACTERIOPHAGES INFECTIVE TO *BRADYRHIZOBIUM JAPONICUM* SEROCLUSTER 123 FROM IOWA SOILS

NATIVE BACTERIOPHAGES INFECTIVE TO *BRADYRHIZOBIUM JAPONICUM* SEROCLUSTER 123 FROM IOWA SOILS

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ABSTRACT

Members of Bradyrhizobium japonicum serocluster 123 native to Iowa are highly competitive in forming nodules on soybean [Glycine max (L.) Merr.] and have been reported in recent studies to be heterogenous. The methods to determine heterogeneity, however, are time consuming. The objective of this study was to establish a quick method by using virulent phages to distinguish a particular isolate from other isolates within this diverse population. Twenty-one phage strains were isolated by an enrichment method against 37 B. japonicum serocluster 123 Iowa isolates and four USDA reference strains: 122, 123, 127, and 129. A phage-typing pattern was constructed based on the lytic activity of virulent phages against the bradyrhizobia tested, where each bacterium was identified based upon its sensitivity to the phages. From the 41 bradyrhizobia tested, nine did not react with any phages, 14 reacted with one phage, seven reacted with two phages, and 11 had multiple receptors for more than two phages. Transmission electron micrographs of randomly chosen phages showed similar morphology to Escherichia coli phage T_4 (Bradley's "morphological group A"), and all phages had similar sizes. Phage DNA endonuclease-restriction patterns revealed genotypic variation among phages even though no morphological differences were observed. Comparison of the digested phage DNA bands with Lambda/Hind III showed phage genomes to be approximately 50 kilobase pairs (kb) in length.

INTRODUCTION

Bacteriophages specific to rhizobial species (rhizobiophages) have been detected in soils with a history of legume cultivation (Vincent, 1970). Kowalski et al. (1974) isolated phages from rhizosphere soil and nodules of Iowa field-grown soybean and examined the relationships between the phages and bradyrhizobial hosts. The presence of phages in soil has been reported to decrease rhizobial populations, or to increase the proportion of ineffective strains in existing populations (Evans et al., 1979a, 1979b). Nutman (1946) also reported increases in the population of ineffective strains when rhizobia were incubated in soil for long periods of time, but effective strains did not form from ineffective strains with long incubation. Phage selection for effectivity among members of B. japonicum serocluster 123 has not been studied.

Several techniques have been used to established diversity among members of serocluster 123. Berg et al. (1988) surveyed soils across Iowa for native bradyrhizobia and found isolates belonging to serocluster 123 were the predominant strains occupying soybean nodules (68% occupancy). A greenhouse study showed an existing variability of isolates in their competitiveness and N_2 fixation. Other phenotypic variations also were observed by Hickey et al. (1987), when sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein was used to examine diversity among 78 B. japonicum serocluster 123 isolates from a 22.8-m^2,

intensively sampled area in central Iowa, and 98 isolates from other locations within the state. Protein profiling revealed 74 identifiable patterns among the 176 tested isolates, and only 18% of the isolates belonged to the pattern represented by USDA 123. Diversity was as great among isolates from within an intensively sampled area as across areas.

Genetic diversity among members of serocluster 123 from midwestern states also has been shown. Sadowsky et al. (1987) studied genotype-specific nodulation of soybean and found different nodulation patterns among the 20 isolates examined. Sobral et al. (1990) evaluated the intact genomes of 11 B. japonicum 123 isolates by using Field Inversion Gel Electrophoresis (FIGE). Although these techniques showed that differences existed, the methods used were laborious.

Phage-typing may be a viable alternative for rapid differentiation among serocluster 123 members. Bromfield et al. (1986) used this procedure to determine the composition of indigenous Rhizobium meliloti strains in two field sites. Fifty-five and 65 unique phage types, respectively, were observed when 1920 nodule isolates were typed. Lesley (1982) earlier had used phage-typing patterns to differentiate among R. meliloti field strains. Kowalski et al. (1974) related phage types to the serological classification of B. japonicum strains. From 51 phage isolates, 45 showed lytic activities against serologically related rhizobial strains and the

distribution of rhizobia and their specific phages were pH dependent.

In this study, we used phage specificity to identify and characterize Iowa field isolates of B. japonicum serocluster 123. We also studied the morphology and genetic diversity of native phages.

MATERIALS AND METHODS

Phage Isolation

Five soil samples were collected from Iowa fields where soybean previously had been grown (Table 1). The soils were placed in plastic bags, transported to the laboratory, stored at 4 °C, and equal volumes were mixed together just before enrichment. The enrichment procedures of Somasegaran and Hoben (1985) were used to isolate phages. Thirty-seven native B. japonicum 123 isolates collected from many diverse soils across Iowa (Berg, 1987) were cultured in 100 mL of Yeast Sucrose Phage Broth (YSPB) to late log growth (five to seven days). Cultures were then inoculated with 1 g of the soil mixture and incubated for 24-48 hours on a rotary shaker at 30 °C.

Table 1. Soils used to isolate phages in the enrichment technique.

Soil series	County	Surface texture	pH[a]
Dinsdale	Butler	Silty Clay Loam	6.6
Ladoga	Warren	Silty Clay Loam	6.6
Nicollet	Boone	Clay Loam	6.6
Nicollet	Story	Clay Loam	6.8
Webster	Boone	Clay Loam	6.1

[a]1:1 ratio of soil to water used to determine the pH.

The enriched bacterial cultures were vacuum filtered to remove soil particles. Ten mL of filtrate was serially

transferred to new log-growth cultures of bradyrhizobia, incubated 24-48 hours, and cells were removed by centrifugation at 12,000 G for 15 min. This was repeated two more times to obtain a relatively pure phage stock culture of about 10^6-10^8 plaque-forming units/mL (PFU/mL). When a clear lysate was obtained, the supernatant was filtered through a 0.22-μm Millipore membrane. Each phage stock culture received 0.5-1.0 mL of chloroform and was stored in sterile bottles at 4 °C.

Phage Typing

The double layer agar method (Adams, 1959) was used to score for phage susceptibility of bacterial isolates. Twenty-five milliliters of Yeast Extract Mannitol Agar (YEMA) (1.5% Difco agar) was poured into square phage-typing petri plates (each plated divided into 16 equal areas), and the plates were left to dry overnight. Yeast Sucrose Phage Agar (YSPA) was prepared with 0.7% Difco Agar (soft agar) and kept at approximately 50 °C. Two mL of bacterial broth, late-log growth phase, was added to the soft agar and mixed thoroughly by using a vortex mixer and poured on the solidified YEMA plates. After 15 min, plates were inverted and incubated at 25-30 °C for two-to-four days until a turbid and uniform lawn of bacteria was observed. Next, 0.1-0.2 mL of each phage stock culture was added by capillary pipette over each of the 16 areas of the plate, and the plates were incubated for 24-48 hours. Cleared zones (plaques) formed by the phages indicated

a positive response. Routine Test Dilution (RTD) (Adams, 1959) was not performed on the phage stock cultures because the original titers were not high after purification (10^6-10^8 PFU/mL). The occurrence of multiple infectivity of one phage culture over a number of bacterial isolates was infrequently observed. Reproducibility of the host-range patterns was examined by replicating the double layer agar plates four or more times.

Electron Microscopy

High-titer phage suspensions (> 10^{10}) were prepared by ultracentrifugation of the stock culture at 30,000 G for 2 hours, and the pellet was then resuspended in 10 mg/L ammonium acetate (Kowalski et al., 1974). The negative-stain solutions used were 12 mg/L potassium phosphotungstate (pH=7.0) or 12 mg/L uranyl acetate (pH=4.5) (Barnet, 1972), with bovine serum albumin (0.5 mg/L) as the wetting agent. A 1:1 mixture of phage suspension-to-staining reagent was prepared, and a drop was placed on a 400-mesh carbon-coated formvar copper grid. Vacuum drying prevented crystallization of the reagents (quick fixed). Grids were examined at 100 kV on a CRX 100 Jewel Transmission Electron Microscope (TEM). The size of the phage particles was estimated by averaging at least two different micrographs.

Phage Genomic Analysis

Four 1-L flasks containing 500 mL of YSPB were prepared for each phage strain. Exponentially growing bacteria were

inoculated with the corresponding phage, and incubated on a rotary shaker until a clear lysate appeared in each flask (24-48 hours). Bacterial components were removed by low-speed centrifugation (8000 G). Phage precipitation by using polyethyleneglycol (PEG) 8000 was done according to the procedure by Maniatis et al. (1982). Resuspended particles were spun at 40,000 G for 24 hours in a CsCl density gradient to obtain highly purified and concentrated phage lysate. Phage-coat disruption, DNA isolation, and DNA restriction were done according to Maniatis's procedures devised for phage lambda of E. coli bacteria. Proteinase K, DNAse I, and restriction endonucleases Bam HI, Eco RI, Hind III, Kpn I, Pst I, and Sma I were obtained from the Bethesda Research Laboratory (BRL, Gaithersburg, MD). Digested phage DNA electrophoresis was done on 0.6% agarose gel containing 10 μL of 5 mg/mL ethidium bromide (EtBr) per 100 mL of agarose. The gel slabs were photographed under UV light after exposure to 16 hours of 2 V/cm high-voltage current.

RESULTS AND DISCUSSION

Phage Typing

Twenty-one phages were obtained from the composite soil and named according to the bacterial hosts against which they were isolated. The phages were isolated from different soils than were the bradyrhizobia to reduced the frequency of isolating polyvalent phages lytic to a large number of bacterial isolates. Lysogenic reactions of bradyrhizobia with phages were occasionally observed, but only confluent growth of phages in double layer agar plates was reported. Bacterial host-controlled modifications were minimized by using the original host for reisolation of the phage stocks (Luria and Human, 1959). Host-induced modifications (altering phage susceptibility) and phage-induced host modifications (changing bacterial recognition sites) is always a concern and likely is essential for survival of both organisms in the soil environment (Kleczkowska, 1950; Gupta and Kleczkowska, 1962).

Phage-sensitivity patterns were recorded by typing each bacterial isolate against all 21 phages; bacteria reacted with zero to seven phages (Table 2). A positive reaction was recorded if confluent lysis was detected in three or more of the replicated plates. Of the 41 bradyrhizobia tested, nine did not react with any phages, 14 reacted with only one phage, seven reacted with two phages, and the remainder reacted with more than two phages. This reactivity confirms the variable nature of native B. japonicum 123 isolates and the specificity

Table 2. Phage-sensitivity patterns of members of *B. japonicum* serocluster 123.

Phage[a] strain	Bacterial isolate[b]						
	H1.4	H1.5	H1.9	H1.10	H1.11	H1.12	H1.14
H1.4	+[c]						
H1.11					+		+
H1.12						+	
H1.19							
H1.20							
H2.6			+				
H2.13							
S1.1							
S2.2							
S2.4							
S2.10							
S2.11							
S3.5							
S3.6							
S4.9							
G1.6							
G5.2						+	
G6.1							+
G6.6							
G6.7							
G6.10							
Reactions	1	0	1	0	1	2	2

[a]Phage strains are designated by using the bacterial identification from which they were isolated.

[b]Bacterial isolates are designated by soil types and locations from which they were isolated.

[c]+ indicates confluent lysis only, blank indicates no confluent lysis when reacted with the identified phage strain.

Table 2. Phage-sensitivity patterns of members of B. japonicum serocluster 123 (continued).

Phage[a] strain	Bacterial isolate[b]						
	H1.19	H1.20	H2.2	H2.6	H2.11	H2.13	G1.2
H1.4							
H1.11							
H1.12							
H1.19	+						
H1.20		+					
H2.6				+			
H2.13						+	
S1.1							
S2.2							
S2.4				+			
S2.10							
S2.11				+			
S3.5							
S3.6							
S4.9							
G1.6			+				+
G5.2							
G6.1							
G6.6	+						
G6.7		+		+			
G6.10							
Reactions	2	2	1	4	0	1	1

Table 2. Phage-sensitivity patterns of members of B. japonicum serocluster 123 (continued).

Phage[a] strain	Bacterial isolate[b]						
	G1.6	G3.1	G3.2	G3.5	G5.2	G5.3	G6.1
H1.4							
H1.11							
H1.12	+						
H1.19							
H1.20							
H2.6							
H2.13							
S1.1							+
S2.2							
S2.4							
S2.10							
S2.11		+					
S3.5							+
S3.6							
S4.9							+
G1.6	+				+		
G5.2	+				+		
G6.1							+
G6.6							+
G6.7				+		+	+
G6.10							
Reactions	3	1	0	1	2	1	6

Table 2. Phage-sensitivity patterns of members of B. japonicum serocluster 123 (continued).

Phage[a] strain	Bacterial isolate[b]							
	G6.3	G6.4	G6.6	G6.7	G6.10	S1.1	S1.4	S2.2
H1.4								
H1.11								
H1.12								
H1.19								
H1.20								
H2.6				+				
H2.13								
S1.1						+		
S2.2								+
S2.4								
S2.10								+
S2.11								
S3.5			+			+		
S3.6								
S4.9						+		
G1.6								
G5.2								
G6.1						+		
G6.6			+			+		
G6.7			+	+			+	
G6.10					+			
Reactions	0	0	3	2	1	5	1	2

Table 2. Phage-sensitivity patterns of members of *B. japonicum* serocluster 123 (continued).

Phage[a] strain	Bacterial isolate[b]						
	S2.4	S2.6	S2.10	S2.11	S3.5	S3.6	S4.6
H1.4							
H1.11							
H1.12							
H1.19							
H1.20							
H2.6	+			+			
H2.13							
S1.1					+		
S2.2			+				
S2.4	+			+			
S2.10			+				
S2.11	+	+		+			
S3.5					+		
S3.6		+				+	
S4.9					+		
G1.6							
G5.2							
G6.1					+		
G6.6					+		
G6.7		+				+	+
G6.10							
Reactions	3	3	2	3	5	2	1

Table 2. Phage-sensitivity patterns of members of B. japonicum serocluster 123 (continued).

Phage[a] strain	Bacterial isolate[b]					
	S4.8	S4.9	USDA 122	USDA 123	USDA 127	USDA 129
H1.4						
H1.11						
H1.12						
H1.19						
H1.20						
H2.6						
H2.13						
S1.1				+		+
S2.2	+					
S2.4						
S2.10						
S2.11						
S3.5		+		+		+
S3.6						
S4.9		+		+		+
G1.6						
G5.2						
G6.1		+		+		+
G6.6		+		+		+
G6.7		+				
G6.10						
Reactions	1	5	0	5	0	5

of the phages. Hosts designated S3.5 and S1.1 were lysed by seven and six phages, respectively, and five additional bacterial isolates reacted with five phages. Although Kowalski et al. (1974) reported the isolation of phages specific for B. japonicum serocluster 123 and other serologically related strains, we did not isolate virulent phages against USDA strains 122, 123, 127, and 129. Five phages specific for native B. japonicum 123 members, however, effectively lysed USDA 123 and 129. No lytic activity was observed for strains USDA 122 and 127 against any of the phages tested. This finding suggests a relatedness of USDA 123 and 129 with native isolates, and also supports the conclusion of Schmidt et al. (1986) that USDA 123 and 129 are more closely related to each other than are USDA 122 and 127 within the 123 serocluster.

All phages reacted positively with the bacteria from which they were isolated, and bacterial host H1.11 showed lysogenic reactions with most of the phages tested (data not shown). The phage designated S2.11 behaved as a temperate phage and lysogenized most bacterial isolates it infected (data not shown). Twelve out of 21 phages reacted with at least three or more bacterial isolates (polyvalent phages) and phage G6.7 lysed 13 different isolates. Of the remaining phages, three lysed only two isolates, and six lysed only one.

Phage Morphology

Transmission electron micrographs were obtained randomly from over 50% of the negatively stained (uranyl acetate) phage stock cultures. Potassium phosphotungstate solution had a highly disruptive effect on phage particles and the procedure was eliminated. We do not know whether this was an artifact in specimen preparation but this phenomenon also was reported for certain R. trifolii phages found in New Zealand soils (Patel and Craig, 1984).

All micrographs revealed phages with similar size and morphology, including an icosahedral head and a conventional contractile tail, thus, they belonged to Bradley's "Morphological Group A" phages (Bradley, 1967) (Fig. 1a). Unusual fibrous appendages of the tail end were observed on several micrographs (Fig. 1b); this also was previously reported by Barnet (1972) on the non-contractile tail of phage NT3 of R. trifolii. Base plates and spikes, whose functions presumably are attachment and stabilization of the phages during infection, were not observed in our micrographs. Perhaps the fibrous appendages in phages served the same functions as the base plate and spikes. Tails were approximately 110 nm long and 17.5 nm wide, whereas heads were 75 nm long and 75 nm wide.

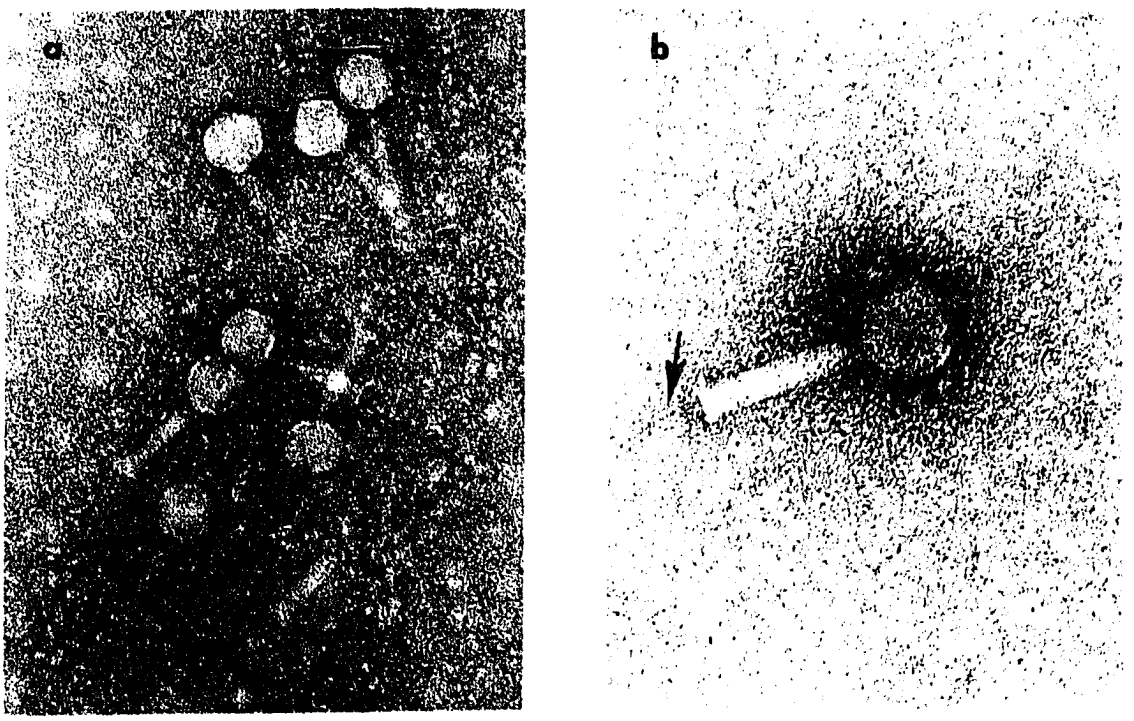

Figure 1. Electron micrographs of uranyl acetate negatively stained phage stock.

a. Phage G6.1 stock culture representing the phage morphotypes examined. Magnification 140,000x.

b. Phage H1.11 stock culture showing unique fibrous appendages. Magnification 200,000x.

Phage Genomic Analysis

Because morphological differences were not detected by TEM among the particles examined, we next evaluated phage genetic compositions. We were only partially successful in using CsCl density gradients (Maniatis et al., 1982) to concentrate and purify phages. Only eight intact phages were purified by CsCl, the rest were severely disrupted. The DNAs were isolated and digested by restriction endonucleases. Of the six restriction endonucleases evaluated, Eco RI and Kpn I gave the best contrasts among the gel patterns. Differences in restriction DNA patterns among those samples with the best resolution (G6.1, S2.2, S2.10, and S4.9) (Fig. 2) verified that phages were not identical, although morphologically they belonged to the same phage group. The genomic size of the phages determined by using Hind III Lambda digestion as a size standard was approximately 50 kb.

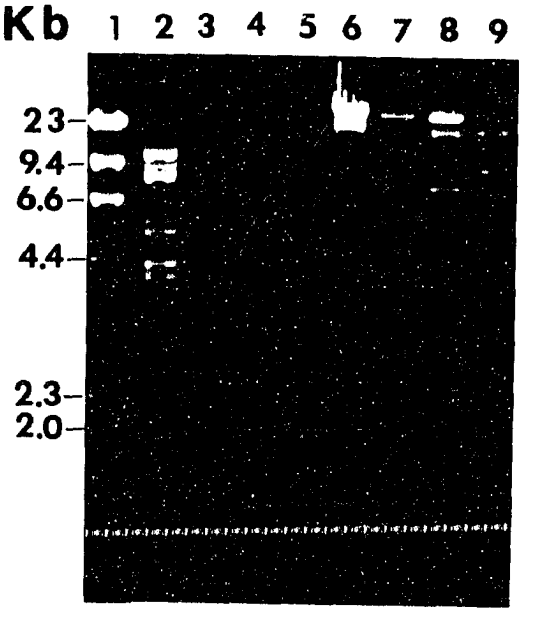

Figure 2. Agarose gel electrophoresis of phage DNAs G6.1, S2.2, S2.10, S4.9. Lane 1 is Lambda DNA digested with Hind III. Lanes 2, 3, 4, and 5 are Eco RI digestions; Lanes 6, 7, 8, and 9 are Kpn I digestions.

SUMMARY

Unique phage-sensitivity patterns among native 123 bacterial isolates suggest that this technique is useful as a relatively quick method to distinguish specific isolates within a diverse population. Phages reacting with few bacterial isolates and especially those posessing monvalent properties (reacting only with one isolate), may be used as markers for quick identification, tracing, and determination of the percentage of isolates in nature and in laboratory settings. Even though morphologies were similar, genetic analyses of DNA confirmed the differences among phages observed with the sensitivity patterns.

A method that quickly identifies diversity within a native population may be useful to evaluate potential responses to inoculation. For example, suppose that phage-sensitivity patterns suggest that a soil has primarily strains known to be ineffective in N_2 fixation. A farmer may be willing to invest in a "heavy inoculation" to displace the native organisms if the potential for a positive response is great. Before this becomes useful, correlations between phage-sensitivity patterns verses N_2 fixation, and soil competiveness of native bradyrhizobia are needed. The potential is to develop a biological soil test to determine the likelihood of a positive inoculation response.

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PAPER 2.

INTERSTRAIN COMPETITION AND N_2-FIXATION EVALUATION AMONG *BRADYRHIZOBIUM JAPONICUM* SEROCLUSTER 123 MEMBER ISOLATES

INTERSTRAIN COMPETITION AND N_2-FIXATION EVALUATION AMONG *BRADYRHIZOBIUM JAPONICUM* SEROCLUSTER 123 MEMBER ISOLATES

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ABSTRACT

The predominant native bacteria nodulating soybean [Glycine max (L.) Merr.] in soils of midwestern United States belong to Bradyrhizobium japonicum serocluster 123. Members of this heterogenous serocluster occupy 60-80% of the nodules of field-grown soybean, and are very competitive against more effective strains introduced as inoculum into these soils. Our objectives were to study interstrain diversity among the serocluster members and to identify isolates that retained the competitive traits unique to this serocluster but possess an effective N_2-fixation capacity similar to strain USDA 110, a common strain used in commercial inoculants. A screening study evaluated the N_2-fixation abilities of 37 native B. japonicum serocluster 123 member isolates collected from Iowa soybean fields. Eight isolates (four having poor fixation and four having good fixation) were selected for further study. We evaluated their effectiveness in the greenhouse on two cultivars ('Williams 82', 'Corsoy 79') against the standard strains USDA 110 and USDA 123. Test strains reacted differently on the two cultivars. Furthermore, strain S2.6 was found to be a competitive strain when challenged with an antibiotically-marked strain (I110 ARS) on both cultivars (56.3% nodule occupancy on Corsoy 79 and 50% on Williams 82). This strain, however, tended to produce low yields on Williams 82 in three of the four experiments. All experiments showed highly significant strain-by-cultivar interactions, possibly

indicating the heterogeneous nature of members of the native serocluster 123. There was no correlation found between bacteriophage susceptibility and either effectivity or competitiveness. The data presented suggest potential for the selection of a native serocluster 123 isolate as a possible commercial inoculum.

INTRODUCTION

Higher yield of the economically significant legume soybean (Glycine max. [L.] Merr.) in midwestern states may be dependent upon effective N_2 fixation from its bacterial endosymbiont, Bradyrhizobium japonicum. Members of the indigenous B. japonicum serocluster 123 are known to be highly competitive, occupying nearly 60-80% of the field-grown soybean nodules, but are thought, based on studies with USDA 123, to be less effective N_2 fixers when compared with other strains of B. japonicum (Berg et al., 1988; Ham, 1980). The introduction and establishment of more effective strains of B. japonicum (i.e., USDA 110) into midwestern soils have been unsuccessful due to their inability to compete against the native serocluster 123 members (Ham, 1980). Moawad et al. (1984) demonstrated that the majority of soybean nodules were occupied by members of this serocluster although an equal number of bacterial cells from others strains were present in the soil.

In recent years, other alternatives have been sought to displace ineffective native 123 strains. Cregan and Keyser (1986) first suggested screening soybean plant introductions (PI) for genotypes capable of restricting nodulation by certain B. japonicum serogroups. When two USDA 123-PI-restricting genotypes and the cultivar Williams 82 were inoculated with both USDA 123 and a more effective strain, nearly 80% of the nodules on Williams 82 were occupied by USDA

123, while less than 10% of the nodules on the PI genotypes were occupied by USDA 123. When the PI genotypes, however, were inoculated with 20 serocluster 123 members isolated from fields across the United States, nodulation was only partly inhibited and the PI genotypes were not effective in restricting all serocluster 123 members (Cregan and Keyser, 1988).

Other alternatives also have been examined. Fast-growing Rhizobium fredii were isolated from Glycine soja and unimproved soybean lines (Glycine max cv. Peking) and evaluated for competition against ineffective native strains for nodulation and N_2 fixation (Keyser et al., 1982). It was later shown that these bacteria were unable to successfully compete with most indigenous bradyrhizobial strains in forming an effective nodulation with improved North American soybean cultivars (Israel et al., 1986). McLoughlin et al. (1986) reported that, during the year of inoculation, only 50% of soybean nodules were occupied by R. fredii when competing against indigenous B. japonicum strains in midwestern soils, and during the second year, no nodules were occupied by the fast growers. They suggested that only R. fredii strain USDA 191 was able to effectively nodulate some of the improved soybean cultivars, but lacked competitiveness against members of serocluster 123.

Since Gibson et al. (1971) reported the existing diversity among the members of serocluster 123, many attempts

have been made to better understand this diversity. Phenotypic variation among members has been studied using serology (Schmidt et al., 1986) and protein profiling (Hickey et al., 1987). Berg et al. (1988) reported members of B. japonicum 123 were more competitive in forming nodules than members of other serogroups when introduced as inoculants into Iowa soils. Recently, Abdel Basit et al. (1991) reported phenotypic diversity among strains of B. japonicum belonging to serogroup 110. When soybean plants (Williams 82) were inoculated with these strains, differences in N_2 fixation and nodulation were observed. Additionally, variations in their hydrogenase activity and bacteriophage susceptibility reactions were reported.

Here, we evaluated the diversity for N_2-fixation effectivity and the diversity for competitiveness within native Iowa isolates belonging to serocluster 123. Identifying a competitive and effective native rhizobia may eliminate the need for finding, or engineering, superior strains able to compete against indigenous strains. Additionally, we evaluated whether members of serocluster 123 differing in competitive ability or N_2-fixation efficiency could be categorized based on their phage sensitivities.

MATERIALS AND METHODS

Greenhouse studies were designed to test native isolates of B. japonicum serocluster 123 collected from various Iowa soybean fields (Berg, 1987) and to make comparisons with USDA strains 110, 123, 127, and 129 (obtained from the USDA Agricultural Research Service, Beltsville, MD).

In a screening experiment, soybean seeds (Williams 82) were surface sterilized in 70% ethanol and 5% sodium hypochlorite for 5 min each, and washed five times with sterile water. Two-day-old pre-germinated seedlings were transferred to 0.9-L styrofoam containers filled with sterile vermiculite. Containers were replicated four times and arranged in a completely randomized design.

The seeds were inoculated 1 mL with cell cultures from 37 Iowa isolates grown to log 8 mL^{-1}. Capped glass tubes were inserted to allow nutrient application, and two cm of perlite was poured over the surface of the vermiculite to reduce contamination from the air. Bond's N-free nutrient solution (Vincent, 1970) was provided to the seedlings three times a week. After six weeks, plants were harvested, oven-dried, and weighed.

A second study of similar experimental design was used to determine the effectivity of the native isolates compared with USDA strains 110 and 123. In this study, eight B. japonicum isolates were selected from the preliminary study: four from plants having high dry weights (and approximating the fixation

efficiency of USDA 110) and four isolates from plants having low dry weights (and showing less effectiveness than their serotype representative, strain USDA 123). Styrofoam containers were filled with sterilized vermiculite and two pre-germinated and surface-sterilized seedlings were planted approximately 2 cm below the surface, and inoculated with 1 mL of cells grown to log 9 mL^{-1}. Controls received no inoculum. Pots were replicated five times in a completely randomized design. Soybean plants were harvested after six weeks, oven dried, and weighed.

To study the competitiveness of eight native isolates and strain USDA 123 against antibiotic-marked strain I110 ARS (kindly provided by Dr. van Berkum, USDA-ARS Beltsville, MD), a similar greenhouse study was arranged. Two-day old pre-germinated seedlings were planted in 0.9-L styrofoam containers filled with sterilized vermiculite, which were inoculated 1 mL with bacterial cells grown to log 9 mL^{-1}. Both test strains and I110 ARS (1 mL each) were added to each cup. Controls received no inoculation. Again, glass tubes with caps and perlite were used to supply nutrients and reduce contamination from the surroundings. Five replications were arranged in a completely randomized design.

Six-week-old plants were harvested, plant dry weights were determined, and nodules were counted. Sixteen nodules were randomly selected from each root, surface sterilized in successive 4-min washes of 5% sodium hypochlorite and 70%

ethanol, and washed three times in sterile water. Nodules were typed by using the microtiter-plate technique described by Lieberman et al. (1986). Yeast Mannitol Agar (YMA) plates contained 20 $\mu g\ mL^{-1}$ actidione as an antifungal agent and 1000 $\mu g\ mL^{-1}$ streptomycin sulfate (Sigma Chemical Company, St. Louis, MO). Nodule occupancy of each native isolate and USDA 123 was determined based on the recovery of antibiotic-resistant strain I110 ARS from the plates. No nodules were observed on the control plants.

To confirm that native strains belonged to serocluster 123, a simple agglutination test (Somasegaran and Hoben, 1985) was used.

Phage susceptibility was determined as earlier described (Aharchi and Loynachan, Paper 1).

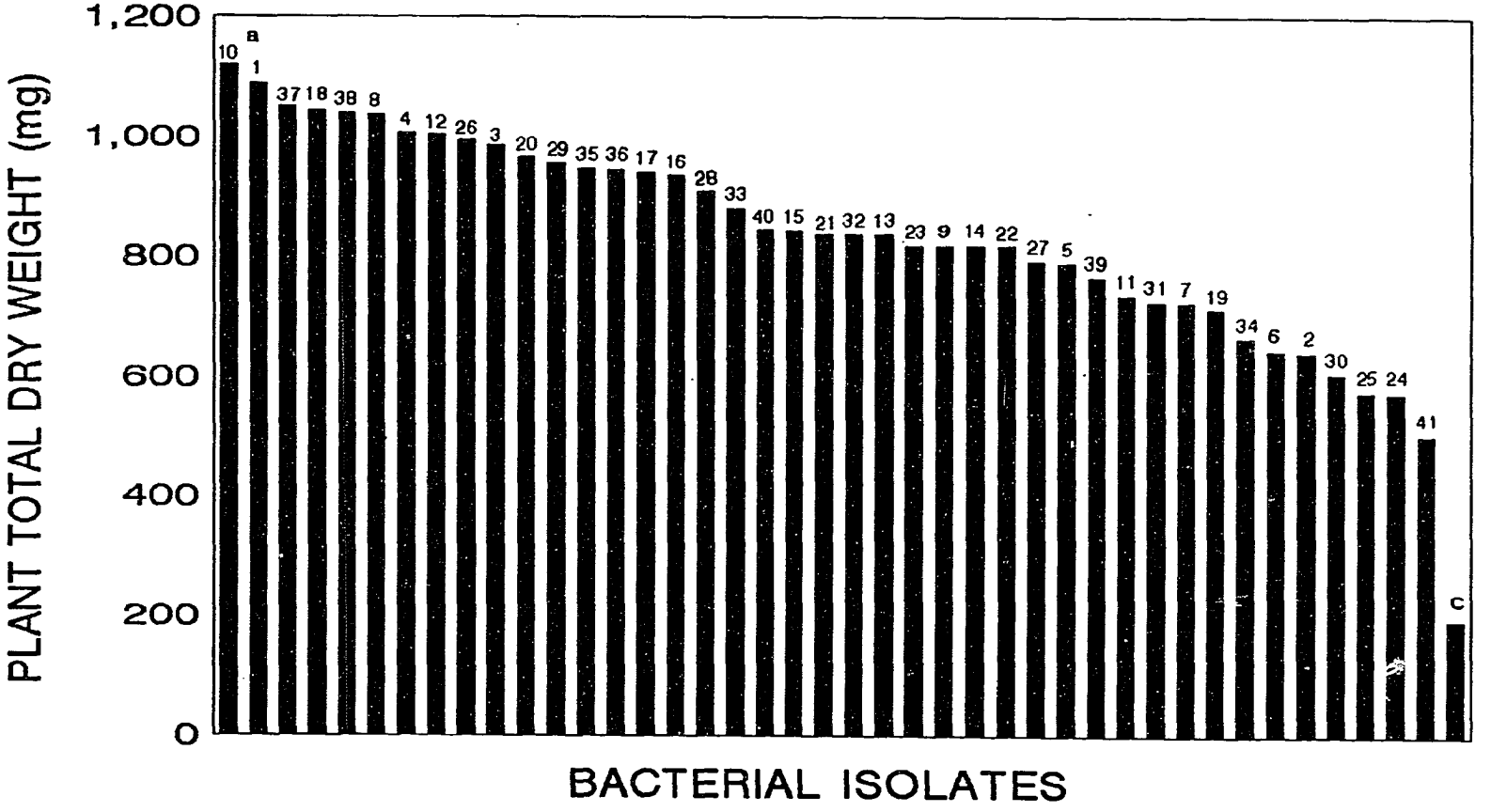
RESULTS AND DISCUSSION

Screening of Isolates

The screening of native B. japonicum serocluster 123 isolates, based on plant dry weights produced (Fig. 1), revealed that the isolates were not homogeneous and that symbiotic properties varied among them. The F-test for treatment effects showed statistical differences at the 0.0002 level. Duncan's multiple ranges tests were used to separate differences. All isolates between 10 and 19 were statistically the same. From the other direction of the histogram, all isolates between 41 and 28 were statistically the same. Therefore, as expected in a gradient of responses, many of the isolates tested were statistically the same, but isolates identified at the extremes of the histogram statistically varied.

Although isolate 38 (USDA 110) produced high plant-dry weights, as was expected, four 123 native isolates had greater absolute yields than this commonly used commercial strain. Strain USDA 123 (isolate 39) showed approximately 75% effectivity of USDA 110. Although all tested isolates did better than the non-inoculated control, strain USDA 129 (isolate 41) produced the poorest absolute growth of the inoculated isolates. These data suggest that members of serocluster 123 are diverse in N_2 fixation and they are not well represented by USDA 123 as their serotype strain. This agrees with the observation of Hickey et al. (1987), who

Figure 1. Plant dry-weight comparisons of 37 B. japonicum 123 native isolates and USDA strains 110, 123, 127, and 129.



[a]Numbers above each bar indicate bacterial isolate. Isolates 38, 39 40, and 41 indicate USDA strains 110, 123, 127, and 129,respectively. The non-inoculated control is represented by "C".

showed high variation in protein profiles within native isolates of serocluster 123. Because of the high variability found within serocluster 123 isolates, we increased the number of replications to five and cultivars to two and concentrated our studies on four isolates that ranked high in plant dry-weight production and four isolates that ranked low (Table 1). These strains were chosen as representative of different ends of the spectrum (Fig. 1). In further studies, we evaluated effectivity, competitiveness, and phage susceptibility.

Effectiveness Study

The effectiveness of the isolates presented in Table 1 was reevaluated using two soybean cultivars (Corsoy 79 and Williams 82) (Table 2). (These cultivars were suggested by Dr. Walter Fehr, Iowa State University, as being genetically diverse.) Inoculated treatments were nodulated and all produced significantly higher plant dry matter than did the controls. There were no significant differences among treatments within cultivars in the first trial. Because this was different than what was expected, we repeated the study a second time.

In the second trial, all treatments on both cultivars produced significantly higher plant dry weights than did non-inoculated controls. This suggested that N was indeed limiting the plant growth. On Williams 82, strains G6.4 and S2.6 produced less growth than six of the tested strains;

Table 1. B. japonicum serocluster 123 isolates selected for further study.

Isolate number[a]	Strain[b]	N_2-fixation property	Bacteriophage susceptibility[c]
1	G1.2	High	1
8	G6.1	High	6
10	G6.4	High	0
18	H1.11	High	1
6	G.52	Low	2
24	H2.11	Low	0
25	H2.13	Low	1
30	S2.6	Low	3

[a]Bacterial isolate identified in Fig. 1.

[b]Notation from Berg, 1987.

[c]Indicates number of lytic reactions of each isolate to an array of bacteriophages (Paper 1, this dissertation).

however, one of these strains was from the "high" producing group and one was from the "low" producing group in the screening study. Thus, the results expressed in Fig. 1 were not reproduced. No significant growth differences were observed with Corsoy 79 among any of the tested strains. Strain USDA 123 seemed to be as effective in N_2 fixation as strain USDA 110. Data in Table 2 showed strong interactions ($P = 0.001$) between strains and cultivars for the first and second trials.

We determined the number of virulent bacteriophages lytic to each of the test strains (Lesley et al., 1982) and found no

Table 2. Plant dry weights of two greenhouse-grown soybean cultivars in two subsequent trials inoculated with eight native B. japonicum serocluster 123 isolates and USDA strains 110 and 123.

	First trial		Second trial	
Strain	Corsoy 79	Williams 82	Corsoy 79	Williams 82
	Plant dry wt. (mg plant^{-1})			
G1.2	2108a[a]	2692a	1602ab	2010a
G6.1	2358a	2732a	1400ab	1998a
G6.4	2144a	2482a	1450ab	1592b
H1.11	2762a	2766a	1452ab	2112a
G5.2	2240a	2500a	1586ab	1780ab
H2.11	2280a	2692a	1780a	1904ab
H2.13	2432a	2570a	1660ab	2018a
S2.6	2610a	2648a	1840a	1564b
USDA 110	2524a	2794a	1676ab	2168a
USDA 123	2238a	2509a	1742ab	2170a
Control[b]	980b	1196b	926c	944c
		Interaction		
Str x Cul	***	***	***	***

***Significant at the 0.001 probability level.

[a]Each value is the mean of five replications. Means within a column not followed by the same letter differed significantly (P = 0.05) by Duncan's multiple range test.

[b]Control received no inoculation.

correlation with the yield data (Table 3). Lesley et al. (1982) reported the identification and characterization of 80 R. meliloti field isolates based on the development of bacteriophage-susceptibility patterns. The effect of bacteriophages on bacterial density and on changes in population ratios have been reported (Kleczkowska, 1950). Ineffective strains have been described to arise in soil with the introduction of their specific bacteriophages (Evans et al., 1979), suggesting an alteration in the effectivity of the rhizobial population. Susceptibility to a greater number of bacteriophages may reduce the symbiotic properties of a specific rhizobial strain. To our knowledge, this was the first attempt to determine a relationship between bacteriophage-susceptibility patterns and phenotypic responses on plants.

Table 3. Pearson correlation coefficients between effectivity[a] and bacteriophage susceptibility[b] of native B. japonicum serocluster 123 isolates.

Corsoy 79		Williams 82	
First trial[a]	Second trial	First trial	Second trial
0.20	-0.19	0.32	0.06

[a]Yield data based on plant dry weights (Table 2).

[b]Susceptibility to lytic bacteriophages given in Table 1.

Competition Study

In the competition study, we evaluated the competitiveness of the isolates and related this to N_2-fixation effectivities and bacteriophage susceptibilities. The native B. japonicum serocluster 123 isolates varied in nodule numbers and nodule occupancies (Table 4). Gibson et al. (1971) earlier had showed interstrain diversity among the native B. japonicum serogroup 123 by using serology, which later was confirmed by Hickey et al. (1987) by using protein profiles. On Corsoy 79, strain G1.2 produced an average of 66.6 nodules, whereas strain H2.13 only produced 48.6 nodules. Because the strains were challenged by a common strain (I110 ARS), the number of nodules formed may be a function of both strains. The percentage nodule occupancy was more variable. Strain G1.2 had 46.0% nodule occupancy, whereas the H2.13 strain had only 12.5% occupancy.

Strain S2.6 was found to be a very competitive strain, with the highest occupancy (Table 4) of any of the test strains on both cultivars (56.3% on Corsoy 79 and 50% on Williams 82), but tended to produce low yields (Table 5). This low yield production by S2.6 was observed in the screening study (Fig. 1), the second trial on Williams 82 (Table 2), and in this study, especially on Williams 82 (Table 5). These results suggest the competitive nature of S2.6, as well as the ineffectiveness of this strain.

Table 4. Nodule number and nodule occupancy of greenhouse-grown soybean inoculated with eight native *B. japonicum* serocluster 123 isolates and USDA strain 123 against I110 ARS.

	Cultivar			
	Corsoy 79		Williams 82	
Strain	Nod. no.	% Occ.	Nod. no.	% Occ.
G1.2	66.6a[a]	46.00ab	65.0ab	42.50a
G6.1	55.0ab	35.00abc	61.4ab	37.50a
G6.4	55.8ab	26.25bcd	61.6ab	43.75a
H1.11	53.2ab	26.25bcd	63.2ab	26.25ab
G5.2	51.8ab	34.38abc	56.0b	43.75a
H2.11	51.8ab	40.00abc	62.6ab	43.75a
H2.13	48.6b	12.50cd	61.2ab	10.00bc
S2.6	55.2ab	56.25a	66.6ab	50.00a
USDA 123	66.0ab	38.75abc	77.0a	45.00a
Control[b]	0.0c	0.00d	0.0c	0.00c
		Interaction		
Str x Cul	***	***	***	***

***Significant at the 0.001 probability level.

[a]Each value is the mean of five replications. Means within a column not followed by the same letter differed significantly (P = 0.05) by Duncan's multiple range test.

[b]Control received no inoculation.

Table 5. Plant dry weights of greenhouse-grown soybean inoculated with eight native B. japonicum serocluster 123 isolates and USDA strain 123 against I110 ARS.

	Cultivar	
Strain	Corsoy 79	Williams 82
	----------Plant dry wt. (mg plant^{-1})----------	
G1.2	928a[a]	1564a
G6.1	1002a	1384ab
G6.4	972a	1302a
H1.11	1106a	1366ab
G5.2	1060a	1450ab
H2.11	952a	1392ab
H2.13	1002a	1276b
S2.6	876a	1298b
USDA 123	888a	1430ab
Control[b]	396b	600c
	Interaction	
Str x Cul	***	***

***Significant at 0.001 probability level.

[a]Each value is the mean of five replications. Means within a column not followed by the same letter differed significantly (P = 0.05) by Duncan's multiple range test.

[b]Control received no inoculation.

Statistical correlation analysis between the competition data and percentage nodule occupancy revealed a significant (0.1 level) and negative relationship with Corsoy 79 (Table

6). This means that the treatments with high nodule occupancy tended to produce the lowest yield. In other words, when I110 ARS had a high nodule occupancy (and low occupancy by the test strain), yields were higher. This is consistent with the notion that I110 ARS is an effective N_2-fixing strain. The correlation between the competition data and percentage nodule occupancy with Williams 82 was not significant (Table 6). The significance on Corsoy 79 but not on Williams 82 suggests differences of cultivars on bacterial behaviors (significant interactions also shown in Tables 2, 4, and 5).

Table 6. Pearson correlation coefficients between competitiveness and bacteriophage susceptibility of native B. japonicum serocluster 123 isolates.

	Corsoy 79			Williams 82		
	Nod. no.[a]	% Occ.[a]	Yld[a]	Nod. no.	% Occ.	Yld
Ph. Sus.[a]	-0.01	0.26	-0.03	-0.03	0.11	-0.01
Nod. No.		0.51	-0.41		0.16	-0.07
% Occ			-0.65[b]			0.30

[a]Nod. No. = nodule numbers (Table 4); % Occ. = percentage nodule occupancy (Table 4); Yld = yield (Table 5); Ph. Sus. = phage susceptibility (Table 1).

[b]Significant at the 0.1 level

Highly significant stain-by-cultivar interactions, in addition to the general inconsistency over trials of our effectivity data, suggest the heterogenity that exists among these isolates. The sensitive nature of these isolates may also be reflected by minor changes in abiotic factors such as light, temperature and humidity, or by handling and nutrient preparation of cultures. Care must be taken when characterizing native isolates. High levels of variability existed among the isolates, and if this diversity is a dynamic process within the serocluster 123, other variants could be generated from our "pure" isolates, which may also differ in their symbiotic properties.

A significant strain-by-cultivar interaction clearly complicates studies involving different rhizobia and cultivars and clearly complicates selection of suitable rhizobia for use in commercial inoculates.

SUMMARY

The results from our study indicate that it may be possible to select effective and competitive native Bradyrhizobium japonicum serocluster 123 isolate(s) for use as inoculum for better yields of field-grown soybean, but the cultivars used may determine inoculant suitability. Strains varied in their competitiveness when challenged by a common test strain. Strain G1.2 had 46.0% nodule occupancy on Corsoy 79, whereas the H2.13 strain had only 12.5% occupancy. Strain S2.6 was a very competitive strain, with the highest occupancy of any of the test strains on both cultivars (56.3% on Corsoy 79 and 50% on Williams 82). This strain, however, tended to produce low yields. There was no correlation between bacteriophage susceptibility and either effectivity or competitiveness.

The highly significant strain-by-cultivar interactions and the inconsistency in effectivity and competition studies may reflect existing diversity within serocluster 123.

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SUMMARY AND CONCLUSIONS

We evaluated 37 native Bradyrhizobium japonicum serocluster 123 isolates from Iowa soils based on their bacteriophage susceptibility, N_2-fixation effectivity, and competitiveness.

In the first study, a bacteriophage-sensitivity pattern was developed discriminating among the isolates. An examination by transmission electron microscope revealed that all isolates had a similar morphology and belonged to the Escherichia coli T_4 (Bradley's Group 'A') bacteriophage classification. Endonuclease digestion patterns of bacteriophage genomic DNAs showed differences among them. The length of genomic DNAs was estimated at approximately 50kb, by comparison with a Lambda/Hind III digestion as a size standard.

In the second study, all isolates were tested in the greenhouse for their effectiveness on cultivar Williams 82. A spectrum of isolates from low to high effectivity was produced, based on plant dry weights. Four isolates from each end of the spectrum were selected for further study in the greenhouse on two cultivars, Corsoy 79 and Williams 82. The next two effectivity trials did not agree with the results obtained from the screening study. Highly significant strain-by-cultivar interactions, however, were observed on all trials, indicating the heterogeneous nature of serocluster

123. This heterogenity confirms the results of the bacteriophage sensitivity patterns.

A competition study also was conducted in the greenhouse using Corsoy 79 and Williams 82. The isolate designated S2.6 was found to be competitive in displacing I110 ARS in nodules of both cultivars (56.3% and 50.0% nodule occupancy on Corsoy 79 and Williams 82, respectively), but produced poor growth of soybean. The bacteriophage-sensitivity pattern of the eight isolates had no correlation with either effectivity or competition data.

Results from these studies suggest that it may be possible to identify a particular isolate within serocluster 123 to be used as a field inoculant that is competitive against other members of this serocluster and is effective in N_2 fixation. Care must be used, however, in matching the inoculant strain with the soybean cultivar used.

Results do not support the notion that the effectivity or competitiveness of native rhizobia in a specific soil can be determined by analyzing bacteriophage-sensitivity patterns. Unfortunately, this prevents the quick assessment of the native rhizobia, and prevents development of a "soil test" to evaluate the likelihood of a response to inoculation based on the effectivity and competitiveness of the rhizobia residing in a specific soil.

These studies clearly show that diversity in effectiveness and competitiveness exists within native Iowa

isolates. We recommend that a larger pool of isolates from serocluster 123 be evaluted, since more competitive and more effective N_2 fixers may be found than identfied in the present studies.

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APPENDIX

Table A.1a. Plant dry weight of greenhouse-grown soybean cultivar 'Williams 82' inoculated with Bradyrhizobium japonicum serocluster 123 and strains USDA 110, 123, 127, and 129.

Bacterial Isolates	R1[a]	R2	R3	R4
	----------grams plant^{-1}----------			
G1.2	0.99	1.00	1.07	1.30
G1.6	0.74	0.76	0.39	0.68
G3.1	0.96	1.13	0.98	0.88
G3.2	1.13	0.78	1.02	1.10
G3.5	1.00	0.49	0.56	1.11
G5.2	0.61	0.64	0.86	0.48
G5.3	0.77	0.99	0.41	0.73
G6.1	1.29	0.96	1.03	0.87
G6.3	0.74	1.02	0.77	0.75
G6.4	0.81	1.26	1.33	1.08
G6.6	0.36	0.41	1.10	1.08
G6.7	0.96	1.06	0.86	1.14
G6.10	0.66	0.97	0.69	1.03
H1.4	1.04	0.50	1.10	0.64
H1.5	1.00	0.85	1.02	0.51
H1.9	0.58	1.05	1.39	0.73
H1.10	0.98	1.11	0.77	0.90
H1.11	0.98	1.18	1.09	0.93
H1.12	0.60	0.86	0.68	0.72
H1.14	1.28	0.96	1.38	0.25
H1.20	0.59	0.97	1.06	0.73
H2.2	0.92	0.71	0.90	0.75
H2.6	0.78	1.02	0.72	0.76
H2.11	0.37	0.83	0.45	0.65
H2.13	0.34	0.22	0.77	0.98

[a]Replication.

Table A.1b. Plant dry weight of greenhouse-grown soybean cultivar 'Williams 82' inoculated with Bradyrhizobium japonicum serocluster 123 and strains USDA 110, 123, 127, and 129 (continued).

Bacterial Isolates	R1[a]	R2	R3	R4
	grams plant^{-1}			
S1.1	1.24	0.63	0.82	1.29
S1.4	0.76	0.98	1.00	0.43
S2.2	0.96	0.95	0.99	0.74
S2.4	0.96	1.05	1.00	0.82
S2.6	0.97	0.21	0.73	0.52
S2.10	0.65	1.12	0.49	0.65
S2.11	1.00	0.53	0.90	0.92
S3.5	0.46	1.01	0.91	1.15
S3.6	0.86	0.32	0.88	0.61
S4.6	1.08	1.05	0.65	1.01
S4.8	0.70	1.00	1.07	1.01
S4.9	0.81	1.25	1.42	0.72
USDA 110	1.32	0.84	0.97	1.03
USDA 123	0.80	0.66	0.90	0.71
USDA 127	1.44	0.68	0.58	0.69
USDA 129	0.64	0.52	0.35	----[b]
CONTROL	0.22	0.19	0.20	0.17

[a]Replication.

[b]Missing data.

Table A.2. Plant dry weights of two greenhouse-grown soybean cultivars in the first trial inoculated with eight native B. japonicum serocluster 123 isolates and USDA strains 110 and 123.

Bacterial Isolates	Williams 82					Corsoy 79				
	R1[a]	R2	R3	R4	R5	R1	R2	R3	R4	R5
	Plant dry weight (grams plant^{-1})									
G1.2	3.03	2.62	2.50	2.79	2.52	1.94	1.93	2.50	2.32	1.85
G6.1	2.71	2.70	2.35	2.93	2.97	3.02	1.82	2.14	2.06	2.75
G6.4	2.40	2.28	2.66	2.26	2.81	2.48	2.17	2.35	2.23	1.49
H1.11	2.72	3.47	2.77	2.32	2.55	2.25	3.23	2.87	3.22	2.24
G5.2	2.68	2.23	2.15	2.98	2.45	1.26	2.09	2.81	2.32	2.72
H2.11	2.68	2.23	3.10	2.86	2.59	2.18	2.35	2.07	2.11	2.69
H2.13	2.67	2.31	1.88	2.93	3.06	2.18	2.35	2.07	2.11	2.69
S2.6	3.08	2.22	2.96	2.64	2.34	2.44	2.23	2.20	2.85	3.33
I110 ARS	3.01	2.93	2.46	2.59	2.98	2.53	3.02	2.26	2.65	2.16
USDA 123	2.77	2.02	2.75	2.97	2.89	2.11	2.88	2.15	2.43	1.62
Control	1.78	0.52	1.62	1.42	0.64	0.71	2.06	0.45	0.96	0.72

[a]Replication.

Table A.3. Plant dry weights of two greenhouse-grown soybean cultivars in the second trial inoculated with eight native B. japonicum serocluster 123 isolates and USDA strains 110 and 123.

Bacterial Isolates	Williams 82					Corsoy 79				
	R1[a]	R2	R3	R4	R5	R1	R2	R3	R4	R5
	Plant dry weight (grams plant^{-1})									
G1.2	2.04	2.31	1.90	1.85	1.95	1.63	1.42	1.76	1.60	1.60
G6.1	2.21	2.35	2.02	1.20	2.21	1.42	1.45	1.50	1.33	1.30
S6.4	1.79	1.37	1.87	1.37	1.65	1.49	1.50	1.31	1.58	1.37
H1.11	2.28	1.96	1.88	2.08	2.36	1.30	1.41	1.15	1.67	1.73
G5.2	1.80	1.77	2.18	1.52	1.63	1.57	1.31	1.81	1.73	1.51
H2.11	1.78	2.28	1.97	1.43	2.06	1.65	2.03	1.66	2.13	1.73
H2.13	1.84	1.85	2.51	1.96	1.93	1.43	1.28	1.65	1.94	2.00
S2.6	1.69	1.59	1.43	1.75	1.36	1.68	2.09	1.60	1.81	1.02
I110 ARS	2.00	2.65	2.35	2.24	1.60	1.53	1.65	1.11	1.56	1.53
USDA 123	2.45	1.84	2.18	1.71	2.17	1.62	1.78	1.86	1.57	1.88
Control	0.63	1.39	1.01	0.74	0.95	1.06	0.53	0.84	0.87	1.33

[a]Replication.

Table A.4. Nodule numbers of two greenhouse-grown soybean cultivars inoculated with eight native B. japonicum serocluster 123 isolates and USDA strain 123 against I110 ARS.

Bacterial Isolates	Williams 82					Corsoy 79				
	R1[a]	R2	R3	R4	R5	R1	R2	R3	R4	R5
	Nodule numbers plant^{-1}									
G1.2	50	66	72	72	65	59	79	87	64	59
G6.1	45	90	46	38	88	73	40	60	32	70
G6.4	75	54	52	86	41	43	58	40	60	78
H1.11	61	94	64	49	48	30	80	56	52	48
G5.2	38	70	58	54	60	52	49	58	58	42
H2.11	59	61	57	64	72	43	55	62	40	59
H2.13	62	42	73	64	65	44	48	44	57	50
S2.6	74	65	72	66	56	44	48	57	42	85
I110 ARS	67	82	73	77	90	70	57	65	66	72
Control	0	0	0	0	0	0	0	0	0	0

[a]Replication.

Table A.5. Nodule occupancy rates of two greenhouse-grown soybean cultivars inoculated with eight native B. japonicum serocluster 123 isolates and USDA strain 123 against I110 ARS.

Bacterial Isolates	Williams 82					Corsoy 79				
	R1[a]	R2	R3	R4	R5	R1	R2	R3	R4	R5
	% Occupancy									
G1.2	31.3	75.0	37.5	31.8	37.5	50.0	81.3	37.5	56.3	----[b]
G6.1	25.0	93.8	37.5	18.8	12.5	31.3	25.0	43.8	12.5	62.5
G6.4	37.5	68.8	50.0	25.0	37.5	18.8	00.0	12.5	18.8	81.3
H1.11	31.3	31.3	31.3	6.3	31.3	12.5	18.8	50.0	18.8	31.2
G5.2	43.8	56.3	18.8	31.3	68.8	18.8	31.2	56.2	31.2	50.0
H2.11	68.8	37.5	56.2	00.0	6.2	37.5	31.2	50.0	31.2	50.0
H2.13	18.8	6.2	12.5	12.5	00.0	00.0	00.0	31.2	12.5	18.8
S2.6	75.0	25.0	56.2	56.2	37.5	37.5	25.0	62.5	68.8	87.5
I110 ARS	50.0	43.8	25.0	43.8	62.5	37.5	43.8	10.8	56.2	32.5
Control	0	0	0	0	0	0	0	0	0	0

[a]Replication.

[b]Missing data.

Table A.6. Plant dry weights of two greenhouse-grown soybean cultivars inoculated with eight native B. japonicum serocluster 123 isolates and USDA strain 123 against I110 ARS.

Bacterial Isolates	Williams 82					Corsoy 79				
	R1[a]	R2	R3	R4	R5	R1	R2	R3	R4	R5
	Plant dry weight (grams plant^{-1})									
G1.2	1.52	1.56	1.42	1.35	1.95	0.77	0.77	0.97	1.35	0.78
G6.1	1.28	1.39	1.57	1.43	1.25	0.70	1.18	0.88	1.10	1.15
G6.4	1.20	1.40	1.38	1.20	1.33	1.01	0.82	0.75	0.95	1.33
H1.11	1.34	1.25	1.15	1.49	1.60	0.77	1.27	1.21	0.92	1.36
G5.2	1.53	1.48	1.60	1.40	1.24	1.01	0.75	0.94	1.55	1.05
H2.11	1.38	1.45	1.45	1.22	1.46	1.01	0.82	0.78	0.90	1.25
H2.13	1.40	1.14	1.49	0.82	1.53	1.23	1.11	0.92	0.81	0.94
S2.6	1.59	1.22	1.30	1.15	1.23	1.06	0.70	0.72	0.96	0.94
USDA 123	1.45	1.54	1.42	1.39	1.35	1.33	0.76	0.76	0.81	0.78
Control	0.60	0.43	0.78	0.71	0.48	0.51	0.36	0.45	0.30	0.36

[a]Replication.