SURVIVAL AND PERSISTENCE OF GENETICALLY MODIFIED *SINORHIZOBIUM MELILOTI* STRAIN 104A14 IN SOIL

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FORMAT OF THESIS

This thesis is presented in combination of Applied Soil Ecology, Microbial Ecology, and formats outlined by the Oklahoma State University graduate college style manual. This format allows the independent chapters to be suitable for submission to scientific journals. A complete chapter contains an abstract, introduction, materials and methods, results, discussion, and reference section.

Chapter I

INTRODUCTION

In Symbiotic Nitrogen Fixation (SNF), the host plant provides the microorganism with reduced carbon (C) as an energy source and the microorganism use this energy to convert atmospheric dinitrogen (N_2) to ammonia. This is a process used by some microorganisms and plants to acquire atmospheric N for growth. It was estimated that SNF fixes one-third to half of the global N fixation (Wang and Martinez, 2000) that account for more than 65% of the N used in agriculture (Graham, 1998).

The most common SNF system encountered in agriculture is the legume-rhizobia symbiosis. It contributes more than 100 million metric tons of combined N per year to the global N supply (Graham, 1998). The best-studied examples of rhizobia are the soil bacteria in the genera of *Rhizobium, Sinorrhizobium, Mesorhizobium, Bradyrhizobium, Allorhizobium* and *Azorhizobium* (Martineez-Romerro and Caballero-Mellado, 1996). However, N-fixation rate varies among host, rhizobia species, and the environment. Some native soil rhizobia are not effective in N fixation (Segovia et al., 1991). Thus, inoculation of highly effective N-fixing rhizobia is a common practice in agricultural production. Strains of rhizobia have been grown under laboratory conditions and released into the environment since their role in inducing the formation of N-fixing root nodules was first demonstrated in 1888 (Hellriegel and Wilfarth, 1888). For example,

approximate 12-20 $X10^6$ acres of land are inoculated worldwide for soybean production each year (Catroux et al., 2001).

Therefore, effective N fixation requires successful survival and establishment of inoculated rhizobia in the soil environment. Meanwhile successful survival and establishment of inoculated rhizobia requires soil conditions that are conducive for rhizobia growth, including optimal nutritional supplies.

Phosphorus (P) is one of the nutrients that are crucial in legume-rhizobia symbiosis. It has been demonstrated that legume plants require high levels of P for optimal growth and performance of symbiosis (Israel, 1987). Virtually every aspect of the symbiosis is significantly enhanced by P addition (Pereira and Bliss, 1987). Unfortunately, little information is available on the role of P in relation to the establishment and function of the N symbiosis.

Creation of phosphatase-negative mutants of *Sinorhizobium meliloti* (Deng *et al.*, 1998) provided the opportunity to reveal the role of phosphatases and organic P in relation to legume-rhizobia symbiosis. It has been demonstrated that single mutation of each of the two acid phosphatases in *S. meliloti* did not affect symbiotic performance significantly regardless of P levels (Deng *et al.*, 1998). However, it is not clear whether these two enzymes are critical for P acquisition of rhizobia.

Of the extensive studies conducted to evaluate the ability of rhizobia to survive in soil, much of the effort was focused on factors such as rhizobium competition, soil pH, moisture, temperature, and predation (Ramirez and

Alexander, 1980; Soa-Afiana and Alezander, 1982; Moawad et al., 1984; Brockwell et al., 1991). Little information is available on effect of nutrient supply in survival of inoculated rhizobia in soil. Even less is known about P nutrition and the role of phosphatases on the survival and persistence of rhizobia in the soil environment.

In addition, many genetic improvements on rhizobium have made significant contributions to agricultural production (Schmidt and Robert, 1985; Esperanza M.R. and Rosenblueth M., 1990; Bosworth et al., 1994). Releases of engineered *Sinorhizobium meliloti* with an extra copy of both *nif*A and *dct*ABD (a regulatory N fixation gene and C_4-dicarboxylic acid transport gene, respectively) into soil have demonstrated increase of alfalfa yield by 12.9% (Bosworth et al., 1994). Unfortunately, genetic modification also raised concerns. Although genetically modified microorganisms (GMMs) have been developed for commercial pest control application, pollution abatement, and frost protection etc. (Keeler, 1988; Lindow, 1992; Lorenz M.G. and Wackernagel W. 1993), potentially, GMMs could alter balance of fundamental processes and exchange DNA with the indigenous microorganisms (Lorenz and Wackernagel, 1990; Smit *et al.*, 1991; Walter *et al.,* 1991; Crawford et al., 1993). The concerned processes include energy, carbon, and nutrient cycling. Gene exchange in the environment could be achieved via conjugation, transduction and/or transformation.

The benefit and risk related to the release of GMMs depend on their establishment in the environment. Attempts in application of GMMs failed due to

poor understanding on their survival and persistence in the environment (Stotzky and Babich, 1984; Hirsch and Spokes, 1994). Leser et al., (1995) reported that the introduced genetically engineered *Pseudompnas* sp. strain B13 (FR1) decreased from 10^6 to 10^2 cells ml^{-1} within 10 days in the sediment and to undetectable level in three days in a water column. Only if GMMs are able to survive and multiply within an ecosystem, will they have impact on the environment and possibly cause significant perturbations. The long-term survival of deliberately released GMMs in the environment is generally considered undesirable, although limited survival is essential for GMMs to carry out their allotted tasks. Therefore, survival and persistence within an ecosystem are key elements in assessing the benefit and risk of releasing GMMs into the environment.

The objectives of this study, therefore, are: (1) To assess mutation of acid phosphatase genes in relation to survival of *S. meliloti* in soil; (2) To determine the effect of alfalfa growth on establishment, survival and persistence of *S. meliloti* in the soil environment; and (3) To evaluate the impact of genetically modified *S. meliloti* on soil microbial community structure. Understanding P nutrition in relation to survival of rhizobia in soil would help us understand N-fixation process and maximize the beneficial trait of this natural phenomenon in application. In addition, information on persistence of GMMs in soil and their potential impact on soil microbial activities may be useful in guiding the safe release of GMMs in the environment.

Reference

Bosworth A.H., Williams M.K., Albercht K.A., Kwiatkowski R., Beynon J. Hankinson T.R., Ronson C.W., Cannon F., Wacek T.J., and Triplett. E.W. 1994. Alfalfa yield response to inoculation with recombinant strain of *Rhizobium meliloti* with an extra copy of *dct*ABD and /or modified *nif*A repression. *Applied and Environmental Microbiology* 60: 3815-3832.

Brockwell J., Pilka A., and Holliday R.A. 1991. Soil pH is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils in central New South Wales. *Australian Journal of Experimental Agriculture* 31: 211-219.

Catroux G., Hartmann A., and Reveellin C. 2001. Trends in rhizobial inoculant production and use. *Plant and Soil* 230: 21-30

Crawford D.L., Doyle J.D., Wang Z., Hendricks C.W., Bentjen S.A., Bolton H. Jr., Fredrickson J.K., and Bleakley B.H. 1993. Effects of a lignin peroxidase-expressing recombinant, Streptomyces lividans TK23.1, on biogeochemical cycling and the numbers and activities of microorganisms in soil. *Applied and Environmental Microbiology* 59: 508-518.

Deng S.P., Summers M.L., Kahn M.L., Mcdermott T.R. 1998. Cloning and characterization of *rhizobium meliloti* nonspecific acid phosphatase. *Archives of Microbiology* 170: 18-26.

Esperanza M.R., and Rosenblueth M. 1990. Increased bean (Phaseolus vulgaris L.) nodulation competitiveness of genetically modified Rhizobium strains. *Applied and Environmental Microbiology* 56: 2384-2688.

Graham P.H. 1998. Biological dinitrogen fixation: symbiotic. In: Sylvia et al. (ed.). Principles and application of soil microbiology. Prentice Hall Inc., New Jersey.

Hellriegel H., and Wilfarth H. 1888. Untersuchungen uber die stickstoffnhung de gramineen und leguminosen. Beilagehefin Eeitschrift Versammlung Rubenzucker Industrie Deutschen Reichs 234.

Hirsch P.R., and Spokes J.D. 1994. Survival and dispersion of genetically modified rhizobia in the field and genetic interactions with native strains. *FEMS Microbiology Ecology* 15: 147-160.

Israel D.W. 1987. Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* 84: 835-840.

Keeler K.H. 1988. Can we guarantee the safety of genetically engineered organisms in the environment? *Critical Review in Biotechnology* 8: 85-97.

Leser T., Boye M., and Hendriksen N.B. 1995. Survival and activity of Pseudomonas sp. strain B13 (FR1) in a marine microcosm determined by quantitative PCR and rRNA-Targeting probe and its effect on the indigenous bacteriaplankton. *Applied and Environmental Microbiology* 61: 1201-1207.

Lindow S.E. 1992 Ice strain of *Pseudomonas syringae* introduced to control ice nucleation active strains on potato, p. 169-174 .In Tjamos E.S. (ed), Biological control of plant diseases. Plenum Press, New York.

Lorenz M.G. and Wackernagel W. 1993. Bacterial gene transfer in the environment. In: Worhrmann K. and Tomiuk J. (ed.) Transgenic Organisms: risk assessment of deliberate release. 43-64.

Lorenz M.G., and Wackernagel W. 1990. Natural genetic transformation of *Pseudomonas stutzeri* by sand-absorbed DNA. *Archives of Microbiology* 154: 380-385.

Martineez-Romerro E., and Caballero-Mellado J. 1996 *Rhizobium* phylogenies and bacterial genetic diversity. *Critical Reviews in Plant Sciences.* 15: 113-140.

Moawad H.A., Ellis W.R., and Schmidtt E.L. 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field grown soybeans. *Applied and Environmental Microbiology* 47: 607-612.

Pereira P.A.A., and Bliss F.A. 1987. Nitrogen fixation and plant growth of common bean at different level of phosphorus availability. *Plant and Soil* 104: 79-84.

Ramirez C., and Alexander M. 1980. Evidence suggesting protozoan predation on *Rhizobium* associated with germinating seeds and in the rhizosphere of beans. *Applied and Environmental Microbiology* 40: 492-499.

Schmidt E.L, Robert F.M. 1985. Recent advances in the ecology of *rhizobium.* p. 279-385. *In*: Nitrogen fixation research process, Evans HJ, Bottomley PJ, Newton WE (eds). Martinus Nijhoff, Dordrecht, The Netherlands.

Segovia L., Pinero D., Palacios R., and Martinez-Romero E. 1991. Genetic structure of a soil population of non-symbiotic *Rhizobium leguminosarum. Applied and Environmental Microbiology* 57: 426-433.

Smit E., van Elsas J.D., van Veen J.A., and de Vos W.M. 1991. Plasmid transfer from *Pseudomonas fluorescents* to indigenous bacteria in soil by using bacteriophage R2f for donor counter selection. *Applied and Environmental Microbiology* 57: 3482-3488.

Soa-Afiana L.O., and Alezander M. 1982. Differences among cowpea rhizobia in tolerance to high temperature and desiccation in soil. *Applied and Environmental Microbiology* 43: 435-439.

Stotzky G., and Babich H. 1984. Fate of genetically engineered microbes in natural environments. *Recombination DNA Technology Bulletin* 7: 163-188.

Walter M.V., Porteous L.A., Prince V.J., Ganio L., and Seidler R.J. 1991. A microcosm for measuring survival and conjugation of genetically engineered bacteria in rhizosphere environment. *Current Microbiology* 22: 117-121.

Wang E.T., and Martinez-Romero E. 2000. Phylogeny of root-and stem-nodule bacteria associated with legumes. In: Triplett E.W. (ed.) Prokaryotic Nitrogen Fixation: A model System for the Analysis of a Biological Process. pp: 177-186 Horizon Scientific Press, Wymondham, England.

Chapter II

LITERATURE REVIEEW

Nitrogen (N) is an essential nutrient that is commonly deficient in available form for plant uptake, contributing to reduced agricultural production throughout the world (Berg, 1990; Haby et al., 1991). Virtually all the N used in agricultural production comes from atmospheric N_2 that composes approximately 78% of atmospheric gases. Inorganic N fertilizer used in agricultural production comes from atmospheric N_2 fixed by a high-energy input chemical process. This process is costly and holds potential to pollute the environment with a potent "greenhouse" gas, nitrous oxide (N_2O). On the other hand, symbiotic N fixation contributes significantly to agricultural production (Graham P.H. 1998; Wang and Martinez, 2000) and is also favored over chemical N fixation because of increasing concerns for energy and the potential of groundwater and air pollution.

Symbiosis between legume plant and rhizobium results in the formation of root nodules. Effective N fixation requires active participation of a healthy plant and successful establishment of rhizobia in soil. It is, therefore, important to understand the survival and persistence of rhizobia in soil, nutritional needs for rhizobial growth, as well as impact of genetic modified rhizobia on soil biological environment.

Survival and persistence of rhizobia in soil

Highly effective rhizobia, following their introduction into soil, often do not

survive and persist in significant numbers (Vidopr and Miller, 1980; Dudeja Khurana, 1989; Slatiery et al., 1993). Field studies showed that population of inoculated *Bradyrhizobium sp.* dropped to 0.01% of original population within one month in soil (Dudeja Khurana, 1989). Various environmental stresses prevent the N-fixing microorganisms from surviving and establishing in soil, including biotic and abiotic stresses.

Biological stresses

Major biological stresses encountered by introduced rhizobium in soil include competition, biologically formed toxins, and predation. Competition with the indigenous microbial community is inevitably encountered when a microbe is first introduced to a new environment. Some rhizobium strains are poor competitors in the soil (Hely et al., 1957; Kvien et al., 1981; Moawad et al., 1984). It has been reported that the native microbial communities of certain Australian soils could prevent colonization of inoculated *Rhizobium trifolii* to plant roots (Hely et al., 1957). However, establishment was possible when the size of indigenous microbial community was reduced by applying antimicrobial agents (Li and Alexander, 1986).

The basis of rhizobium competition is not well understood. Ames and Bergaman (1981) suggested that bacterial motility might be involved. An ethyl methanesulfonate-induced motility deficient mutant of *Rhizobium meliloti* was less competitive compared to parental strain in soil (Ames and Bergaman, 1981). Later, the same characteristic was found in motility mutants of *Rhizobium*

leguminosarum bv. *Trifolii* (Mellor et al., 1987).

Biologically formed toxins and predation also influence the survival of rhizobium in the soil. In the early report, extracts of soil in which clover nodulated poorly were found to be toxic to *Rhizobium trifolii* (Chatel and Parker, 1972). The nature and origin of this toxin was unknown. Several studies were carried out on fungi toxins against rhizobia (Chhonkar and Subba-Rao, 1966; Angle et al., 1981). Toxin-producing fungus, *Trichoderma viride*, decreased population of inoculated *Rhizobium japonnicum* in soil to 2% of original density within 64 days (Angle et al., 1981). The protozoa apparently fed on large numbers of rhizobia and appreciably reduced their density (Danso et al., 1975). Another bacterial parasite *Bdellovibrio* are present in many soils, and they were shown to reduce rhizobia numbers (Keya and Alexander, 1975). However, rhizobium persisted in relatively large numbers in the presence of this parasite in soil (Keya and Alexander, 1975).

Abiotic stresses

Since many agricultural soils are acidic, numerous studies have been carried out on survival and growth of rhizobium strains at low pH (Bryan, 1923; Brockwell et al., 1991; Watkin et al., 1997;). Sensitivity of a given species of rhizobium to pH varied up to 1 pH unit depending on individual strains (Graham and Parker, 1964). The number of *R. meliloti* increased with increasing soil pH (Brockwell et al., 1991). In the laboratory, the mean generation times of *R. leguminosarum* bv. *trifolii* was 50-60% slower when the culture pH was changed

from 7.0 to 5.0 (Watkin et al., 1997). In addition, low pH induced increase of heavy metal solubility that may be deleterious to rhizobia survival (Keyser and Munns, 1979; Hirsch et al., 1993). Population of *R. leguminosarum* bv. *trifolii* showed a dramatic decline in soils contaminated with heavy metal due to receiving sludge over a long period of time (Hirsch et al., 1993).

Moisture and temperature are two other main abiotic factors affecting persistence and survival of rhizobia in soil. Moisture has a marked influence on the rhizobium even at moderate soil temperatures (Danso and Alexander, 1974; Chao and Alexander, 1982; Trotamn and Weaver, 1995). One out of ten commercial *R. leguminosarum* bv. *trifolii* strains could be maintained over 1% of its inoculated population for 1 week in dry soil, while most of them could not be maintained at 1% of their population for one day (Trotman and Weaver, 1995). Drought tolerance of rhizobia has been shown to vary among species. Slow-growing rhizobium, such as *R. lupini*, and *R. japonicum* have greater inherent ability to survive under dry soil condition than the fast-growing species including *R. leguminosarum* and *R. meliloti* (Bushby and Marshall, 1977a). The cause of rhizobium death by desiccation may be due to changes of membrane permeability (Bushby and Murshall, 1977b). Relatively less work has been done to examine the survival of rhizobia under flooded condition. An early report indicated that it was not a problem (Vandecaveye, 1927), while a recent report showed dramatic decrease of *B. japonicum* population under waterlogged soil (Beringer and Kay, 1993). The conflicting results could be due to species variation.

Rhizobia are not heat-resistant and cannot survive at soil temperatures above 45°C that are found in some tropical soil (Dudeja and Khurana, 1989; Roughley et al., 1995). In general, rhizobium strains vary greatly in their susceptibility to high temperatures. The maximum survival temperature averaged 44°C for slow-growing *Bradyrhizobium* spp. compared with 47.1°C for fast-growing *Rhizobium* spp. (Mpepereki et al., 1996). It was suggested that extracellular polysaccharide (EPS) play a role in rhizobium growth and survival under high temperatures (Mpepereki et al., 1996). Survival of rhizobium in high temperature soils also depended on soil moisture content. Rhizobium is more sensitive to heat in moist soil than in dry soil, and bacterial population dropped markedly in moist soil even at a moderate 36 °C (Wilkin, 1967; Danso and Alexander, 1974). Few studies have been carried out to demonstrate cold-tolerance of rhizobium. Rhizobia adapted to low temperatures not only grew but also nodulated at lower temperatures than strains from a temperate region (Lipsanen and Lindstrom, 1986).

Salinity could potentially influence survival and establishment of rhizobia in soil. Approximately 4 x 10^6 Km^2 worldwide was affected by the excessive salt that was not good for growth of most legume plants and their root-nodule bacteria (Balasubramanian and Sinha, 1976; Flowers et al., 1977; Rai, 1983). Rhizobia in fact survive better in saline soils than their legume hosts (Craig et al., 1991). *Rhizobium meliloti* could grow in media containing 500 mM NaCl (Hua et al., 1982). Thus, It was suggested that attention should be given to the influence of salinity on aspects of symbiosis other than the survival of the bacteria

(Singleton et al. 1982).

In addition, fungicides, widely applied as leguminous seed coatings to prevent plant pathogens, have been shown to be deleterious to rhizobia bacteria. Decline of viable rhizobia number on the fungicide treated seed has been reported (Kyei-Boahen et al., 2001). Furthermore, fungicides were shown to have negative impacts on the synergistic relationship between rhizobia and root-associated mycorrhizal fungi (Redente and Reeves, 1981). The fungicide captafol is toxic to *Rhizobium trifolii* and its resistant mutant lost the ability of nodulation (Ruiz-Saninz et al., 1984). Fungicide toxicity continues as an issue for rhizobium application because of widespread application and continued introduction of new fungicides.

In summary, soil is a heterogeneous mixture of solid, gas, and liquid phases with a multitude of difficult situations that rhizobia may encounter as stress factors. These include temperature extremes, pH extremes, oxygen (lack of, or presence of), and water stress. In addition, rhizobia also face a great deal of competition from indigenous rhizobia and non-rhizobia microbes for food, habitat, and space. The introduced rhizobium is an invader into the soil system and may not survive and establish in the environment, but rather be just a food source for other predators. The survival and persistence of the introduced rhizobium depends on biotic factors, such as the indigenous microbial community, and the specific soil abiotic conditions.

Phosphorus nutrition in relation to rhizobia symbiosis

The successful biological N fixation of rhizobium-legume symbiosis also depends on nutrient supplies to symbiotic partners. Mineral nutrients may limit N fixation by effecting rhizobia survival, infection and nodule development as well as by host plant growth. Phosphorus, a major essential macronutrient required for cell structure, replication, and energy, is a limiting nutrient in the soil. Understanding of the influence and role of phosphorus on symbiosis should be of considerable important to successful nitrogen fixation.

Global phosphorus effects on symbiosis

Every aspect of symbiosis from symbiotic partner, plant and rhizobium to symbiosis processes is influenced by P. Studies on alfalfa (Al-Niemi, 1997), soybean (Israel, 1987), clover (Robson, 1981), common bean (Pereira and Bliss, 1987; 1989), and chickpea (Itoh, 1987) have consistently shown a positive response to P fertilization. Addition of P increased whole plant N and P concentration, plant dry matter, nodule number, and nodule mass (Pereira and Bliss, 1987; Sa and Isreal, 1991; Isreal, 1993; Reibet and Drevon, 1995). Field studies have shown that P concentrations in stems and seeds of nodulating peanuts were higher than those in non-nodulating plants (Sahrawat, 1988). This infers that the plant P uptake was affected when legume plants grow symbiotically.

Phosphorus affects rhizobium growth (Keyser and Munns, 1979; Cassman, 1981a; 1981b; Watkin, 1997). The number of *Bradyrhizobium radicicola* was doubled after P was added to soil (Truesdell, 1917). Limiting P in culture media decreased P storage capacity of rhizobium, changed pattern of periplasmic enzymes and increased rate of P uptake (Cassman, 1981b; Samrt, 1884a; 1984b). More recent studies demonstrated that the structure and biosynthesis of rhizobia cell surface carbohydrates such as exopolysaccharides II, lipoplysacchardes and cyclic ß-(1,2)-glucan was strongly influenced by extracellular P concentration (Zhan et al., 1991; Tao et al., 1992; Breedveld et al., 1995). These results imply that phenotype of rhizobium may change in response to P limitation.

Phosphorus limitation also affects symbiotic processes such as infection, nodule formation and nodule function. Efficiency of soybean root infection by *Bradyrhizobium japonicum* was reduced when the inoculated strain had been P stressed previously (Mullen et al., 1988). The attachment of rhizobium to plant root was affected by external P concentration (Howieson, et al., 1993). The attachment of *Rhizobium melliloti* to *Medicage polymorpha* increased 1.1 fold with P concentration in media increasing from 20 to 200 μM (Howieson, et al., 1993). Additional, excretion of lipooligosaaccharides (Nod metabolites) was significantly reduced when media P dropped from 1 μM to 0.5 μM (McKay and Djordjevic, 1993). Moreover, the activity of N-fixing enzyme, nitrogenase was affected by P limitation (Sa and Isreal, 1991) and was reported to decrease 28% of soybean nodule nitrogenase over a 16-day experiment period when P level

was 0.05 mM (Sa and Isreal, 1991). All these results suggest that rhizobium infection activities and symbiotic processes in soil are inhibited by P limitation.

Phosphorus source and availability in soil

Total P in soil ranged from 200 to 5000 mg kg^{-1} soil with an average of 600 mg kg^{-1} of soil (Lindsay, 1979). Rocks and deposits, such as primary apatites and other primary minerals, are the major reserves of P in the world. For example, there is about 140 million tons of phosphate rock distributed in different states of India (Farmwal, 1989). However, solubility of phosphate rock-P is very low though it can be solubilized under appropriate conditions and become available for plant and microbial growth (Goldsterin, 1986). One of the major mechanisms in mineralizing P is the action of organic acid synthesized by soil microorganisms (Banik and Dey, 1982; Halder, 1990). Halder et al. (1990) showed that the amount of P solubilized by purified 2-ketogluconic acid was equivalent to that by the whole cell culture of *Rhizobium leguminosarum*.

Another major form of soil P is organic matter (Dalal, 1977). Organic forms of P may constitute 20-80% of the total P in soil, ranging from 5% to 90% (Dalal, 1977). Organic P in soil is largely in the form of inositol phosphate (phytate), which accounts for up to 50% of the total organic P (Dalal, 1977). Other organic P compounds in soil are in various forms including phosphomonoesters, phosphodiesters, such as phospholipids and nucleic acids, and C-P compound, such as alkyiphophonates (Dalal, 1977; Beever and Burns, 1986). To be assimilated by plant and microorganism cells, many of these high molecular-

weight organic P compounds must first be converted to either soluble ionic P (Pi, HPO_4^{2}, $H_2PO_4^{-}$), or low molecular-weight organic P (Bieleski, 1973; Beever and Burns, 1986). Plants and microorganisms take several forms of Pi dominating by HPO_4^{2-} or $H_2PO_4^{-}$ (Bieleski, 1973; Beever and Burns, 1986).

Phosphorus transportation, assimilation and regulation in rhizobia

Inorganic P has been identified to be a major P source taken up by rhizobium (Bardin and Finan, 1998), demonstrated by studies using radioactive labeled Pi (Werner and Berghauser, 1976; Tan and Broughton, 1982). Uptake of P by rhizobia involves a P inorganic transport (Pit) system. Understanding of the Pit system requires understanding of the structure of a root nodule. The membrane of plant origin in a root nodule is perbactroid membrane. Within the perbactroid membrane, bacteria divide and differentiate to form N-fixing bacteroids. A structure comprised of both perbactroid membrane and bacteroids is a symbiosome (Kinnback and Werner, 1991). It has been shown that Pi moved across the symbiosome membrane from the soil solution around nodules and accumulated significantly in nodule bacteroids (Al-Niemi et al., 1998), while *S. meliloti* mutant that was deficient of Pit system formed nodules that were largely devoid of bacteria and failed to fix N (Bardin and Finan, 1998).

An efficient Pit system is essential for optimal symbiotic performance under P limiting conditions. In general, Pi concentration in soil solutions is low, ranging from 10^{-5} to 10^{-7} M (Dalal, 1977; Bieleski, 1973), and that in the rhizosphere is even lower, around 10^{-8} M (Nye. 1979). Two distinct Pit systems

have been identified in *Sinorhizobium meliloti* (Voegele et al., 1997), a high P_i affinity permease (K_m, 0.2μM) and a lower Pi affinity system (K_m, 1.5μM). The high-affinity system is encoded by the *phoCDET* operon, while the low-affinity system is encoded by *pit* in the *orfA-pit* operon (Bardin et al., 1998). Under high P conditions, the low affinity Pit permease is expressed and is responsible for Pi uptake. Under P limiting conditions, the low affinity Pit system is repressed, while the high affinity phoCDET system is induced (Voegele et al., 1997). *Rhizobium tropici* also has two Pit systems that have been identified as high- and low-affinity system (Botero et al., 2000). However, the *R. tropici* transport systems expressed constitutively at low level with enhanced expression under P stress (Botero et al., 2000). Other rhizobium strains such as *Rhizobium* NGR234, *Bradyrhizobium lupini* WU8, and *Rhizobium trifolii* WU95 have only one single repressible Pit system (Samrt, 1984a). For these free-living strains, rate of Pi uptake was repressed under sufficient Pi, and only reaches its maximum rate under P-stress conditions (Samrt, 1984a).

Organic P may also serve as a P and energy source for rhizobium growth. *Rhizobium spp.* was able to use glucose-1-phoaphate, ATP and some extent β-glycero-phoahate for growth (Abd-Alla, 1994). *Rhizobium huakuii* has also been reported to use phosphomycin as a sole P and C sources (McGrath et al., 1998). *Bradyhizobium japonicum* bacteroids were also able to take up small molecular weight organic P such as UDP-glucose as an energy source (Salminen and Streeter, 1987).

However, inorganic P is considered the major form taken up by rhizobia.

Conversion of organic to inorganic P requires a group of enzymes, phosphatases (Cosgrove D. J. 1967; Fox and Comerford, 1992). In soil, phosphatases are widely distributed (Kirchner, 1993; Feller et al., 1994), particularly in the rhizospheres (Fox and Comerford, 1992), and are mostly of microbial origin (Hernandez et al., 1993; Tabatabai, 1994).

Two acid phosphatase genes have been cloned from *Sinorhizobium meliloti* 104A14 and both are expressed constitutively (Deng et al., 1998; 2001). *Rhizobium tropici* also has two constitutively expressed acid phosphatases (Al-Niemi, 1997a). A single mutation of each of the two acid phospatases in *S. meliloti* did not affect symbiosis significantly regardless of P levels (Deng et al., 1998). The role of these acid phosphatases in symbiotic performance remains to be understood. However, cloning of genes encoding phosphatases allows us to evaluate its potential regulation systems. Evidence suggested that phosphatase activities and Pi transportation rate of free-living rhizobium are regulated by external P condition (Samrt, 1984a; Bardin et al., 1996; Al-Niemi et al., 1997a). The activity of alkaline phosphatases of *S. meliloti* increased 160 fold under P limited conditions (< 0.05 mM Pi) compared to that under P rich conditions (> 10 mM) (Samrt, 1984a).

A phosphate regulator, *Pho*B, has been identified in *Sinorhizobium meliloti* (Al-Niemi et al., 1997b), which is shown to be a positive regulator of *pho*CDET encoding the high affinity P system (Bardin and Finan, 1998). Transcriptions of *pit* and *orfA* were 4.5-5.5 fold higher in a *Pho*B mutant than in a wild type under external P_i–rich condition (2mM) (Bardin and Finan, 1998), suggesting that *Pho*B

negatively regulate expression of *orfA-pit*. Under high P concentrations, *Pho*B is inactive and *orfA-pit* is expressed, while under P starvation, *Pho*B is activated and *phoCDET* expression is induced (Voegele et al., 1997; Bardin and Finan, 1998). The gene *Pho*B has been cloned and sequenced from *Bradyrhizobium japonicum* (Minder, 1998). The protein produced by *B. japonicum Pho*B share 71.4% identical amino acids with *Pho*B protein of *S. meliloti*. *Pho*B mutant of *B. japonicum* showed a growth defect when the P concentration in media was less than 25 μm. The *S. meliloti Pho*B mutant also showed that alkaline phosphatase activity was not induced under Pi -stress condition (Al-Niemi et al., 1997b), imply that *Pho*B is also a regulator for rhzobium alkaline phosphatase. However, the principal mechanism for the regulation of rhizobia acid phosphatase remains largely unknown.

In summary, legume-rhizobium symbiosis has shown to be a P dependent N-fixation process. Rhizobia are able to use both organic and inorganic P to serve their nutritional needs, but favor inorganic P and small molecular weight organic P. One or two Pit systems were detected in rhizobia. The role of phosphatases in symbiosis remains to be understood. A comprehensive understanding of P acquisition in the N fixation process is critical for optimizing SNF. Microbiologists are hunting for superior strains or mutants for their unique traits, such as highly competitive, persistent in soil, high N_2 fixation rate and efficiency, etc. With the advance and development in molecular techniques, it is possible to engineer a microorganism of desired traits and properties, opening a new era in application of soil microbiology. However, introduction of superior

strains to the field will succeed only if these strains can survive and persist in the soil environment.

Risk assessment of releasing genetically modified microorganisms into the environment

Since biotechnology became a practical application in the early 1970's because of the discovery of restriction enzyme, recombinant DNA technology used for genetic modification increasingly plays an important role in industry, agriculture and food processing throughout the world (Keeler et al., 1988; James et al., 1998). Genetically modified microorganisms (GMMs) have been developed commercially for field applications of pest control, pollution abatement, frost protection, and stimulation of N fixation (Keeler et al., 1988; Bosworth et al., 1994; James et al., 1998).

However, genetic manipulation of bacteria and deliberate release of GMMs into the environment could potentially have certain risks. Some fear that application of GMMs may spread modified DNA or genes in the natural microbial community. Consistent evidence demonstrated that microorganisms have the capacity not only for intraspecific but also for interspecific DNA transfer via conjugation, transduction and/or transformation (Lorenz and Wackernagel, 1990; Walter *et al.,* 1991; Lorenz and Wackeragel, 1993; Selbitschka et al., 1995). It is even possible to transfer DNA from prokaryotes to eukaryotes and vice versa (Lorenz and Wackeragel, 1993). These exchanges could potentially lead to development of pathogenic or undesirable microbes that might be deleterious to

plants, animals or humans. Introduction of GMMs into natural environment may facilitate such processes, which result in increasing potential risks (Lorenz and Wackeragel, 1993).

Introduction of GMMs might also alter balance of fundamental processes, such as carbon, energy, and nutrient cycling that mainly relies on bacterial species or products (Doyle et al. 1991; Crawford et al. 1993). The genetically engineered *Pseudomonas putida* retarded the rate of carbon dioxide evolution in indigenous microorganisms (Doyle et al. 1991). Studies demonstrated that a recombinant actinomycete, *Streptomyces lividans* TK23.1 had a transient enhancement of organic C mineralization, altered soil pH, the incorporation rate of C into soil humus fractions, N cycling, and also certain soil enzyme activities (Crawford et al., 1993).

Much of the current biotechnology risk assessment was based on concepts originally put forward by the "chemical" risk assessment (Seidler and Settel, 1991). However, microorganisms have unique capabilities to grow and multiply, to exchange genes with members of the indigenous bacteria and potentially to move actively through an ecosystem (Bushan, 1986; Trevors et al., 1990; Selbitschka et al., 1995). This raises serious difficulties for microbial risk assessment as compared to that of chemicals where physico-chemical laws of dissipation and decay tend to resign. Various governments and agencies including the United States Environmental Protection Agencies (EPA), have proposed guidelines that necessitate industry to collect fundamental, ecological information on the recombinant products (Levin et al., 1987). These parameters

include survival time, potential gene exchange, dispersal, and potential effects on beneficial non-targeted organisms and on the ecosystem (Levin et al., 1987).

GMM population introduced to the soil environment usually progressively declines and its establishment is often hindered due to poor survival (van Elsas *et al.*, 1989, 1991; Compeau *et al.*, 1988). The population of inoculated genetically modified *Pseudomonas fluoreescens* decayed at 0.7 log CFU g^{-1} soil per 10 days (Compeau *et al.*, 1988). This was partially attributed to the extra metabolic load of the additional genes or loss of expression of some traits (van Elsas *et al.*, 1991). Soil conditions, such as moisture temperature, pH, organic matter content, and presence of protozoa may also affect the survival of introduced GMMs (van Elsas and Trevors, 1991).

Gene transfer, include transduction, transformation, and conjugation, has been detected in soil and sediments (Germida and Khachatourians, 1988; Zeph *et al.*, 1988). However, transduction of indigenous soil bacteria was not unequivocally demonstrated (Zeph *et al.*, 1988). This is not surprising because of host specificity. Transformation, uptake of naked DNA from their environment, has been suggested to play a dominant role in gene transfer in the environment (Lorenz and Wackeragel, 1990; Stewart and Sinigalliano, 1990; Paul *et al.*, 1991). Sand-absorbed plasmid DNA can be taken up by *Pseudomoinas stutzeri* (Lorenz and Wackeragel, 1990). There are evidence suggesting that transformation can be environmentally regulated (Lorenz and Wackeragel, 1990).

On the other hand, conjugation appears to be of potential ecological significance. Conjugation has been observed in a wide species of Gram-negative

and Gram-positive bacteria (Van Elsas *et al.*, 1987; Henschke and Schmidt, 1990; Lorenz and Wackeragel, 1993). Frequency of plasmid transfer from *Bacillus cereus* to *B. subtilis* was 7×10^{-8} (number of transconjugant CFU per number of donor CFU) in sterile soil and 9×10^{-8} under similar condition in non-sterile soil amended with bentonite clay (van Elsas *et al.*, 1987). Further more, plasmid may be mobilized into indigenous soil bacteria. Genetically modified plasmid pFL67-2 was found in soil indigenous *Pseudomonas fluorescens* after the *Eschericjhis coli* with pFL67-2 was inoculated in the soil (Henschke and Schmidt, 1990).

Releasing GMMs could also affect the soil ecosystem as well as non-target organisms. As shown by Bej et al. (1991), soil bacteria diversity indices increased initially and declined to the control level after inoculation of an engineered *P. cepacea* for 6 weeks. Similar results were observed by De Leij et al. (1995) who found a transient perturbation in the indigenous microbial community with the introduction of wild type and genetically marked *P. fluorescens* to the rhizosphere of wheat. Fungi population was found to decline upon introduction of genetically engineered *P. putidas* PPO301 (Doyle et al., 1991). Interestingly, introduction of GMMs into soil did not significantly alter population of total bacteria, fluorescent pseudomonads, actinomycetes and salt-tolerant bacteria (Lacy and Stromberg, 1991).

Nevertheless, risk assessments should be considered as the determination of the biological safety of released GMMs in connection to the biological effectiveness or beneficial effect. The more comprehensive knowledge

of the consequences of such a release into the environment must be provided before they can be utilized.

In summary, survival and persistence of introduced rhizobium is the critical step enhancing biological N fixation, while P is an essential element affecting this process. Phosphatase may play a vital role for P acquisition in relation to survival of rhiozobia under P limiting conditions. Information on persistence and survival of GMMs in soil and their potential impact on the soil microbial communities may guide us for safe release of GMMs in environment.

Reference:

Abd-Alla M.H. 1994. Use of organic phosphorus by *Rhizobium leguminosarum* biovar *viceae* phosphatases. *Biology and Fertility of Soils* 18: 2016-218.

Al-Niemi T.S., Kahn M.L., and McDermott T.R. 1998. Phosphorus uptake by bean nodules. *Plant and soil* 198: 71-78.

Al-Niemi T.S., Kahn M.L., and McDermott T.R. 1997a. P metabolism in the *Rhizobium tropici*- bean symbiosis. *Plant Physiology* 113: 1233-12452.

Al-Niemi T.S., Summers M.L., Elkins J.G., Kahn M.L., and McDermott T.R. 1997b. Regulation of the phosphate stress response in Rhizobium meliloti. *Applied and Environmental Microbiology* 63: 4978-4981.

Ames P., and Bergman K. 1981. Competitive advantage provided by bacterial motility in the formation of nodules by Rhizobium meliloti. *Journal of Bacteriology* 148: 728-729.

Angle J.S., Pugasshetti B.K., and Wagner G.H. 1981. Fungal effects on Rhizobium japonicum –soybean symbiosis. *Agronomy Journal* 73: 301-306

Balasubramanian V., and Sinha S.K. 1976. Effects of salt stress on growth, nodulation and nitrogen fixation in cowpea and mung beans. *Physiologia Plantarum* 36: 197-200.

Banik S., and Dey B.K. 1982. Available phosphate content of an alluvial soil is influenced by inoculation of some isolated phosphate solubilizing microorganisms. *Plant and Soil* 69: 353-364.

Bardin S., Dan S., Osteras M., and Finan T.M. 1996. A phosphate transport system is required for symbiotics nitrogen fixation by Rhizobium meliloti. *Journal of Bacteriology* 178: 4540-4547.

Bardin S.D., and Finan T.M. 1998. Regulation of phosphate assimilation in *Rhizobium (Sinorhizobium) meliloti. Genetics* 148: 1689-1700.

Bardin S.D., Voegele R.T., and Finan T.M. 1998. Phosphate assimilation in *Rhizobium (Sinorhizobium) meliloti*: identification of a *pit*–like gene. *Journal of Bacteriology* 180: 4219-4226.

Beever R.E., and Burns D.J.W., 1986. Phosphorus uptake, storage and utilization by fungi. *Advance in Botanical Research* 8: 127-219.

Bej A.k., Perlin M., and Atlas R.M., 1991. Effect of introducing genetically engineered microorganisms on soil microbial community diversity. *FEMS Microbiology Ecology* 86: 169-176

Berg, W.A. 1990. Herbage production and nitrogen accumulation by alfalfa and

cicer milkvetch in the Southern Plains. *Agronomy journal.* 82: 224-229.

Beringer J.E., and Kay H,E. 1993. Monitoring the survival of *Bradyrhizobium japonicum* released into Italian soils. In: Bridre/Biotech Final sectorial meeting on biosafety and first sectorial meeting on microbial ecology. Granada, October 24-27.

Bieleski R.L. 1973. Phosphate pools, phosphate transports, and phosphate availability. *Annual Review of Plant Physiology* 24:225-252.

Bosworth A.H., Williams M.K., Albercht K.A., Kwiatkowski R., Beynon J. Hankinson T.R., Ronson C.W., Cannon F., Wacek T.J., and Triplett. E.W. 1994. Alfalfa yield response to inoculation with recombinant strain of *Rhizobium meliloti* with an extra copy of *dct*ABD and /or modified *nif*A repression. *Applied and Environmental Microbiology* 60: 3815-3832.

Botero L. M., Al-Niemi T.S., and Mcdermott T.R. 2000. Characterization of two inducible phosphate transport system in Rhizobium tropici. *Applied and Environmental Microbiology* 66: 15-22.

Breedveld M.W., Benesi A.L., Marco M.L., and Miller K.J. 1995. Effect of phosphate lilitation on synthesis of periplasmic cyclic ß-(1,2)-glucans. *Applied and Environmental Microbiology.* 61: 1045-1053.

Brockwell J., Pilka A., and Holliday R.A. 1991. Soil pH is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils in central New South Wales. *Australian Journal of Experimental Agriculture* 31: 211-219.

Bryan O.C. 1923. Effect of acid soils on nodule-forming bacteria. *Soil Science*

15:37-40.

Bushan Y. 1986. Migration of the rhizobium bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* towards wheat roots in the soil. *Journal of General Microbiology* 132: 3407-3414.

Bushby H.V.A., and Marshall K.C. 1977a. Some factors affecting the survival of root-nodule bacteria on desiccation. *Soil Biology and Biochemistry* 9: 143-149.

Bushby H.V.A., and Marshall K.C. 1977b. Desiccation induced damage to the cell envelope of root-nodule bacteria. *Soil Biology and Biochemistry* 9: 149-152.

Cassman K.G., Munns D.N., and Beck D.P. 1981a. Growth of *Rhizobium* strains at low concentration phosphate. *Soil Science Society of America Journal* 45: 520-523.

Cassman K.G., Munns D.N., and Beck D.P. 1981b. Phosphorus nutrition of Rhizobium japonium: strain differences in phosphate storage and utilization. *Soil Science Society of America Journal* 45: 517-520.

Chao W.L., and Alexander M. 1982. Influence of soil characteristic on the survival of Rhizobium in soil undergoing drying. *Soil Science Society of America Journal* 46: 949-952.

Chatel D.L., and Parker C.A. 1972. Inhibition of rhizobia by toxic soil-water extracts. *Soil Biology and Biochemistry* 4:289-294.

Chhonkar P.K., and Subba-Rao N.S. 1966. Fungi associated with legume root nodules and their effect on rhizobia. *Canadian Journal of Microbiology* 12:

1253-1261.

Compeau G., Jadoun Al-achi B., Platsouka E., and Levy S.B. 1988. Survival of rifampicin-resistant mutants *of Pseudomonas fluorescens* and *Pseudomonas Putida* in soil systems. *Applied and Environmental Microbiology* 54: 2432-2438.

Cosgrove D.J. 1967. Metabolism of organic phosphates in soil. In: McLaren A.D. and Peterson G.H. (eds). Soil biochemistry. Vol. 1 pp. 216-228. Dekker. New yoork.

Craig G.F., Atkins C.A., and Bell D.T. 1991 Effects of salinity on growth of four strains of Rhizobium and their infectivity and effectiveness on two species of Acacia. *Plant and Soil* 133: 253-262.

Crawford D.L., Doyle J.D., Wang Z., Hendricks C.W., Bentjen S.A., Bolton H. Jr., Fredrickson J.K., and Bleakley B.H. 1993. Effects of a lignin peroxidase-expressing recombinant, Streptomyces lividans TK23.1, on biogeochemical cycling and the numbers and activities of microorganisms in soil. *Applied and Environmental Microbiology* 59: 508-518.

Dalal R.C. 1977. Soil organic phosphorus. *Advances in Agronomy* 29: 83-117.

Danso S.K.A., and Alezander M. 1974. Survival of two strains of Rhizobium in soil. *Soil Science Society of America Journal* 38: 86-89.

Danso S.K.A., Keya S.O., and Alexander M. 1975 Protozoa and the decline of *Rhizobium* populations added to soil. *Canadian Journal of Microbiology* 21: 884-895.

De Leij F.A.A.M., Sutton E.J., Whipps J.M., Fenlon J.S., and Lynch J.M. 1995. Impact of field relesae of genetically modified on indigenous microbial population of wheat. *Applied and Environmental Microbiology* 61:3443-3453

Deng S.P., Elkins J.G., Da H.N., Botero L.M., and McDermott T.R. 2001. Characterization of a Second Acid Phosphatase From *Sinorhizobium meliloti* Strain 104A14. *Archives of Microbiology* 176: 255-263.

Deng S.P., Summers M., Kahn M.L. and McDermott T.R. 1998. Cloning and characterization of a *Rhizobium meliloti* nonspecific acid phosphatase. *Archives of Microbiology* 170: 18-26.

Doyle J.D., Short K.A., Stotzky G., King R.J. Seidler R.J., and Olsen R.H. 1991. Ecologically significant effects of *Pseudomonas Putida* PPO301 genetically engineered to degrade 2,4-dichlorophenoxyacetate, on microbial populations and process in soil. *Canadian Journal of Microbiology* 37: 682-691.

Dudeja S.S., and Khurana A.L. 1989. Persistence of Bradyrhizobium sp. in a sandy loam. *Soil Biology and Biochemistry* 21: 709-713.

Farmwal N.S. Singh R.B. and Rai R. 1989. Isolation of phosphate solubilizers from different sources. *Current science* 58: 570 -571.

Feller C., Frossard E., and Brossard M. 1994. Phosphatase activity in low activity tropical clay soils. *Canadian journal of soil science* 74: 121-129

Flowers T.J., Troke P.F., and Yeo A.R. 1977 The mechanism of salt tolerance in halophytes. *Annual Review of Plant Physiology* 28: 89-121.

Fox T.R., and Comerford N.B., 1992. Rhizosphere phosphatase activity and phosphatase hydrolysable organic phosphorus in two forested spodosols. *Soil biology and Biochemistry* 24: 579-583.

Germida J., and Khachatourians C.G. 1988. Transduction of *Escherichia coli* in soil. *Canadian Journal of Microbiology* 34: 190-193

Goldsterin A.H. 1986. Bacterial solubilization of mineral phosphate: historical perspective and future prospects. *American Journal of Alternative Agriculture* 1:51-57

Graham P.H. 1998. Biological dinitrogen fixation: symbiotic. In: Sylvia et al. (ed.). Principles and application of soil microbiology. Prentice Hall Inc., New Jersey

Graham P.H., and Parker C.A. 1964. Diagnostic feature in the characterization of the root-nodule bacteria of legumes. *Plant and Soil* 20:383-396.

Haby V.A., Davis J.V., Leonard A.T., Patten K.D. 1991. Rabbiteye blueberry plant response to nitrogen and phosphorus. *Journal of plant nutrition*. 14: 1081-1090.

Halder A.K., Mishra A.K., Bhattacharyya P., and Chakrabartty P.K. 1990 Solubilization of rock phosphate by *Rhizobium and Bradyrhizobium. The Journal of General and Applied Microbiology* 36: 81-92.

Hely F.W., Bergersen F.J., and Brockwell J.1957. Microbial antagonism in the rhizosphere as a factor in the failure of inoculation of subterranean clover. *Australian Journal of Agriculture Research* 8:24-44.

Henschke R.B., and Schmidt F.R.J. 1990. Plasmid mobilization from genetically engineered bacteria to members of the indigenous soil microflora. *Current Microbiology* 20: 105-110.

Hernandez C.G., Costa F., Ceccanti B., and Masciandaro G. 1993. Kinetics of phosphatases activity in organic wastes. *Soil biology and Biochemistry* 25: 561-565.

Hirsch P.R., Jones M.J., Mcgrath S.P., and Giller K.E. 1993. Heavy metals from past application of sewage sludge decrease the genetic diversity of *Rhizobium leguminosarum* bv. *trifolii* populations. *Soil Biology and Biochemistry* 25: 1485-1490.

Howieson J.G., Robson A.D., and Ewin M.A. 1993 External P_i and calcium concentration and pH but not the product of rhizobial nodulation genes affect the attachment of *Rhizobium meliloti* to roots of annual medies. *Soil Biology and Biochemistry* 25: 567-573.

Hua S.S., Tsai V.Y., Lichens G.M., and Noma A.N. 1982. Accumulation of amino acid in *Rhizobium sp.* strain WR1001 in response too sodium chloride salinity. *Applied and Environmental Microbiology* 44: 135-140.

Israel D.W. 1987 Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* 84: 835-840.

Isreal D.W. 1993. Symbiotic dinitrogen fixation and host-plant growth during development of and recovery from phosphorus deficiency. *Physiologia Plantarum.* 88:294-300.

Itoh S. 1987. Characteristics of phosphorus uptake of chickpea in comparison with pigeon pea, soybean and maize. *Soil science and plant nutrition* 33:417-422.

James, R.R., DiFazio, S.P., Brunner, A.M., and Strauss, S.H. 1998. Environmental effects of genetically engineered woody biomass crops. *Biomass and Bioenergy* 14: 403-414.

Keeler, K.H. 1988 Can we guarantee the safety of genetically engineered organisms in the environment? *Critical Review in Biotechnology* 8: 85-97.

Keya S., and Alexander M. 1975. Regulation of parasitism by host density: the *Bdellovibrio-Rhizobium* interrelationship. *Soil Biology and Biochemistry* 7: 231-237.

Keyser H.H., and Munns D.N. 1979. Tolerance of Rhizobia to acidity, aluminum and phosphate. *Soil Science Society of America Journal* 43: 519-523.

Kinnback A., and Werner D. 1991. Glucosidases (α, β-) and trehalase (α-) in the peribacterioid space and the bacteroids periplasm of *Glycine max* root nodules. *Plant Science* 77: 47-55.

Kirchner M.J., Wolum A.G., and King L. D. 1993. Soil microbial population and activities in reduced chemical input agroecosystems. *Soil Science Society of America Journal* 57: 1289-1295.

Kvien C.S., Ham G.E., and Lambert J.W. 1981. Recovery of introduced strains by soybean genotypes. *Agronomy Journal* 73: 900-905.

Kyei-Boahen S., Slinkard A.E., and Walley F.L. 2001. Rhizobium survival and nodulation of chickpea as influenced by fungicide seed treatment.

Canadian Journal of Microbiology 47: 585-589.

Lacy G.H., and Stromberg V.K. 1991. Pre-release microcosm tests with a genetically engineered plant pathogen. In: Biological monitoring of genetically engineered plant and microbes, proceedings of the Kiawah island conference (Mackenzie D.R., and Henry S.C. Eds.). P 81-98. Agricultural Research Institute Bethesda, Bethesda, MD.

Levin M.A., Seidler R.J., Bourquin A.W., Fowle J.R., and Barkay T. 1987. EPA developing methods to assess environmental release. *Biotechnology* 5: 38-45.

Li D.M., and Alexander M. 1986. Bacterial growth rates and competition affect nodulation and root colonization by *Rhizobium meliloti*. *Applied and Environmental Microbiology* 52:807-811

Lindsay W.L. 1979. Chemical equilibria in soils. John wiley & Sons, New York.

Lipsanen P., and Lindstrom K. 1986. Adaptation of red clover rhizobia to low temperatures. *Plant and Soil* 92: 55-62.

Lorenz M.G., and Wackernagel W. 1993. Bacterial gene transfer in the environment. In: Worhrmann K. and Tomiuk J. (ed.) Transgenic Organisms: risk assessment of deliberate release. 43-64.

Lorenz M.G., and Wackernagel W. 1990. Natural genetic transformation of *Pseudomonas stutzeri* by sand-absorbed DNA. *Archives of Microbiology* 154: 380-385.

McGrath J.W., Hammerschmidt F. and Quinn J.P. 1998. Biodegradation of phosphonomycin by *Rhizobium huakii* PMY1. *Applied and Environmental Microbiology* 64: 356-358.

McKay I.A., and Djordjevic M.A. 1993. Production and excretion of *nod* metabolites by *Rhizobiummm leguminosarum bv. trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. *Applied and Environmental Microbiology* 59: 3385-3392

Mellor H.Y., Glenn A.R., Arwas R., and Dilworth M.J. 1987. Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* TA1. *Archives of Microbiology* 148: 34-39.

Minder A.C., NarberHaus F., Fischer H., and Hennecke H. 1998. The *Bradyrhizobium japonicum pho*B gene is required for phosphate limited growth but not fro symbiotic nitrogen fixation. *FEMS Microbiology Letters* 161: 47-52.

Moawad H.A., Ellis W.R., and Schmidtt E.L. 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field grown soybeans. *Applied and Environmental Microbiology* 47: 607-612.

Mpepereki S., Wollum A.G., and Makonese F. 1996. Growth temperature characteristics of indigenous *Rhizobium* and *Bradyrhizobium* isolates from Zimbabwean soils. *Soil Biology and Biochemistry* 28: 1537-1539.

Mullen M.D., Lssrael D.W., and Wollum A.G. 1988. Effects of *Bradyrhizobium japonicum* and soybean (*Glycine Max Merr.*) phosphorus nutrition on

nodulation and dinitrogen fixation. *Applied and Environmental Microbiology*. 54: 2387-2392.

Nye P.H. 1979. Soil properties controlling the supply of nutrients to root surfaces. In: Harley J.L. and Russel R.S. (eds), The soil root interface. Academic Press Inc., London.

Paul J.H., Frischer M.E., and Thurmond J.M. 1991. Gene transfer in marine water column and sediment microcosms by natural plasmid transformation. *Applied and Environmental Microbiology* 57: 1509-1515.

Pereira P.A., and Bliss F.A. 1987. Nitrogen fixation and plant growth of common bean *(Phaseolus vulgaris L.)* at different level of phosphorus availability. *Plant and Soil* 104: 79-84.

Pereira P.A., and Bliss F.A. 1989. Selection of common bean *(Phaseolus vulgaris L.)* for N2 fixation at different level of availability phosphorus under field and environmentally controlled conditions. *Plant and Soil* 115: 75-82.

Rai R. 1983 The salt tolerance of Rhizobium strains and lentil genotypes and the effect of salinity on aspects of symbiotic N-fixation. *Journal of agriculture Science* 100: 81-86.

Redente E.F., and Reeves F.B. 1981. Interactions between vesicular-arbuscular mycorrhiza and Rhizobium and their effect on sweetvetch growth. *Soil Science* 132:410-415.

Reibet J., and Drevon J.J. 1995. Increase in permeability to oxygen and in oxygen uptake of soybean nodule under limiting phosphorus nutrition. *Physiologia Plantarum.* 94:298-304.

Robson A.D., O'Hara G.W., and Abbott L.K. 1981. Involvement of phosphorus in nitrogen fixation by subterraneum clover (*Trifolium subterraneum L.) Australian Journal of Plant Physiology.* 8: 427-436.

Roughley R.J., Simanungkalit R.D.M., Gemell L.G. Hartley E.J. and Cain P. 1995. Growth and Survival of root-nodule bacteria in legume inoculants stored at high temperatures. *Soil Biology and Biochemistry* 27: 707-712.

Ruiz-Saninz J.E., Beringer J.E., and Gutierrez-Navarro A.M., 1984. Effect of the fungicide captafol on the survival and symbiotic properties of *Rhizobium trifolii. Journal of Applied Bacteriology* 57: 361-367.

Sa T.M., and Isreal D.W. 1991 Energy status and functioning of phosphorus-deficient soybean nodules. *Plant Physiology* 97:928-935.

Sahrawat K.L., Rao B.S. and Nambiar P.T.C. 1988. Macro-and Micronutrient uptake by nodulating and non-nodulating peanut lines. *Plant and Soil* 109: 291-293.

Salminen S.O., and Streeter J.G. 1987. Uptake and metabolism of carbohydrates by *Bradyrhizobium japonium* bacteroids. *Plant Physiology* 83: 535-540.

Samrt J.B., Dilworth M.J., and Robson A.D. 1984a. Effects of phosphorus supply on phosphate uptake and alkaline phosphatase activity in Rhizobia. *Archives of Microbiology* 140: 281-286.

Samrt J.B., Dilworth M.J., and Robson A.D. 1984b. Effects of phosphorus supply on Periplasmioc protein profiles in Rhizobia. *Archives of Microbiology* 140: 287-290.

Seidler R.J., and Settel J. (eds) 1991. The use and development of environmentally controlled chambers (mesocosms) for evaluating biotechnology products. US EPA/600/9-91/013.

Selbitschka W., Jording D., Mieman S., Schmidt R., Puhler A., Mendum T., and Hirsch P. 1995. Construction and Characterization of a *Rhizobium leguminosarum* biovar *viciae* strain designated to assess horizontal gene transfer in the experiment. *FEMS Microbiology Letters* 128: 255-263.

Singleton P.W., Elswaify S.A., and Bohlool B.B. 1982. Effect of salinity on *Rhizobium* growth and survival. *Applied and Environmental Microbiology* 44: 884-890.

Stewart G.J., and Sinigalliano C.D. 1990. Detection of horizontal gene transfer by natural transformation in native and introduced species of bacteria in marine and synthetic sediments. *Applied and Environmental Microbiology* 56: 1818-1824

Tabatabai M.A. 1994. Soil enzymes. In: Methods of Soil Analysis. Part2-Microbiological and Biochemical Properties. Weaver R.W., Angel J.S., and Bottomley P.S. (eds). Soil Science Society of America, Inc. Wisconsin. Pp. 775-833.

Tan I.K.P., and Broughton W.J. 1982. Rhizobia in tropical legumes – XIV. Ion uptake differences between fast – and slow – growing strains. *Soil Biology and Biochemistry*. 14: 295-299.

Tao H., Brewin N., and Noel K.D. 1992. *Rhizobium leguminosarum* CFN42 lipoplysaccharde antigenic changes induced by environmental conditions. *Jouranl of Bacteriology* 174:2222-2229.

Trevors J.T., Van Elsas J.D., Van Overbeek L., and Starodub M.E. 1990. Transport of a genetically engineered *Pseudomonas fluorescens* strain through a soil microcosm. *Applied and Environmental Microbiology* 56: 401-408.

Trotman A.P., and Weaver R.W. 1995. Tolerance of clover *rhizobia* to heat and desiccation stress in soil. *Soil Science Society of America Journal* 59: 466-470.

Truesdell H.W. 1917. The effect of phosphorus on alfalfa and alfalfa bacteria. *Soil Science* 3: 77-98.

Van Elsas J.D., and Trevors J.T. 1991. Environmental risks and fate of genetically engineered microorganisms in soil. *Journal of Environmental Science and Health* 26:981-1001.

Van Elsas J.D., Govaert J.M., and Van Veen J.A. 1987. Transfer of plasmid pFT30 between bacilli in soil as influenced by bacterial population dynamics and soil conditions. *Soil Biology and Biochemistry* 19: 639-645

Van Elsas J.D., Trevors J.T., Van Overbeek L.S., and Starodub M.E. 1989. Survival of *Pseudomonas fluorescenscontaining* plasmids RP4 and

pRK2501 and plasmids stability after introduction into two soils of different texture. *Canadian Journal of Microbiology* 35: 951-959.

Van Elsas J.D., Van Overbeek L.S., Feldman A.M., Dullmans A.M., and De Leeuw O. 1991. Survival of genetically engineered *Pseudomonas fluorescenscontaining* in soil in competition with parent strain. *FEMS Microbiology Ecology* 85: 53-64.

Vandecaveye S.C. 1927. Effect of soil moisture, temperature, and other climatic condition on *R. leguminosarum* in the soil. *Soil Science* 23: 355-362.

Vidopr C., and Miller R.H. 1980. Relative saprophytic competence of *Rhizobium japonicum* strain in soils as determined by the quantitative fluorescent antibody technique. *Soil Biology and Biochemistry* 12:483-487.

Voegele R.T., Bardin S., and Finan T.M. 1997. Characterization of the *Rhizobium (Sinorhizobium) meliloti* high- and low- affinity phosphate uptake systems. *Journal of Bacteriology* 179: 7226-7232.

Walter M.V., Porteous L.A., Prince V.J., Ganio L, Seidler R.J. 1991. A microcosm for measuring survival and conjugation of genetically engineered bacteria in rhizosphere environment. *Current Microbiology* 22: 117-121.

Wang E.T., and Martinez-Romero E. 2000. Phylogeny of root-and stem-nodule bacteria associated with legumes. In: Triplett E.W. (ed.) Prokaryotic Nitrogen Fixation: A model System for the Analysis of a Biological Process. pp: 177-186 Horizon Scientific Press. Wymondham. England

Watkin E.L.J., O'Hara G.W., and Glenn A.R. 1997. Calcium and acid stress interact to affect the growth of *Rhizobium leguminosarum* bv. *trifolii*. *Soil Biology and Biochemistry* 29: 1427-1432.

Werner D., and Berghauser K. 1976. Discrimination of *Rhizobium japonicum*, *Rhizobium lupini*, *Rhizobium leguminosarum* and bacterioids by uptake of 2-Ketoglutaric acid, glutamic acid and phosphate. *Archives of Microbiology* 107: 257-262.

Wilkins J. 1967. The effects of high temperature on certain root-nodules bacteria. *Australian Journal of Agriculture Research* 18: 299-304.

Zeph L.R., Onaga M.A, and Stotzky G. 1988. Transduction of *Escherichia coli* by bacteriophage P1 in soil. *Applied and Environmental Microbiology* 54: 1731-1740.

Zhan H., Lee C., and Leigh J.A. 1991. Induction of the second exopolysaccharide (EPSb) on *Rhizobium meliloti* SU-47 by low phosphate concentration. *Journal of Bacteriology* 173: 7391-7394.

Chapter III

SURVIVAL AND PERSISTENCE OF *SINORHIZOBIUM MELILOTI* STRAIN 104A14 IN SOIL

Abstract

Survival and persistence of *Sinorhizobium meliloti* 104 A14 and its two acid phosphatase-negative mutants in soil were evaluated. Three soils with different P levels ranging from 8 to 57 mg P kg^{-1} soil (Mehlic 3-Murphy Riley P) were sterilized, inoculated with *S. meliloti* and incubated for up to 64 days. The low-P soil has not been fertilized, while the high-P soil has been applied 14.6 kg P ha^{-1} for over 70 years. The medium-P soil was treated with cattle manure for over a century. Results indicated that *S. meliloti* recovered was about 100% immediately after inoculation, but decreased to undetectable level in the low-P soil within 32 days, and the high-P soil within 64 days. Approximately 2-3% of the mutants and 23% of the wild-type inocula were recovered from the medium-P soil after 64 days. When *S. meliloti* was introduced with alfalfa seedlings in sterile and non-sterile soils, 83 to 91% of bacteria isolated from nodules grown in the sterile soil and 15 to 48% from those in the non-sterile soils were the inoculated strains. However, results obtained from repeating these experiments in the same set of soils one month after harvest without additional inoculation demonstrated dominance of the inoculated strains in both sterile and non-sterile soils, 85 to

100%. These results suggested that inoculated strains out competed the indigenous *S. meliloti* in nodule formation after 70 days of initial inoculation.

Introduction

Symbiotic nitrogen (N) fixation between rhizobium and legume plant is a phosphorus depended process. Every aspect of symbiosis, from symbiotic partners, plant and rhizobium to symbiosis processes, is influenced by phosphorus (Cassman et al., 1981b; Samrt et al., 1984b; McKay and Djordjevic, 1993; Reibet and Drevon, 1995). Addition of fertilizer-P not only increased nutrient uptake and dry matter of plants but also the formation, number, mass and function of the nodules (Pereira and Bliss 1987; Howieson et al., 1993; Isreal, 1993; McKay and Djordjevic, 1993; Reibet and Drevon, 1995). Phosphate affects rhizobia by enhancing bacteria growth and capacity of P storage, and altering pattern of periplasmic enzymes and phenotypes of the cell (Cassman et al., 1981b; Samrt et al., 1984b; Zhan et al., 1991; Tao et al., 1992). Moreover, symbiotic legume plants require more P than those that gain N from fertilizer (Israel, 1987).

Some rhizobia, such as *Rhizobium huakuii* and *Bradyhizobium japonicum*, have been shown to use organic P as a sole source of P (Salminen and Streeter, 1987; McGrath et al., 1998;). However, inorganic P is believed to be the most common and available form used for plant and microbial growth (Bieleski, 1973; Beever and Burns, 1986). With 20-80% of the total P in the organic form in most soils (Dalal, 1977), phosphatases may play a crucial role converting organic P to

inorganic forms for plant and microbial uptake. This is evidenced by increased activities of alkaline and /or acid phosphatases of rhizobium under limiting inorganic P conditions (Samrt 1984a; Al-Niemi, 1997;). Naturally, assumption was made that phosphatases may affect legume-rhizobia symbiotic performance under P limiting condition. Interestingly, single mutation of each of the two acid phosphatases identified in *S. meliloti* did not affect symbiotic performance significantly regardless of P levels in the growth medium (Deng *et al.*, 1998 & 2001). This leads to the question whether mutation of acid phosphatase impaired its ability to survive and persist in the soil environment under limiting available P conditions.

Although survival and persistence of rhizobia in soil have been studied extensively, most of the studies focused on biotic factors, such as competition with indigenous microbes, predation, and abiotic factors, such as soil pH, moisture, temperature, and fungicide (Ramirez and Alexander, 1980; Soa-Afiana and Alezander, 1982; Moawad et al., 1984; Ruiz-Saninz et al., 1984; Brockwell et al., 1991). Host plants have been shown to play a positive role in promoting rhizobial growth. Studies demonstrated that rhizobial population increased from less than 30 to 10^3 CFU g^{-1} dry soil due to the presence of its host plant (Vlassak, 1996). However, the ability of rhizobium to grow and survive at low P concentrations varied among strains (Cassman et al., 1981a; Beck and Munns, 1984). Among 5 rhizobia strains tested, two of them were able to grow at as low as 0.06 μM P, while others, such as *R. trifolii* and *R. meliloti* stopped growth (Beck and Munns, 1984). Organic P has been shown to be an important P

source under limiting available P condition, suggesting that phosphatases play a crucial role (Salminen and Streeter, 1987; McGrath et al., 1998). Nevertheless, little information is available on the role of phosphatases in relation to survival and persistence of rhizobia in the soil environment. With the creation of acid phosphatase-negative mutants, it is now possible to fill in this gap.

Therefore, the objectives of this study are: (1) To assess mutation of acid phosphatase genes in relation to survival of *S. meliloti* in soil; and (2) To determine effect of alfalfa growth on establishment and persistence of *S. meliloti* in the soil environment.

Material and methods

Survival of *S. meliloti* strains in sterile soils

Soil

Surface soil (0-15cm) samples were taken from a long-term winter wheat (*Triticum aestivum L*) experiment located in central Oklahoma, U.S.A. The soil is a Kirkland (fine, mixed, thermic Udertic Paleustolls) silt loam with a mean particle-size distribution of 37.5% sand, 40.0% silt, and 22.5% clay. Soil 1 has not been applied fertilizer for over a century, soil 3 has been applied 14.6 kg P ha^{-1} every year for 70 years, and soil 2 was treated with cattle manure every four years at approximately 81 kg P ha^{-1} for over a century. These treatments resulted in varied soil properties (Table 1).

Table 1. Properties of soils used.

Soil[†]	pH[‡]	Organic C[§]	Total N[§]	MRP*	ICP-P[#]
		(g C kg $^{-1}$ soil)	(g N kg $^{-1}$ soil)	------- (mg P kg $^{-1}$ soil) -------	
1	4.85	6.70	0.67	8	21
2	5.70	9.00	0.86	22	33
3	4.70	7.80	0.77	57	65

[†]Soil used in this study are Kirkland silt loam that have been under different fertilizer treatment for over 70 years. Soil 1 is an untreated control; soil 2 was treated with cattle manure every four years at 269 kg N ha^{-1} (approx. 89.7 kg P ha^{-1}) for over a century; and soil 3 was applied 14.6 kg P ha^{-1}yr^{-1} for 70 years.

[‡]Soil : 0.1M $CaCl_2$ ratio = 1: 2.5

[§]Organic C and total N were detected by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Surphur Analyzer (Schepers et. al., 1989).

*MRP=P concentration in Mehlich-3 (Mehlich, 1984) extracts determined by Murphy and Rilry method (1962).

[#]ICP-P = P concentration in Mehlich 3 (Mehlich, 1984) extracts determined by Inductively Coupled Plasma Spectrometer (ICP).

Field-moist soils were ground, sieved through a 2-mm screen, air-dried and stored at room temperature. The pH value was determined by using a combination glass electrode followed the procedure described by Thomas G.W. (1996). The organic C and total N was detected by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Surphur Analyzer (Schepers et. al., 1989). Phosphorus level in Mehlich-3 extracts was determined by both Murphy and Riley method (MRP) and by Inductively Coupled Plasma Spectrometer (ICP-P) (Murphy and Riley, 1962; Mehlich, 1984).

Bacteria strain and preparation of inoculants

Wild-type *S. meliloti* 104 A14 and two acid phosphatase-negative mutants derived from this strain, NapD and NapE, were used in this study. Creation and characterization of the mutants have been reported by Deng et al. (1998 & 2001). Bacteria were cultured in YMB media for 48 hrs at 28°C shaking at 200 rpm. The cells were pelleted by centrifuging for 5 min at 5,000 rpm, washed twice with 0.85% NaCl (W/V) solution, and then, re-suspended to approximately 10^8 CFU g^{-1} soils based on estimation from culture density. The actual cell concentrations were determined by plate count on YMA plates.

Incubation

20-g of soil was placed in a 50-ml flask and covered with aluminum foil. The total weight was recorded. Soil 1 and 3 were adjusted to pH 5.7 with modified universal buffer (MUB) (Tabatabai, 1994) with addition of water to reach

60% field capacity. Soil 2 (has pH 5.7) was adjusted to 60% field capacity with water. The soils were autoclaved four times in consecutive four days with 35 minutes each time. After each autoclave, the soil was weighted again and sterile water was added to adjust the loss of water.

Sterile soils were inoculated with the wild-type *Sinorhizobium meliloti* and its two mutants, NapD and NapE, at about 10^8 CFU g^{-1} soils. In the control soil, equivalent volume of sterile 0.85% NaCl (W/V) solution was added. The soils were incubated at 28°C for up to 64 days. Water in an open container was placed inside the incubator to keep the humidity at a near saturation level. Triplicate treatments were performed.

Enumeration of S. meliloti in soil

Survival of *S. meliloti* strains during incubation in the three soils tested was determined by enumerating *S. meliloti* population at day 0, 1, 2, 4, 8, 16, 32, 64 using a modified procedure described by Zuberer (1996). Briefly, the 20-g soil in each flask was transferred aseptically to 500-ml flasks containing 180 ml sterile water and sodium pyrophosphate (0.18% final concentration). The mixtures were shaken at 200 rpm on a rotary shaker for 15 minutes. After settling for about 60 seconds, a 5-ml solution was transferred to a 125-ml flask containing 45-ml of sterile Ringer Solution. Serial dilutions were performed by transferring 1-ml of each dilution mixture to 9-ml of sterile Ringer Solution. Then, 100-ul of a dilution was spread on yeast-mannitol agar (YMA) plates in tetra-plicate. Kanamycine-containing YMA plates were used for the two acid

phosphatases-negative mutants while plain YMA plates for the control and wild type. Colony forming units (CFU) were counted after 3-4 days incubation at 30°C.

Effect of alfalfa growth

Soil

Surface (0-15 cm) Renfrow soil (Udertic Paleustolls, silty clay loam) was taken from Central Oklahoma, USA. Soils were ground, sieved, air-dried and stored at room temperature. The pH value was 6.53, determined by using a combination glass electrode and procedure was followed the procedure described by Thomas G.W. (1996). The organic C was 11.4 g kg^{-1} soil and total N was 1.16 g kg^{-1} soil, determined by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Surphur Analyzer (Schepers et. al., 1989).

Microcosm and plant growth

A soil sample (200 g, < 2 mm) was placed into a Magenta box, which was part of a plant growth unit described by McDermott and Kahn (1992). The soil was adjusted to 60% field capacity with water. Plant growth units in one experiment set were autoclaved for four times in consecutive four days with 60 minutes each time, while those in another experiment set were kept non-sterile. Each plant growth unit was filled with 200 ml nutrient solution for alfalfa growth. The nutrient solution was modified from that described by Wych and Rain (1978) and contained 1.0mM K_2SO_4, 0.5mM KH_2PO_4, 0.25mM K_2HPO_4, 0.5mM

$MgSO_4.7H_2O$, 2.0mM $CaSO_4.2H_2O$, 25uM KCl, 13uM H_3BO_3, 1.0uM $MnSO_4.H_2O$, 1.0uM $ZnSO_4.H_2O$, 0.25uM $CuSO_4.5H_2O$, 2.5uM $CoCl_2.6H_2O$, 20uM $FeCl_3.6H_2O$, and 0.25uM $NaMoO_4.2H_2O$. The solution was sterilized after adjusting to pH 6.5 with KOH and/or HCl.

Alfalfa seeds were surface sterilized with 95% ethanol for 30 seconds, 3% sodium hypochlorite for 5 minutes, rinsed at least six times with sterile, distilled water, and then germinated on YMA plates. Uncontaminated seedlings were transferred to plant growth units with soil (four seedlings per unit) and inoculated immediately with washed cells of *S. meliloti* strains that were prepared as described above. The alfalfa plants were grown in a growth chamber for 5-6 weeks with 16 h of photoperiod and 19/24°C of night/day cycles.

Isolation of rhizobia from alfalfa nodules

Nodules were recovered from the roots, and then surface sterilized by immersing in 70% ethanol for 10 seconds, 1.5% sodium hypochlorite for 1 minutes and sterile distilled water six times. The surface sterilized nodules were homogenized 200 µl 0.85% saline solution in microcentrifuge tubes. Serial dilutions of the nodule suspension were spread on the YMA plates and incubated at 28°C for 3 days. Forty to 50 isolated colonies were picked up randomly from each treatment, purified on fresh YMA plates, and stored in 40% glycerol at –80°C for further studies.

Colony hybridization

A 3-µl of late log-phase YMB culture (approx. 24 hrs) of each isolated bacterium at approximately 10^6 CFU μl^{-1} was placed on an YMA plate. Wild-type *S. meliloti* was used as a positive control and YMB was used as a negative control. The plate was then incubated overnight at 30°C for additional growth. Colonies were lifted using a nylon membrane (Immobilon™-Ny+, Millipore Corp. Bedford, MA). Cells were lysed under a high salt alkaline condition (0.5 N NaOH, 1.5 M NaCl) and the released DNA was fixed with UV crosslinking.

The membrane with fixed bacteria DNA was washed with prewashing solution (5x SSC, 0.5% SDS and 1mM EDTA) for 30 minutes at 50°C with gentle agitation. After washing, membrane was prehybridized with hybridization buffer (0.5M Sodium phosphate (pH 7.1), 2 mM EDTA, 7% (w/v) SDS and 0.1% (w/v) Sodium pyrophosphate) for 2 hours at 65°C, and then hybridized with *Nap*D or *nif*H probe overnight at 65°C with gentle agitation. The chemiluminescent signals that resulted from hybridization between labeled probe and bacteria DNA were developed and detected (Boehiringer Mannheim). Probes were prepared by PCR using primers specific for *Nap*D and *nif*H genes (Table 2) and genomic DNA of wild-type *S. meliloti* as a template, which were labeled by random priming using the DIG-DNA labeling kit (Boehiringer Mannheim).

Polymerase Chain Reaction (PCR)

Unless specified, PCR amplification was performed on an automated thermal cycler (MJ Research Inc. PTC-100) with an initial denaturation (94°C for

120 seconds), following by 30 cycles of denaturation (94°C for 45 seconds), annealing (65°C for 30 seconds) and extension (72°C for 120 seconds), and a single final extension (72°C for 10 minutes).

Amplified ribosomal DNA restriction analysis (ARDRA)

16S rDNA (about 1.6kb) of bacteria were generated by PCR using universal primer (Table 2). Approximately 1.0-1.5 μg 16S rDNA was digested at 37°C for at least 3 hours using *Alu*I, *Hae*III, *Hpa*II, and *Rsa*I, respectively (GIBCOBRL®). The digested DNA was separated by 8% polyacrylamide gel in TBE buffer and banding patterns were visualized by straining in ethidium bromide solution (0.5 μg ml^{-1}). PCR and enzyme restriction digestion were repeated to confirm the obtained results and minimize PCR bias.

Results and Discussion

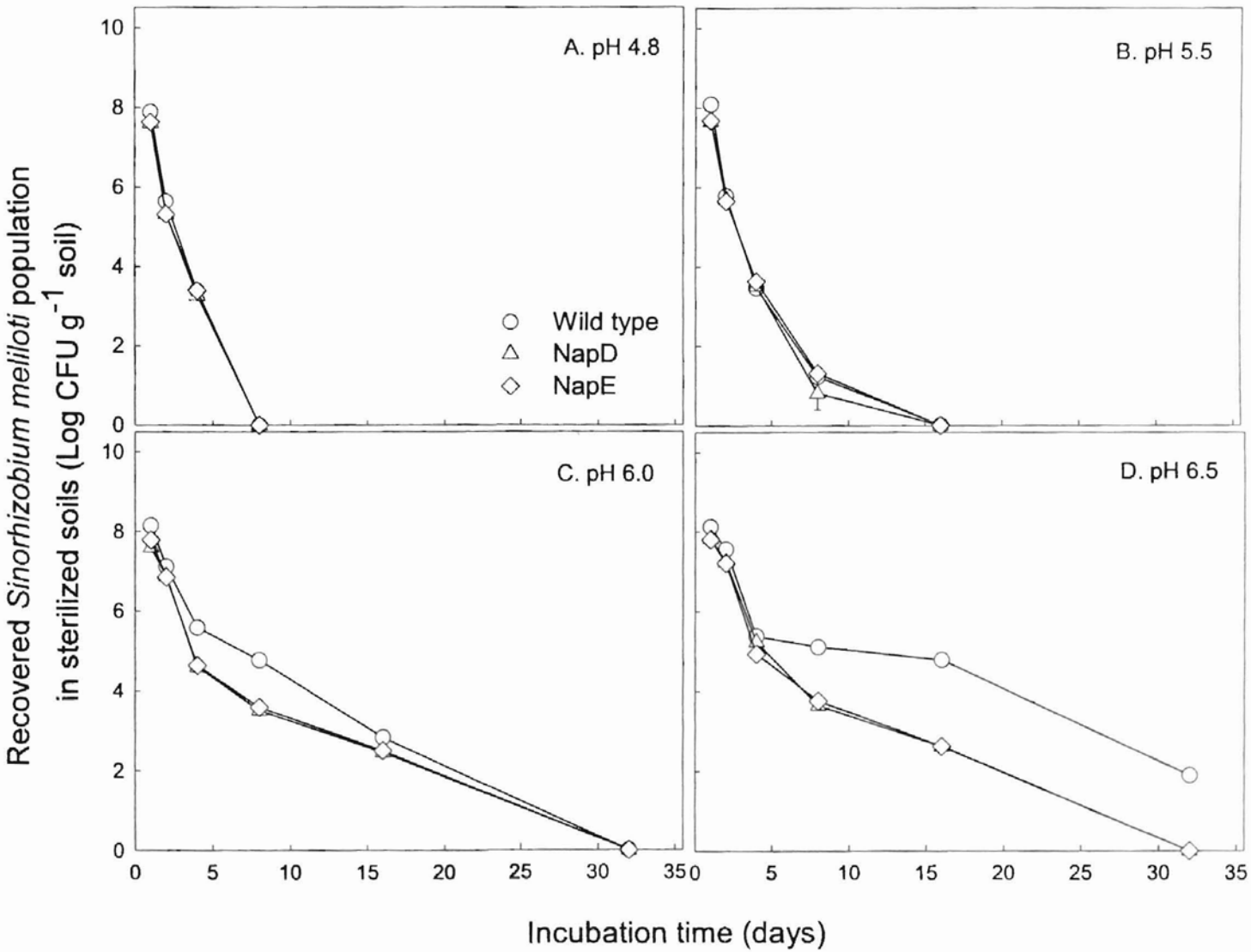
Survival of *S. meliloti* strains in soil

Sinorhizobium meliloti is an acid sensitive species (Graham and Parker, 1964; Robson and Loneragn, 1970). Study on survival of *S. meliloti* in soil 1 at pH values ranging from 4.8 to 6.5 showed that the survivability of *S. meliloti* increased with increasing pH. This strain did not last 10 days when soil was pH 4.8, while it survived more than 30 days when soil pH was increased to 6.5 (Figure 1). These results were consistent with those obtained by Rice et al. (1977). They studied nitrogen fixation by alfalfa in relation to *S. meliloti*

Table 2. Nucleotide sequences of primers used for polymerase chain reactions (PCR) in this study.

Traget gene	Primer	Sequence	Reference
16S rDNA	1	5' - AGA GTT TGA TCC TGG CTC AG - 3'	Toyota et al. (1999)
	2	5'- GGT TAC CTT GTT ACG ACT T - 3'	
Nap D	*3*	5' - GCA AAG AAT ACG GCC AGG AG - 3'	Deng et al. (1998)
	4	5' - ACG CGC CCT ATC CTG TCT CA - 3'	
nif H	*5*	5' - GTT CGG CAA GCA TCT GCT CG - 3'	Eardly et al. (1992)
	6	5' - AAG TGC GTG GAG TCC GGT GG - 3'	

Figure 1. Effect of soil pH on survival and persistence of introduced wild-type and genetically modified *Sinorhizobium meliloti* in sterile soil 1. Soil pH was adjusted to desired pH values using Modified Universal Buffer (Tabatabai, 1994)



population in limed and unlimed soils with pH ranging from 4.5 to 7.2. *Sinorhizobium meliloti* population was found to be around 6 log CFU g^{-1} soil in soils with pH around 7 while it was as low as 1 log CFU g^{-1} soil in soils with pH around 4.5 (Rice et al., 1977). Increasing *S. meliloti* population with increasing pH has been reported for soils with pH values as high as pH 8 (Brockwell et al., 1991).

Among the three soils used in this study, soil 2 has the highest pH values (Table 1). Studies demonstrated that addition of MUB buffer increased the pH values of soil 1 and 3 to as high as 5.9 (Figure 2). Therefore, it is possible to adjust pH values of soil 1 and 3 to that of soil 2 (pH 5.7) and to evaluate effect of factors other than pH on survival of *S. meliloti* in soil.

Results showed that soil properties are among the major factors dictating life of *S. meliloti* in soil (Figure 3). Although introduced *S. meliloti* was recovered about 100% immediately after inoculation in all the soils tested, none of the inoculated *S. meliloti* lasted 32 days in soil 1. However, over 20% of inoculated population survived in soil 2 for over 64 days. The wild-type *S. meliloti* demonstrated significantly higher survivability than the genetically modified mutants (Figure 3).

This lead to the question: What is so unique about soil 2? Since the tested strains included phosphatase mutants, special attentions were placed on soil P levels. As shown in Table 1, available P levels were 8, 22, and 57 mg P kg^{-1} soil in soil 1, 2 and 3, respectively. In addition, these soils also have varied contents of organic C and total N.

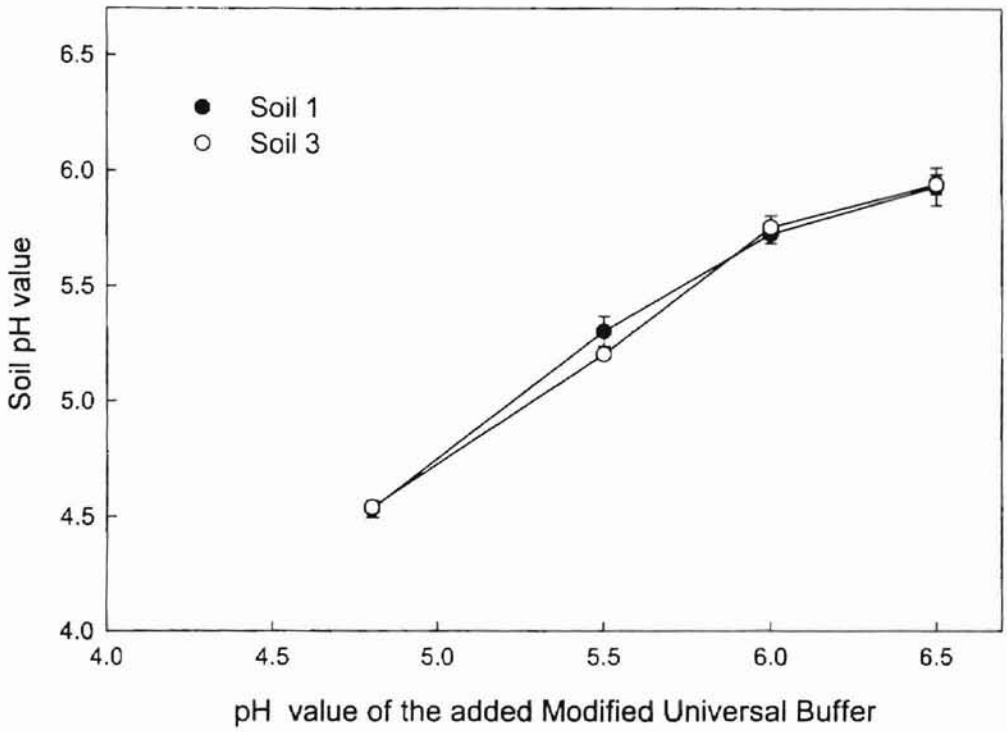


Figure 2. Adjusting soil pH by Modified Universal Buffer (MUB).

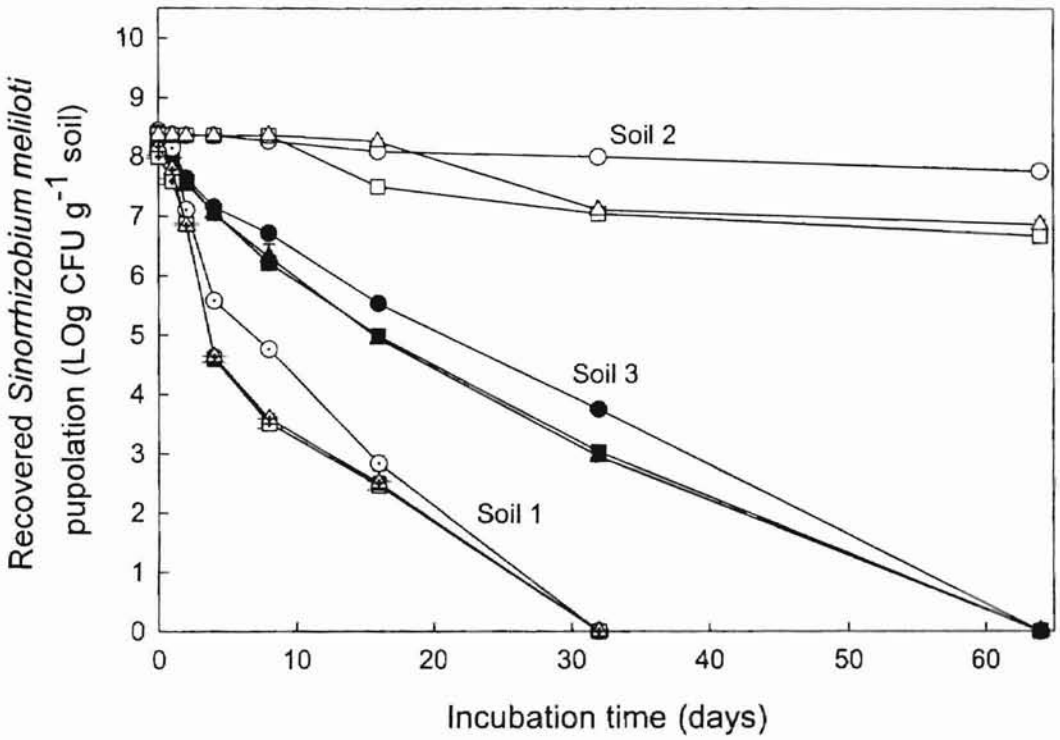


Figure 3. Survival and persistence of inoculated *Sinorhizobium meliloti* (wild-type and its acid phosphatase-negative mutants) in sterile soils at pH 5.7. The circles, triangles, and rectangles indicate inoculation with the wild-type, NapD and NapE, respectively.

Results suggested that likely available P was not limiting in soil 2, but in soil 1. This is supported by results reported by Beck and Munns (1984). Their studies showed that *S. meliloti* could not grow in the media with 0.06 μM P or less (about 10 mg P kg^{-1} soil). Soil 2 was shown to be a more favorable environment for growth of *S. meliloti* could be simply due to higher organic matter content in this soil enriched from century-long application of cattle manure. Since all the soils tested have low organic matter content (< 1%), availability of C in these soils may be at the limiting borderline. It is well known that available C source in the soil is vital for microbial growth and survival. When a culture media was in the short of carbon source, a massive decrease (10,000 times) of the *R. meliloti* number was reported (Clarke et al., 1993).

The results obtained support findings in that GMMs require extra metabolic load for additional genes, which affect their ability to survive and grow in the environment (DaSilva and Bailey, 1986; Orvos et al., 1990; Van Elsas *et al.*, 1991). Under P-limiting condition, such as soil 1, presence of both acid phosphatase genes may also strengthen growth of the wild-type by its greater ability to utilize available P.

The persistence of two mutants was not significantly different in all the soils tested (Figure 3). This is consistent with previous findings in that mutation of either acid phosphatase gene did not result in significantly different symbiotic phenotypes regardless supplemented P concentrations (Deng et al., 1998). It is possible that the two acid phosphatases present two different pathways for P

acquisition in *S. meliloti*. Blockage of one pathway may be compensated by the other. Construction of a double mutant is needed to validate the hypothesis.

Presence of alfalfa plant

Effectiveness in nodule formation and persistence of introduced *S. meliloti* in sterile and non-sterile soils were tested for two alfalfa growth periods with 6 weeks each and one-month interval in between.

After the first planting period, 83% to 91% of bacteria isolated from nodules grown in sterile soil and 15-48% from non-sterile soils were the inoculated strains (Table 3). This percentage increased to 85 to 100% in non-sterile soils when alfalfa seedlings were planted again without inoculation one month after the prior harvesting, suggesting that inoculated strains out competed the indigenous *S. meliloti* in nodule formation after 70 days of initial inoculation.

Dominance of introduced strain in nodule formation in sterile soil was expected, but somewhat surprising in the second growth period in non-sterile soils. In a similar study conducted in the field on nodule occupancy by inoculated *R. tropici*, the reverse situation was reported (Vlassak et al., 1996). Nodule occupancy by inoculated *R. tropici* was dominant (80%) in the first year but not in second year (only 15%) without re-inoculation (Vlassak et al., 1996). It is possible that this difference was partially due to differences in strains tested, soils used, and the length of interval between planting in these two studies. In their study, there was about 10 months interval between planting while only one month in this

Table 3. Recovery of inoculated wild-type and genetically modified Sinorhozobium meliloti 104A14 from nodules of alfalfa growing in sterile and non-sterile soils. The isolates were verified by colony hybridization using an acid phosphatases gene (*NapD*) as a probe.

Experiment[‡]	Treatment[§]		Bacteria isolates from nodule		Isolates tested	Positive single[¥]
	Soil	Strains	Morphology type*	Number of isolates[#]		
Frist	Sterile	W.T.	A	12	6	0
			B	4	2	0
			C	190 (206)	42	38 (83%)
		NapD	B	12	8	0
			C	175 (175)	42	41 (91%)
		NapE	C	179 (179)	40	36 (90%)
	Non-sterile	Control	B	157 (157)	40	0
		W.T.	B	131	25	0
			C	56 (187)	25	25 (15%)
		NapD	C	201 (201)	40	24 (48%)
		NapE	B	128	24	0
			C	88 (216)	26	26 (22%)
Second	Sterile	W.T.	C	156 (156)	40	39 (97%)
		NapD	C	204 (204)	40	40 (100%)
		NapE	C	179 (179)	40	38 (95%)
	Non-sterile	Control	B	119 (119)	40	0
		W.T.	C	224 (224)	40	38 (95%)
		NapD	C	166 (166)	40	40 (100%)
		NapE	B	17	6	0
			C	97 (114)	36	36 (85%)

[†]Colony hybridization was conducted by lifting 24 hr-old bacterial colonies to a nylon membrane from palte. Cells were lysed and DNA release were fix to the membrane for subsequent hybridization using NapD as a probe and chemiluminescent detection.

[‡]First experiment refer to growth of alfalfa in soil immediately following inocualtion of the tested strains. Alfalfa nodules formed were harvested under sterile conditions after 6 weeks of growth. Second experiment refers to alfalfa growth in the same set of soil one month after the first harvest without addition inoculation. Nodules were obtained again after 6 weeks of growth.

[§] W.T., NapD and NapE refer to the wild type *S. meliloti* 104A14 and two acid phosphatase negative mutants, respectively. (Deng et al., 1998). Inoculated cell number of wild-type, NapD, and NapE were 1.18×10^8, 3×10^8 and 1.77×10^8 (CFU g^{-1} soil), respectively.

* A, B, and C indicated the bacteria isolates with different morphology. A-type appeared bright yellow. B and C-type were white colonies.

[#] Figures in the parentheses are the total colonies obtained from each treatment.

[$] Positive isolates refers to those that hybridized with *Nap* D. Figures in the parentheses indicate positive isolates expressed as percentage of total number of isolates obtained.

study (Vlassak et al., 1996). This suggests that host plants play an important role in promoting growth of introduced strains.

However, our results on nodule occupancy by the introduced strains in the first growth period of non-sterile soil are consistent with those reported by Evans et al. (1996). They showed that 92% of the *Pisum sativum* nodules resulted from indigenous rhizobia while only 15% from the inoculated *R. viciae*.

Sinorhizobium meliloti isolated from alfalfa nodules exhibited three distinct morphology, assigned as A, B, and C. A-type isolates appeared to be bright yellow and are smaller in size when compared with B- and C-type isolates. Both B- and C-type isolates appeared to be white. In general, A- and B-type cells grow faster (24 hrs) when compared with C-type cells (48 hrs). B- and C-type cells also produce distinctly more extracellular polysaccharide than A-type cells. It should be noted that A-type cells were observed only in one treatment of sterile soil. This suggests that this strain may not be a strong competitor in the environment.

Unexpectedly, multi-morphological characteristics were observed among isolates obtained from nodules growing in sterile soil (Table 3). Of the 560 isolates obtained from treatments in sterile soils, 28 of them appeared as A- and B-type cells, about 5% of total isolates. None of these isolates contained *Nap*D gene as evidenced by colony hybridization (Figure 4). Of the C-type cells obtained, 2 to 10% of them did not hybridize with *Nap*D, but all contained *nif*H as shown in Figures 5 and 6. This raised questions about the procedure used to sterilize soil. After autoclaving 4 times in consecutive 4 days for 1 h each day at

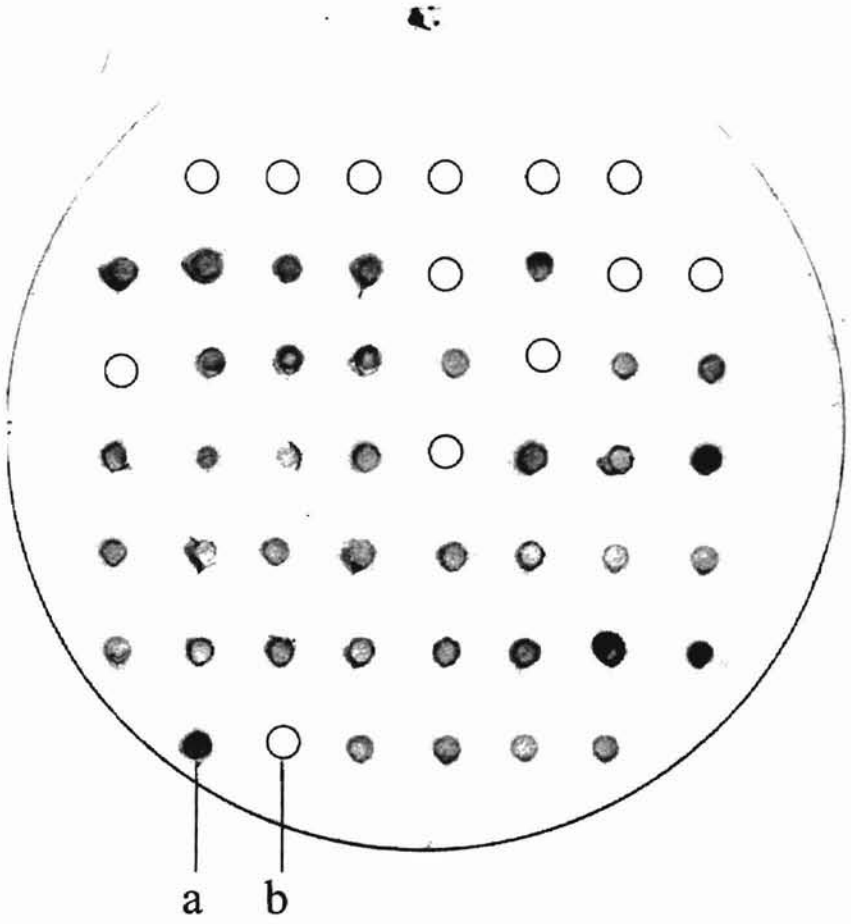


Figure 4. Representative detection of *Sinorhizobium meliloti* in bacteria isolated from nodules of alfalfa growing in soil inoculated with *S. meliloti* by colony hybridization using *Nap*D gene as a probe. The open circles indicate bacteria isolates with negative hybridization signals. (a) *S. meliloti* 104A14 was used as a positive control and (b) YMB media as a negative control.

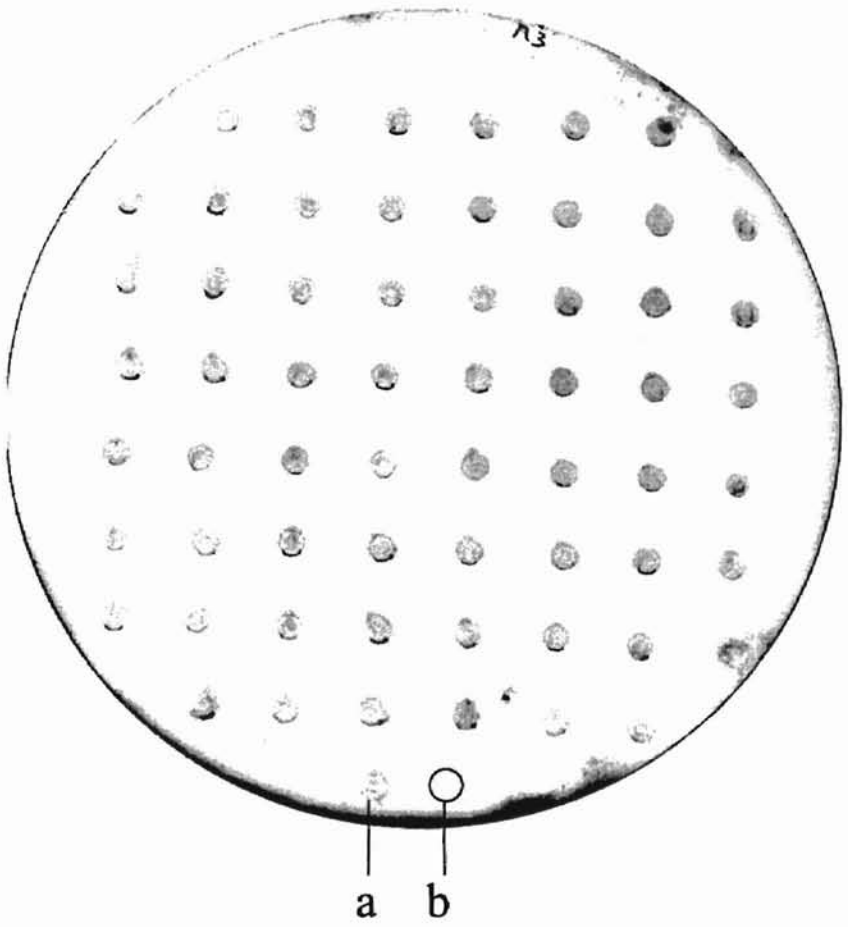


Figure 5. Representative detection of *Sinorhizobium meliloti* in bacteria isolates (60) that showed positive *Nap*D hybridization were hybridized again using *nif*H gene as a probe. The open circles indicate bacteria isolates with negative hybridization signals. (a) *S. meliloti* 104A14 was used as a positive control and (b) YMB media as a negative control.

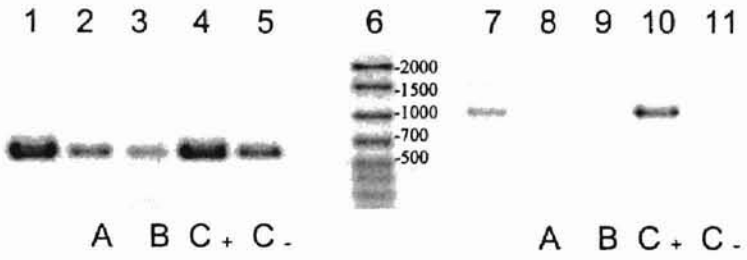


Figure 6. Representative gel for detection of *nif*H and *Nap*D genes in the isolated bacteria from alfalfa nodules by PCR using primers listed in Table 2. These isolates exhibited three morphological appearances, A, B and C. *nif*H PCR products are shown in lane 1-5 (0.6Kb) and *Nap*D in lane 7-11 (1.1Kb). Lane 6 is 50-2000 bp ladder (50ng). The template DNA for lane 1 and 7 were from *S. meliloti* 104A14. Signs +/- indicate corresponding *Nap*D hybridization.

121°C and 20 psi, the soil was still not 100% sterilized. However, nodule formation in sterile soil was not observed in the four controls without inoculation. This may suggest that the introduced strain was more effective in infecting plant root and initiating nodule formation, and the indigenous rhizobia demonstrated certain degree of heat tolerance.

As discussed earlier, a small percentage of C-type cells did not contain *Nap*D. This information was further confirmed by ARDRA of the 16S rDNA of the isolates (Figures 7a & b). These results lead us to conclude that *Nap*D is specific to the introduced *S. meliloti* among the strains isolated from nodules.

Numerous phenotypic, biochemical and molecular methods have been employed in the identification of rhizobium that re-isolated from the nodules of host plants, including the use of antibiotic-resistance mutants (Bushby, 1981; Fettell et al., 1997), serological characterization (Brockwell et al., 1982), and protein analysis (Noel and Brill, 1980). Molecular approaches, such as restriction fragment length polymorphism (RFLP) (Demezas, 1991), ribosomal DNA analysis (Young et al., 1991), the use of specific hybridization probes (Watso and Schofield, 1985; Bjourson and Cooper, 1988) and analyses of DNA amplified through polymerase chain reaction (PCR) (Hebb et al., 1998), have also been widely used. However, these methods are either time-consuming (Noel and Brill, 1980) or lack specificity for differentiating rhizobia strains (Humphrey and Vincent, 1975).

In this study, colony hybridization was used to identify the inoculated *S. meliloti* recovered from nodules by *Nap*D gene as a probe. *Nap*D gene encodes

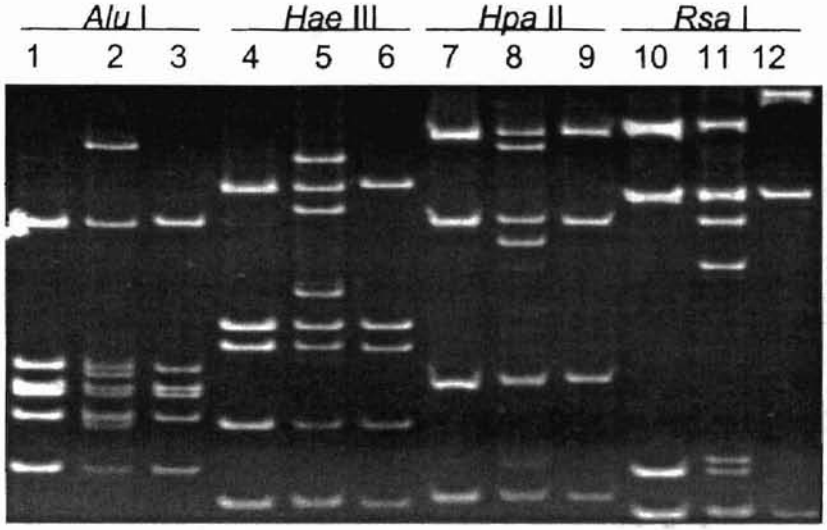


Figure 7a. Amplified ribosomal DNA restriction analysis (ARDRA) of B- and C-type cells isolated from alfalfa nodules. 16S rDNA was amplified with primers shown in Table 2. Approximately 0.2 μg was loaded in each lane. The templates DNA for lanes 1,4,7,10 were from wild type *S. meliloti*, those of lanes 2,5,8,11 were bacteria isolates with B morphology and those of lanes 3,6,9,12 were bacteria isolates with C morphology.

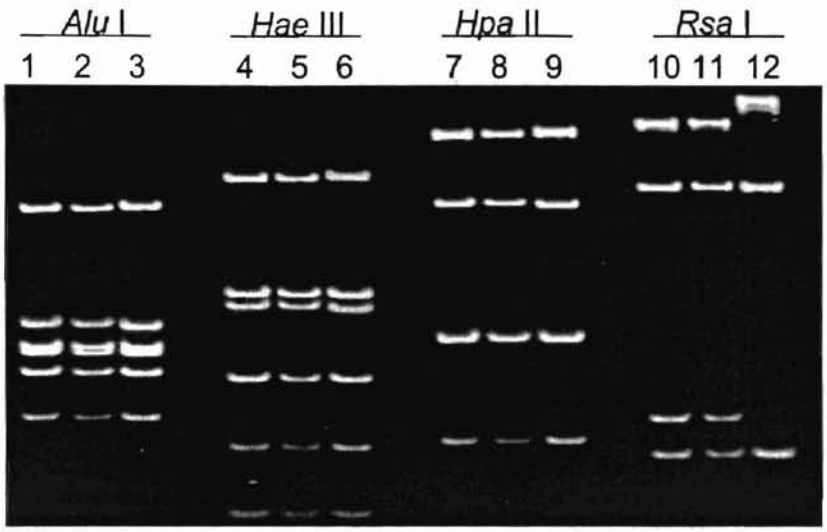


Figure 7b. Amplified ribosomal DNA restriction analysis (ARDRA) of C_+ and C_- morphological bacteria isolated from alfalfa nodules. 16S rDNA was amplified with primers shown in Table 2. Approximately 0.2 μg was loaded in each lane. The template DNA for lanes 1,4,7,10 were from the wild type *S. meliloti*, those of lanes 2,5,8,11 were bacteria isolates with C_+ morphology, and those of lanes 3,6,9,12 were bacteria isolates with C_- morphology.

one of the acid phosphatases in the *S. meliloti* strain 104 A14 (Deng et al., 1998), and its sequence does not match with any known sequences deposited to the GenBank (Deng et al., 1998). If *Nap*D is unique among the strains tested, quick screening methods, such as PCR, may be developed to track the inoculated strain in an nonsterile environment, which would allow a large number of bacterial isolates to be screened quickly and precisely.

To confirm that our surface sterilization technique was effective in minimizing contamination from none-N-fixing bacteria, isolated bacteria were hybridized using *nif*H as a probe. *nif*H gene encodes nitrogenase reductase and has been found to be the most conserved *nif* genes among different N-fixing organisms (Torok and Kondorosi, 1981). This gene has been used as a probe to identify *S. meliloti* for southern hybridizations (Eardly, 1992). Results from this study suggest that the nodule surface sterilization procedure used was effective and all the isolated bacteria from nodules appeared to be N-fixing rhizobia.

In summary, survival and persistence of inoculated *S. meliloti* 104 A14 was enhanced in soil with adequate nutrient supply and high organic matter as well as optimal pH values. Presence of host plant favors the establishment and persistence of inoculated strains. Genetic modification of the wild-type strain impaired its ability to thrive in a new environment. This impact, however, was not as strong as that resulting from presence of host plants or variation in soil properties. Presence of host plants strengthen competitiveness of inoculated *S. meliloti* in soil that resulted in dominance of the inoculated strains, compared with indigenous rhizobia, in nodule formation after 70 days of initial inoculation.

Reference

Al-Niemi T.S., Kahn M.L., and McDermott T.R. 1997. P metabolism in the *Rhizobium tropici*- bean symbiosis. *Plant Physiology* 113: 1233-12452.

Beck D.P., and Munns D.N. 1984. Phosphate nutrition of *Rhizobium* spp. *Applied and Environmental Microbiology* 47: 278-282.

Beever R.E., and Burns D.J.W., 1986. Phosphorus uptake, storage and utilization by fungi. *Advance in Botanical Research* 8: 127-219.

Bieleski R.L. 1973. Phosphate pools, phosphate transports, and phosphate availability. *Annual Review of Plant Physiology* 24:225-252.

Bjourson A.J., and Cooper J.E. 1988. Isolation of Rhiozobium loti strain-specific DNA sequences by subtraction hybridization. *Applied and Environmental Microbiology* 54: 2852-2855.

Brockwell J., Gault R.R., Zorin M., and Roberts M.J. 1982. Effects of environmental variables on the competition between inoculum strains and naturalized populations of *Rhizobium trifolii* for nodulation of *Trifolium subterraneum L.* and on rhizobia persistence in the soil. *Australian Journal of Agricultural Research* 33: 803-815.

Brockwell J., Pilka A., and Holliday R.A. 1991. Soil pH is a major determinant of the numbers of naturally occurring *Rhizobium meliloti* in non-cultivated soils in central New South Wales. *Australian Journal of Experimental Agriculture* 31: 211-219.

Bushby H.V.A. 1981. Quantitative estimation of rhizobia in non-sterile soil using antibiotics and fungicides *Soil Biology and Biochemistry* 13: 237-239.

Cassman K.G., Munns D.N., and Beck D.P. 1981a. Growth of *Rhizobium* strains at low concentration phosphate. *Soil Science Society of America Journal* 45: 520-523

Cassman K.G., Munns D.N., and Beck D.P. 1981b. Phosphorus nutrition of Rhizobium japonium: strain differences in phosphate storage and utilization. *Soil Science Society of America Journal* 45: 517-520.

Clarke L.M., Dilworth M.J., and Glenn A.R. 1993. Survival of *Rhizobium meliloti* WSM419 in laboratory culture: effect of combined pH shock and carbon substrate stress. *Soil Biology and Biochemistry* 25: 1289-1291.

Dalal R.C. 1977. Soil organic phosphorus. *Advances in Agronomy* 29: 83-117. Bieleski R.L. 1973. Phosphate pools, phosphate transports, and phosphate availability. *Annual Review of Plant Physiology* 24:225-252.

DaSilva N.A., and Bailey J.E. 1986. Theoretical growth yield estimates for recombinant calls. *Biotechnology and Bioengineering* 28: 741-746.

Demezas D.H., Reardon T.B., Watson J.M., and Gibson A.H. 1991. Genetics diversity among *Rhizobium leguminosarum* bv. *trifolii* strains revealed by allozyme and restriction fragment length polymorphism analysis. *Applied and Environmental Microbiology* 57: 3489-3495.

Deng S.P., Elkins J.G., Da H.N., Botero L.M., and McDermott T.R. 2001. Characterization of a Second Acid Phosphatase From *Sinorhizobium meliloti* Strain 104A14. *Archives of Microbiology* 176: 255-263.

Deng S.P., Summers M., Kahn M.L. and McDermott T.R. 1998. Cloning and characterization of a *Rhizobium meliloti* nonspecific acid phosphatase. *Archives of Microbiology* 170: 18-26.

Eardly B.D., Young J.P.W., and Selander R.K. 1992. Phylogentic position of Rhizobium sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nif*H genes. *Applied and Environmental Microbiology* 58: 1809-1815.

Evans J., Gregorv A., Dobrowolski N., Morris S.G., O'Connor G.E., and Wallace C. 1996. Nodulation of field-grown *Pisum sativum* and *Vicia faba*: competitiveness of inoculant strains of *Rhizobium leguminosarum* bv. *viciae* determined by an indirect competitive ELISA method. *Soil Biology and Biochemistry* 28: 247-255.

Fettell N.A., O'Connor G.E., Carpenter D.J., Evans J., Bamforth I., Oti-Boateng C., Hebb D.M., and Brockwell J. 1997. Nodulation studies on legumes exotics to Australian: the influence of soil population and inocula of *Rhizoibum leguminosarum* bv. *viciae* on nodulation and nitrogen fixation by field peas. *Applied Soil Ecology* 5: 197-210.

Graham P.H., and Parker C.A. 1964. Diagnostic feature in the characterization of the root-nodule bacteria of legumes. *Plant and Soil* 20:383-396.

Hebb D.M., Richardson A.E., Reid R., and Brockwell. 1998. PCR as an ecological tool to determine the establishment and persistence of *Rhizobium* trains introduced in to the field as seed inoculant. . *Australian Journal of Agricultural Research* 49: 923-934.

Howieson J.G., Robson A.D., and Ewin M.A. 1993 External P_i and calcium concentration and pH but not the product of rhizobial nodulation genes affect the attachment of *Rhizobium meliloti* to roots of annual medies. *Soil Biology and Biochemistry* 25: 567-573.

Humphrey B.A., and Vincent J.M. 1975. Specific and shared antigens in trains of *Rhizobium meliloti. Microbios* 13: 71-76.

Israel D.W. 1987 Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* 84: 835-840.

Isreal D.W. 1993. Symbiotic dinitrogen fixation and host-plant growth during development of and recovery from phosphorus deficiency. *Physiologia Plantarum.* 88:294-300.

McDermott T.R. and Kahn M.L. 1992. Cloning and mutagenesis of the *Rhizobium meliloti* ioscitratee dehydrogenase gene. *Journal of Bacteriology* 174: 4790-4797.

McGrath J.W., Hammerschmidt F. and Quinn J.P. 1998. Biodegradation of phosphonomycin by *Rhizobium huakii* PMY1. *Applied and Environmental Microbiology* 64: 356-358.

McKay I.A., and Djordjevic M.A. 1993. Production and excretion of *nod* metabolites by *Rhizobiummm leguminosarum bv. trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. *Applied and Environmental Microbiology* 59: 3385-3392

Mehlich A. 1984. Mehlich-3 soil test extractant: A modified of Mehlich 2 extractant. *Communication in Soil Science and Plant Analysis* 15: 1409-

1416.

Moawad H.A., Ellis W.R., and Schmidtt E.L. 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field grown soybeans. *Applied and Environmental Microbiology* 47: 607-612.

Murphy J., and Riley J.P. 1962. A modified single solution method fro the determination of phosphate in natural waters. Analytica Chemica Acta 7: 31-36.

Noel D.K., and Brill W.J. 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* population. *Applied and Environmental Microbiology* 40: 931-938.

Orvos D.R., Lacy G.H., and Cairna J.Jr. 1990. Genetically engineered *Erwinia carotovora*: survival, intraspecific competition, and effect upon selected bacterial genera. *Applied and Environmental Microbiology* 56: 1889-1694.

Pereira P.A., and Bliss F.A. 1987. Nitrogen fixation and plant growth of common bean *(Phaseolus vulgaris L.)* at different level of phosphorus availability. *Plant and Soil* 104: 79-84.

Ramirez C., and Alexander M. 1980. Evidence suggesting protozoan predation on *Rhizobium* associated with germinating seeds and in the rhizosphere of beans. *Applied and Environmental Microbiology* 40: 492-499.

Reibet J. and Drevon J.J. 1995. Increase in permeability to oxygen and in oxygen uptake of soybean nodule under limiting phosphorus nutrition. *Physiologia Plantarum.* 94:298-304.

Rice W.A., Penney D.C., and Nyborg M. 1977. Effects of soil acidity on rhizobia number, nodulation and nitrogen fixation by alfalfa and red clover. *Canadican Journal of Soil Science* 57: 197-203.

Robson A.D., and Loneragn J.F. 1970. Nodulation and growth of *Medicago truncatila* on acid soils. II. Colonization of acid soils by *Rhizobium meliloti*. *Australian Journal of Agricultural Research* 21: 435-445.

Ruiz-Saninz J.E., Beringer J.E., and Gutierrez-Navarro A.M., 1984. Effect of the fungicide captafol on the survival and symbiotic properties of *Rhizobium trifolii*. *Journal of Applied Bacteriology* 57: 361-367.

Salminen S.O. and Streeter J.G. 1987. Uptake and metabolism of carbohydrates by *Bradyrhizobium japonium* bacteroids. *Plant Physiology* 83: 535-540.

Samrt J.B., Dilworth M.J., and Robson A.D. 1984a. Effects of phosphorus supply on phosphate uptake and alkaline phosphatase activity in Rhizobia. *Archives of Microbiology* 140: 281-286.

Samrt J.B. Dilworth M.J., and Robson A.D. 1984b. Effects of phosphorus supply on Periplasmioc protein profiles in Rhizobia. *Archives of Microbiology* 140: 287-290

Schepers J.S., Francis, D.D., and Thompson, M.T., 1989 Simultaneous determination of total C, total N and ^{15}N on soil and plant material. Communications in soil science and plant analysis 20, 949-959.

Soa-Afiana L.O., and Alezander M. 1982. Differences among cowpea rhizobia in tolerance to high temperature and desiccation in soil. *Applied and Environmental Microbiology* 43: 435-439.

Tabatabai M.A. 1994. Soil enzymes. In: Methods of Soil Analysis. Part2-Microbiological and Biochemical Properties. Weaver R.W., Angel J.S., and Bottomley P.S. (eds). Soil Science Society of America, Inc. Wisconsin. Pp. 775-833.

Tao H., Brewin N., and Noel K.D. 1992. *Rhizobium leguminosarum* CFN42 lipoplysaccharde antigenic changes induced by environmental conditions. *Jouranl of Bacteriology* 174:2222-2229.

Thomas G.W. 1996. Soil pH and soil acidity. *Methods of soil analysis* Bartels. J.M., Bigham, J.M. (ed.) part 3, p 487-488.

Thurman N.P. and Bromfield E.S.P. 1988. Effect of variation within and between *Medicago* and *Melilotusa* species on the composition and dynamics of indigenous populations of *Rhizobium meliloti. Soil Biology and Biochemistry* 20: 31-38.

Torok I., and Kondorosi A. 1981. Nucleotide sequence of the R. meliloti nitrogenase reductase (nifH) gene. Nucleic Acids Research 9: 5711-5723.

Van Elsas J.D., Van Overbeek L.S., Feldman A.M., Dullmans A.M., and De Leeuw O. 1991. Survival of genetically engineered *Pseudomonas fluorescenscontaining* in soil in competition with parent strain. *FEMS Microbiology Ecology* 85: 53-64.

Vlassak K., Vanderleyden J., and Franco A. 1996. Competition and persistence if *Rhizobium tropici* and *Rhizobium etli* in tropical soil during successive bean (*Phaseolus vulgaris L.)* cultures. Biology and Fertility of Soils 21: 61-68.

Watso J.M., and Schofield P.R. 1985 Species-specific symbiotic plasmid-located repeated DNA sequences in *Rhizobium trifolii*. *Molecular and General Genetics* 199: 279-289.

Wych R.D., and Rain, D.W., 1978. Simultaneous measurement of nitrogen fixation estimated by acetylene-ethylene assay and nitrate absorption by soybean. *Plant physiology* 62: 443 - 448

Young J.P.W., Downes H.L., and Eardly B.D. 1991. Phylogeny of the phototrophic *Rhizobium* train BT Ail by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *Journal of Bacteriology* 173: 2271-2277.

Zhan H., Lee C., and Leigh J.A. 1991. Induction of the second exopolysaccharide (EPSb) on *Rhizobium meliloti* SU-47 by low phosphate concentration. *Jouranl of Bacteriology* 173: 7391-7394.

Zuberer D.A. 1996. Recovery and enumeration of viable bacteria. In: Bartels. J.M., Bigham, J.M. (ed.) *Methods of soil analysis.* part 2. Pp.119-144).

Chapter IV

IMPACT OF GENETICALLY MODIFIED *SINORHIZOBIUM MELILOTI* 104A14 ON SOIL MICROBIAL COMMUNITY STRUCTURE

Abstract

Ecological impacts of genetically modified microorganisms (GMMs) on soil microbial community structure are generating increasing interest, as it may be related to safe release of GMMs to the environment. Impact of wild type *Sinorhizobium meliloti* 104A14 and two acid phosphatase negative mutants derived from this strain on soil microbial community structure in the presence of alfalfa plant were evaluated by culture methods. Total culturable bacteria population in soil inoculated with genetically modified strains increased to a range of 6.58 - 6.69 (Log CFU g^{-1} soil), while that of control soil was about 6.13 (Log CFU g^{-1} soil). But no significantly perturbation was found by wild type. Percentage of r-strategist in *S. meliloti* inoculated soil with alfalfa seeding ranged from 26 to 56%, while that of soil used in this study without seedling ranged from 13% to 18%. The EP-Index of inoculated soil was 0.4-0.6 while that of soil used in this study was 0.3-0.5, indicating more evenly distributed population of increased microbial diversity within the community in the *S. meliloti* inoculated soil with alfalfa. Fungal and yeast population ranged from 3.13 to 3.31 (Log CFU g^{-1} soil) and were similar among the treatments. Lower plant dry matter and fewer nodules were detected in control soil when compared with those in three *S.*

meliloti strain inoculated soils, with little detectable difference between wild type and recombinant strain treatments. In general, the effect of plant was greater than that of inoculants. Thus, in terms of hazard and risk to the environment, the observed impact of these GMMs on soil microbial community may be considered trivial under the tested condition.

Introduction

Genetically modified microorganisms (GMMs) have been developed for commercial application for purposes of pest control, pollution abatement, frost protection, and the stimulation of nitrogen fixation (Bishop et al., 1988; Keeler, 1988; Lindow S.E. 1992; Bosworth et al. 1994). However, the use of GMMs on a wide scale is still hampered by a lack of knowledge of the possible ecological impacts that such organisms might have once they are released into the environment. GMMs could alter balance of fundamental processes, such as carbon, energy, and nutrients cycling that mainly rely on bacterial species or products (Crawford et al., 1993). GMMs may also exchange DNA with the indigenous microorganisms via conjugation, transduction and/or transformation (Lorenz and Wackernagel, 1990; Smit et al., 1991; Walter et al., 1991; Lorenz and Wackernagel, 1993; Selbitschka et al., 1995). Possible consequence of these exchanges includes that the pathogenic or undesirable genes in the introduced GMMs might be deleterious to plants, animals or humans. Various governments and agencies including the United States Environmental Protection

Agencies (EPA), have proposed guidelines requiring industry to collect fundamental, ecological information on the recombinant products (Seidler and Settel, 1991). These parameters include survival time, potential gene exchange, dispersal, and potential effects on beneficial non-target organisms and on the ecosystem (Levin et al., 1987). Therefore, a better understanding of GMMs in the environment is needed before release of functionally active recombinants into the environment,

Recent studies on GMMs demonstrated little environmental problems associated with these organisms tested (Jones and Kerr, 1989; Short et al., 1990; Sandt et al., 1991; Lorenz and Wackernagel, 1993; Evguenieva-Hackenberg et al., 1994; De Leij et al., 1995). All of these studies focused on dissemination, survival of introduced genetically engineered microorganisms, and possible gene transfer of the novel genetic elements with indigenous members of the microbial community. However, the effects of GMMs on indigenous microbial community structure and ecosystem functions have not been well addressed. Bej et al. (1991) measured both phenotypic and genetic diversity after introducing an engineered *Pseudomonas cepacea* that was able to degrade herbicide 2,4,5,-trichlorophenoxyacetic acid. Both diversity indices increased during the incubation period as comparing to the control, and declined to the control level again after 6 weeks. Jones et al. (1991) did not find any effects on either nitrogen transformation or population of nitrogen-transforming microorganisms after addition of a GMM. The work of both Jones et al. (1991) and Bej et al. (1991) did not include plants in the soil systems and thus are not ecologically relevant to

soil-plant-microbe interactions. De Leij et al (1995) reported transient perturbation in the indigenous microbial community with the introduction of a wild type and genetically marked *Pseudomonas fluorescens* to the rhizosphere of wheat. But they did not find significant effects on microbial population and plant growth by these two respective inoculants. Limited available information and reported controversial results hamper application of GMMs into the environment on a large scale. It is, therefore, valuable to conduct further research of GMMs effects on soil microbial community structure.

Soil microbial communities remain one of the most difficult communities to be characterized. There are as many as trillion bacterial species globally and it is impossible to isolate populations representative of the whole community (Atlas, 1983; Dukheiqen et al, 1998). In addition, community-level microbial interactions are complex, with individual species relying on the presence, function, and interaction of many other species. Microbial communities not only change rapidly in time, but also in space in responding to varying environmental conditions. This is especially true for the rhizosphere. Roots are known to excrete different amount and forms of organic materials that changed the microbial community during the plant development period (Lynch, 1990, Doran et al. (1994). So plant–associated microbial communities are even more complicated to characterize.

Several methods have been developed to analyze the structure and diversity of bacterial communities in soil, such as enumerating cultural microorganisms, accessing phospholipid fatty acid (PLFA) and analyzing the extractable soil nuclei acid (Sorheim et al., 1989; De Leij et al., 1993; 1995;

Reichardt et al., 1997; Bossio et al., 1998; øvreas and Torsvik, 1998; Tiedje et al., 1999). Unique fatty acids are indicative of specific groups of living organisms. Analysis of PLFAs was used to assess the structure of soil microbial community by many studies (Reichardt et al., 1997; Bossio et al., 1998). However, appropriate fatty acid molecules are not known for all soil microorganisms, and different amounts and types of fatty acids are produced by microorganisms under various growth conditions (Haack et al., 1994). Oude Elferink et al (1998) demonstrated that PLFA analysis alone couldn't be very accurate, at least in granular sludge. The molecular methods, such as analysis of extracted nucleic acid from soil, have been employed for characterizing soil microbial community (Duineveld et al., 1998; øvreas and Torsvik, 1998; Ritchie et al., 2000). Whereas a number of limitations have been reported such as extraction efficiency, bias of PCR amplification and interference of dead cell DNA (Suzuki et al., 1996; Van Winzingerode et al., 1997; Polz and Cavanaugh, 1998). So far molecular methods do no more than monitor, or detect specific microorganisms and do not give any indication of the actual effect of the inoculants on the ecosystem (Tsushima et al., 1995; Van Winzingerode et al., 1997).

Dilution plate method is a tradition way to quantify microbial community. It uses culture media designed to maximize the recovery of different microbial species. This technique gives several advantages for certain types of microbiological studies, including convenience, easy performance, low cost, and provision for comparison of environments. Plate count population does provide a quantitative procedure, enabling bacterial community structures to be described

in time and space (Torsvik et al., 1990; De Leij et al., 1993). This method has been used to revealed diversity of soil microorganisms in several studies (Bej et al., 1991; De Leij et al., 1993; 1995; Naseby and Lunch, 1998). Population dynamics of microorganisms with different life style strategies, r (rapid colony formation) and K (slow colony formation) strategies, utilized in ecological studies can be evaluated using the plate counting method (De Leij et al., 1993; Panikov, 1994). The objective of this study was to assess the impact of genetically modified *S. meliloti* on soil microbial community structure with alfalfa growth.

Materials and methods

Soil

Surface Renfrow (classified as Kastanozems in the FAO system) soil (0-15cm) is silty clay loam, taken in Central Oklahoma, USA. Soils were ground, sieved, and air-dried and stored at room temperature. The pH value was 6.53, determined by using a combination glass electrode following the procedure described by Thomas G.W. (1996). The organic C was 11.4 g kg^{-1} soil and total N was 1.16 g kg^{-1} soil, detected by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Surphur Analyzer (Schepers et. al., 1989).

Bacteria strain and preparation of inoculants

Wild-type *S. meliloti* 104A14 and two acid phosphatase-negative mutants derived from this strain, NapD and NapE, were used in this study. Creation and characterization of the mutants have been reported by Deng et al. (1998 & 2001).

Bacteria were cultured in YMB media for 48 hrs at 28°C shaking at 200 rpm. The cells were pelleted by centrifuging for 5 min at 5,000 rpm, washed twice with 0.85% NaCl (W/V) solution, and then, re-suspended to approximately 10^8 CFU g^{-1} soils based on estimation from culture density. The actual cell concentrations were determined by plate count on YMA plates.

Microcosm and plant growth

A soil sample (200 g, < 2 mm) was placed into a Magenta box, which was part of a plant growth unit described by McDermott and Kahn (1992). The soil was adjusted to 60% field capacity with water. Plant growth units in one experiment set were autoclaved for four times in consecutive four days with 60 minutes each time, while those in another experiment set kept non-sterile. Each plant growth unit was filled with 200 ml nutrient solution for alfalfa growth. The nutrient solution was modified from that described by Wych and Rain (1978) and contained 1.0mM K_2SO_4, 0.5mM KH_2PO_4, 0.25mM K_2HPO_4, 0.5mM $MgSO_4.7H_2O$, 2.0mM $CaSO_4.2H_2O$, 25uM KCl, 13uM H_3BO_3, 1.0uM $MnSO_4.H_2O$, 1.0uM $ZnSO_4.H_2O$, 0.25uM $CuSO_4.5H_2O$, 2.5uM $CoCl_2.6H_2O$, 20uM $FeCl_3.6H_2O$, and 0.25uM $NaMoO_4.2H_2O$. The solution was sterilized after adjusting to pH 6.5 with KOH and/or HCl.

Alfalfa seeds were surface sterilized with 95% ethanol for 30 seconds, 3% sodium hypochlorite for 5 minutes, rinsed at least six times with sterile, distilled water, and then germinated on YMA plates. Uncontaminated seedlings were transferred to plant growth units with soil (four seedlings per unit) and inoculated immediately with washed cells of *S. meliloti* strains that were prepared as

described above. The alfalfa plants were grown in a growth chamber for 6 weeks with 16 h of photoperiod and 19/24°C of night/day cycles.

Determination of culturable bacteria

After 6 weeks of growth, alfalfa tissues and roots were removed aseptically from the growth units. Soil in the unit was mixed thoroughly and 10 g of the soil (fresh weight) was taken for quantification of bacteria population using a modified method described by Zuberer (1996). Briefly, a 10-g of soil was placed in 90 ml sterile water containing sodium pyrophosphate (0.18% final concentration). The mixture was shaking for 15 minutes at 200 rpm on a rotary shaker. After settling for about 30 seconds, a series of 10-fold dilutions was performed using 0.25-strength sterile Ringer solution. Culturable bacteria were quantified by spreading 0.1 ml of appropriate dilutions on 0.1-strength Tryptone soy agar (TSA) plates. Plates were incubated at 25°C and enumerated daily for 5 days and on the 10^{th} day. TSA has been reported to support the growth of a wide range of bacteria and was used for total cultural bacterial count (Martin, 1975; Miller et al., 1989). Bacteria appearing within 24 hour were designated as r-strategists, and the remaining as K-strategists (De Leij et al., 1993). Fungi were cultured on 0.1-strength malt extract agar (MEA) plates at 23°C for 72 hrs with countable plates of the highest dilution being enumerated. All plate counts were conducted with 4 replicates. Eco-Physiological (EP) Index (H') was calculated using the equation:

$$H' = -\Sigma(p_i * \log_{10} pi)$$

Where p_i representing CFU on each day as a proportion of the total CFU in that sample in 10 days incubation. The control soil was added equivalent amount of 0.85% saline solution to alfalfa seedlings without inoculation.

Data analysis

All results were expressed on soil dry weight basis. Significant differences among treatments were determined using one-way analysis of variance (ANOVA). Comparison of treatment means was done using the least significant difference (LSD) test.

Results

Effect of genetically modified *S. meliloti* on soil microbial community structure

The total culturable bacterial population in alfalfa rhizosphere soils inoculated with NapD and NapE were the highest, ranged from 6.58 to 6.69 (Log CFU g^{-1} soil), while that of the control soil was about 6.13 (Log CFU g^{-1} soil) (Table1). The total bacteria CFU of NapD and NapE inoculated soils were significant greater ($\approx$ 0.45 log unit) than those of the other two treatment soils ($P < 0.05$). The population of fungi and yeast ranged from 3.13 to 3.31 (Log CFU g^{-1} soil) in all treatments (Table 1). Fungal and yeast population in NapD-inoculated soil was the lowest, but little significant difference was detected among treatments evaluated ($P > 0.05$).

The percentage of total cultural bacteria population comprised of r-strategist ranged from 35% to 40% in control and three *S. meliloti* strain

Table 1. Total culturable bacteria, fungi and yeast population in soil as affected by alfalfa plant and inoculation of wild-type and genetically modified *Sinorhizobium meliloti* 104A14.

Treatment[†]	Bacteria Population[‡]	Fungi and Yeast Population[‡]
	(Log CFU g^{-1} soil)	(Log CFU g-1 soil)
Control - alfalfa	6.15 ± 0.15^{b}	3.17 ± 0.37^{a}
Control + alfalfa	6.13 ± 0.08^{b}	3.30 ± 0.13^{a}
W.T.	6.27 ± 0.13^{b}	3.31 ± 0.30^{a}
Nap D	6.69 ± 0.24^{a}	3.13 ± 0.22^{a}
Nap E	6.58 ± 0.25^{a}	3.26 ± 0.23^{a}

[†] Control - alfalfa, no palnt and no inocualtion; Control + alfalfa, treated with 0.85% NaCl; W.T., inoculated alfalfa seedlings with *S. meliloti* 104A14; NapD and NapE, inoculated alfalfa seedlings with acid phosphatase negative mutants (Deng et al., 1998, 2001).

[‡] Different letters indicate significantly different means at $P < 0.05$ according to least significant difference test (n=4).

inoculated soils (Fig. 1A and 1B). The EP-Index was 0.6 in Nap E inoculated soil, with no significant difference detected among those of the control, and soils inoculated with wild type *S. meliloti* and Nap D ($P > 0.05$) (Fig. 2).

Plant dry matter in the control soil was 8.74 mg per plant within average of 1.44 numbers of nodules in each plant, while those in three *S. meliloti* strain inoculated soil ranged from 17.83 – 20.58 mg per plant dry matter and 5.5-6.5 numbers of nodules. The differences in plant dry weights and nodule numbers between wild type *S. meliloti* and recombinant strains was not significant ($P > 0.05$) (Table 2).

Microbial community structure in soil with and without alfalfa growth

The total cultural bacteria population in the soil used in this study was 6.15 (Log CFU g^{-1} soil) and did not significantly differ with soil with alfalfa seeding ($P > 0.05$) (Table1). However, the bacterial community structure of soils without alfalfa markedly differed from that in soil with alfalfa growth (Fig. 1A). A large narrow peak (over 60% of total population) was found in day 2 (48 h). After 48 h, the colony emergence was similar to that of the other treatments.

The EP-Index of soil without alfalfa was 0.4, while that of soil with alfalfa seeding was 0.6 (Fig. 2)

Discussion

Microbial community structure can be characterized in terms of total microbial population, microorganism diversity and distribution within a

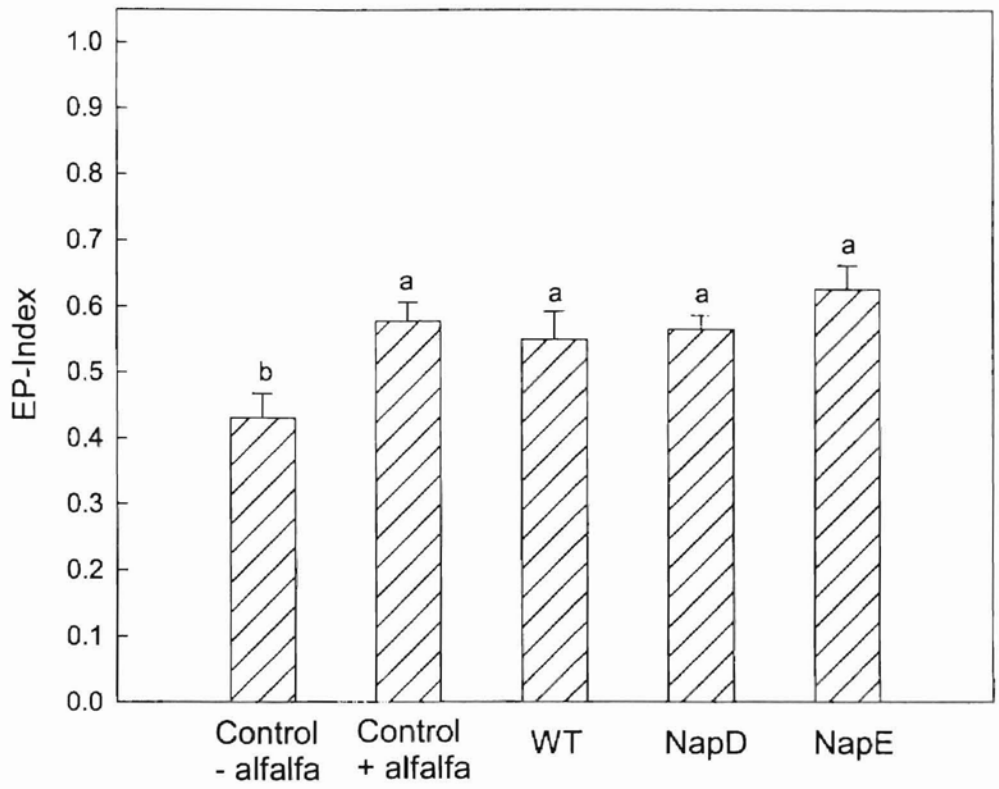


Figure 2. EP-indices in soil. Controls were performed in the presence or absence of alfalfa plants. Alfalfa seedlings were inoculated with the wild-type *Sinorhizobium meliloti* 104A14, NapD or NapE mutant, respectively. Different letters indicate significantly different means at $P < 0.05$ according to least significant difference test.

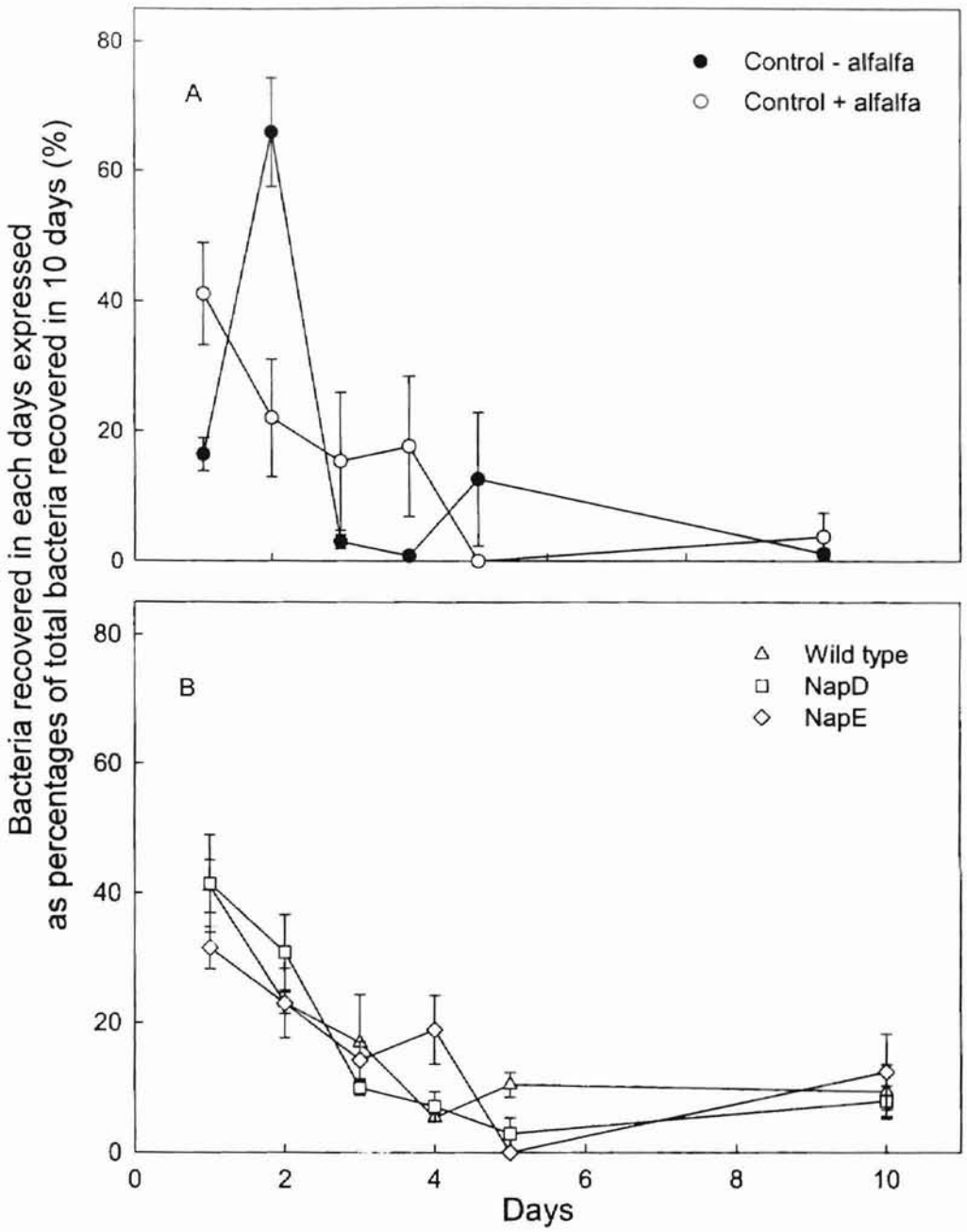


Figure 1. Culturable bacteria in soil: (A) In the presence or absence of alfalfa plant; (B) The alfalfa seedlings were inoculated with the wild-type *Sinorhizobium meliloti* 104A14, NapD, or NapE mutant. Data derived from colony forming units (CFU) appearing on 0.1-strength TSA plates over a period of 10 days (n=4).

Table 2. Mean dry weight of alfalfa plant growing in soils inoculated with the wild-type and genetically modified *Sinorhizobium meliloti* 104A14.

Treatment[†]	Plant dry weight[‡]	Number of nodules
	(mg plant^{-1})	(plant^{-1})
Control	8.74[b]	1.44[b]
W.T	17.83[a]	5.5a
NapD	18.93[a]	6.13[a]
NapE	20.58[a]	6.5[a]

[†] Control , treated with 0.85% NaCl; W.T, inoculated with *S. meliloti* 104A14; Nap D and Nap E, inoculated with acid phosphatase negative mutants (Deng et al., 1998, 2001).

[‡] Different letters indicate significantly different means at $P < 0.05$ according to least significant difference test.

community. Total microbial population can be measured by quantifying total culturable microbial population, and microbial diversity and distribution within a community by determining percentages of r- or K- strategists and Eco-Physiological Index.

In this study, the total cultural bacteria population significantly increased after introduction of genetically modified *S. meliloti* strains, which is consistent with results obtained by Bej et al (1991). Their work showed that the number of total bacteria increased following inoculation of a modified *Pseudomonas cepacea*. We did not observe similar increase in soils inoculated with the wild-type *S. meliloti*, suggesting that the mutants are possibly less persistence than the wild type due to the load of the additional genes by genetically modification as suggested by DaSilva and Bailey (1986). As a result, a portion of the introduced inoculants died, releasing nutrients that could be utilized by indigenous microorganisms and promoted total bacterial population. De Leij et al (1995) found that the total bacteria population was significantly larger in wild type *Pseudomonas fluorescens* treatment soil than that in control when wheat were in the flowering stage (69 days after planting). No significant differences were observed between wild type and control in samples taken at other plant development stage. This is consistent with results of this study in that soil samples used in this study were taken when alfalfa was 42 days growth. Fungi and yeast populations were not affected by any of the *S. meliloti* inoculants ($P > 0.05$), which are in agreement with those reported by Naseby and Lunch (1998). However, Doyle et al. (1991) showed a decline of fungi population in the soil

microcosms after introduction of genetically modified *P. cepacea*. The reason why fungi and yeast population was not changed in this study and Naseby and Lunch's work, may be the effects of plant.

The concept of r and K strategies (r and K selection) was originally introduced in evolution ecology for classifying species according to their reproductive strategy (MacArthur and Wilson, 1967). In the proposed concept, r-strategy organisms were referred to organisms with greater growth rate than K strategists. However, the meaning of these terms has been broadened (Pianka 1970, 1972). Currently, r-strategy is often considered an adaptation for living in unstable or uncrowded environments, e.g., resource-rich ecosystems. On the other hand, K-strategy is considered the adaptation for living in stable or predictable environments, such as old and crowded ecosystems (Hairston et al. 1970; De Leij et al. 1993). The K-strategists shows better survival and competitive ability in harsh environment. In the natural environment, however, distribution of r/k strategists in a community changes continuously because organisms are subjected to changing environmental conditions (Andrews and Harris et al., 1986). When the environmental conditions change to favor either strategists, the distribution of r/K in the whole community change, even though the total microbial population may keep constant. Thus, changes of microbial community structure with environmental conditions can be monitored by changes in distribution of r and K strategists.

Recently, the r/K concept was employed to describe soil microbial community (De Leij et al. 1993). The growth rate of microorganism on the agar

plate was used as a parameter to differentiate r and K strategist. De Leij et al. (1993) defined bacteria that produced visible colonies within 24 h in 0.1-strength TSA plates are r strategists. In this study, the percentage of r-strategist in soil absent of alfalfa growth and inoculation was 17%, which is lower than that in soil with alfalfa growth (about 40%), indicting the soil used in this study favors K-strategists. This soil was taken from field, sieved, air-dried, and then kept at room temperature for about half a year. It is likely that only highly persistence microorganisms remained. This is expected because natural selection should shift toward K-strategy (Pianka, 1970). When alfalfa was introduced to this soil, r-strategist (fast growers) in the microbial community increased to about 40%. This may be attributed to nutrients released to the rhizosphere by plants, that provided an r-like environment for microbial growth. Similar phenomenon was also reported by De Leij et al. (1993). K-strategist reduced their fitness under r-like environment, which leaded to the dominance of r-strategist in this environment. This explanation does not agree with Luckinbill 's finding (1978). No shift between the r and K strategist under the r selection or K selection condition was found when Luckinbill (1978) investigated the behavior of different r and K adapted *Escherichia coli* strains under different r or K selection condition by culture method. In contrary, another pure culture experiment reported that trade-off between r and K strategists were found under r and K-selection condition in *Drosophila* populations (Mueller L. D. and Ayala F.J. 1981). Soil is a complex environment where protections may be provided for some r-strategists to survive and flourish when conditions are favorable for microbial growth, which leaded to

r/K shifting due to alfalfa growth. Among the three tested *S. meliloti* strains, percentages of r-strategist were not significantly difference, suggesting that there is little effect of genetic modified *S. meliloti* on soil microbial community structure under the conditions evaluated.

Species diversity and distribution within community may be evaluated by several ecology diversity indices including Shannon diversity index. Shannon diversity index is expressed as $H' = -\Sigma(p_i * \log_{10} p_i)$ where p_i is the proportion of the community belonging to the *i*th species (Pielou, 1966). This index is developed by taxonomic analysis of randomly selected representative organism (Horowitz et al., 1983; Tate and Mill, 1983). It measures species richness and evenness within the community (Pielou, 1966; Lloyd et al., 1968). The more species in community and the more even their distribution, the higher index of the community (Pielou, 1966). Shannon diversity index has been widely used for characterizing microbial community in sea, sediment, and soil (Kaneko et al., 1977; Miller and Wassel, 1980; Horowitz et al., 1983).

Eco-Physiological (EP) Index was derived from Shannon diversity index and was suggested to be more suitable for characterizing microorganisms with similar development characteristics on agar (De Leij et al., 1993). In this study, EP-index of the soil used in this study was significant lower than those of treated soils, suggesting that the evenness of population distribution and richness of the microbial community were enhanced by alfalfa growth. Although other studies (Atlas, 1983) demonstrated that the diversity of microbial communities generally decreases in response to environmental disturbances due to population balance

interruption within community. The increased richness in this study may be attributed to nutrients released from plant. This is further evidenced by shifting of r- and K-strategists due to introduction of alfalfa to the environment. Interestingly, the EP-index among wild type and two mutants studied were not significantly different, suggesting that the detected changes in microbial diversity and species eveness were resulted from introduction of alfalfa and *S. meliloti* to the environment rather than modifications of phosphatase genes. On the contrary, Bej et al (1991) reported that the evenness of population distribution within the community was increased by inoculating recombined *p.cepacia*, leading to slight increase of the Shannon diversity.

Most studies focus on the impact of GMMs on soil microbial community structure alone (Bej et al., 1991; Jones et al., 1991), although this is not always true in practical applications. It is, therefore, important to link microbial community to other aspects of ecosystem functions, such as plant growth, plant health and soil structure. The plant health heavily relies on the activity of microorganisms in the rhizosphere. Thus, alfalfa growth reflects the change of microbial community around the root. Results from this study indicated that introduction of genetically modified *S. meliloti* did not result in altered alfalfa growth and health. This is supported by little differences detected among the changes in community structure described above. This is also supported by previously studies of Deng et al. (1998, 2000), which showed that mutation of acid phosphatase genes resulted in little changes in N-fixation symbiosis. In fact,

De Leji et al (1995) reported similar result after evaluated the effects on wheat by genetically modified *Pseudimonass fluorescens*.

In summary, this study demonstrated that genetically modified *S. meliloti* strain104A14 promoted bacterial growth in soil, but not growth of fungi and yeast. The perturbation of microbial community structure in soil with introduction of the *S. meliloti* and its two mutants was not significant. Little effects of bacteria inoculation on plant growth and health were found. Thus, in terms of hazard and risk to the environment, the observed impact of these GMMs on soil microbial community may be considered trivial under the tested condition.

Reference

Andrews J. H., and Harris R.F. 1986. r-and K- selection and microbial ecology. In: Marshall K.C. (ed) Advances in Microbial Ecology. Plenum Press, New York. London, pp. 99-47.

Atlas R.M. 1983. Diversity of microbial communities. *Advance in Microbial Ecology* 7: 1-47.

Bej A.K., Perlin M., and Atlas R.M., 1991. Effect of introducing genetically engineered microorganisms on soil microbial community diversity. *FEMS Microbiology Ecology* 86: 169-176

Bishop D.H.L., Entwistle P.F., Cameron I.R., Allen C.J., and Possee R.D. 1988. Field trial of genetically engineered baculovirus insecticides. In: Sussman M. et al. (ed) The Release of Genetically-Engineered Micro-organisms. pp.143-179.

Bossio D.A., Scow K.M., Gunapala N., and Graham K.J. 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. *Microbial Ecology* 36: 1-12.

Boeworth A.H., Williams M.K., Albrecht K.A., Kwiatkowski R., Beynon J., Hankinson R.R., Ronson C.W., Cannon F., Wacek R.J., and Triuplett E.W. 1994. Alfalfa yield response to inoculation with recombinant strains of R*hizobium meliloti* with an extra copy of *dct*ABD and/or modified *nif*A expression 60: 3815-3832.

Crawford D.L., Doyle J.D., Wang Z., Hendricks C.W., Bentjen S.A., Bolton H. Jr., Fredrickson J.K., and Bleakley B.H. 1993. Effects of a lignin peroxidase-expressing recombinant, Streptomycin lividans TK23.1, on biogeochemical cycling and the numbers and activities of microorganisms in soil. *Applied and Environmental Microbiology* 59: 508-518.

DaSilva N.A., and Bailey J.E. 1986. Theoretical growth yield estimates for recombinant calls. *Biotechnology and Bioengineering* 28: 741-746.

De Leij F.A.A.M., Sutton E.J., Whipps J.M., Fenlon J.S., and Lynch J.M. 1995. Impact of field release of genetically modified on indigenous microbial population of wheat. *Applied and Environmental Microbiology* 61:3443-3453

De Leij F.A.A.M., Whipps J.M., and Lynch J.M. 1993. The use of colony development for the characterization of bacterial communities in soil and on roots. *Microbial Ecology* 27: 81-97.

Deng S.P., Elkins J.G., Da H.N., Botero L.M., and McDermott T.R. 2001. Characterization of a Second Acid Phosphatase From *Sinorhizobium meliloti* Strain 104A14. *Archives of Microbiology* 176: 255-263.

Deng S.P., Summers M., Kahn M.L. and McDermott T.R. 1998. Cloning and characterization of a *Rhizobium meliloti* nonspecific acid phosphatase. *Archives of Microbiology* 170: 18-26.

Doran J.W., Coleman D.C., Bezdicek D.F., and Stewart B.A. 1994. Defining soil quality for a sustainable environment. *American Society of Agronomy, SSA Special Publication* No. 35, Madison, WI

Doyle J.D., Short K.A., Stotzky G., King R.J. Seidler R.J., and Olsen R.H. 1991. Ecologically significant effects of *Pseudomonas Putida* PPO301 genetically engineered to degrade 2,4-dichlorophenoxyacetate, on microbial populations and process in soil. *Canadian Journal of Microbiology* 37: 682-691.

Duineveld B.M., Rosado A.S., van Elsas J.D., and van Veen J.A. 1998 Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum vie denaturing gradient gel electrophoresis and substrate utilization patterns. *Applied and Environmental Microbiology* 64: 4950-4957.

Evguenieva-Hackenberg E., Selenska-Pobell S., and Klingmuller W. 1994. Persistence and stability of genetically manipulated derivatives of *Enterobacter agglomerans* in soil microcosms. *FEMS Microbiology Ecology* 15: 179-192.

Haack S.K., Garchow H., Odelson D.A., Forney L.J., and Klug M.J. 1994. Accuracy, Reproducibility, and Interpretation of Fatty Acid Methyl Ester Profiles of Model Bacterial Communities *Applied and Environmental Microbiology* 60: 2483-2493.

Hairston N. G., Tinkle, D. W. & Wilbur, H. M. 1970. Natural selection and the parameters of population growth. *Journal of Wildlife Manage* 34,681-690.

Horowitz A., Krichevsky M.I., and Atlas R.M. 1983 Characteristics and diversity of subarctic marine oligotrophic, stenoheterotrophic, and euryheterotrophic bacterial population. *Canada Journal of Microbiology* 29: 527-535.

Jones D.A., and Kerr A. 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. *Plant Disease*. 73: 15-18

Jones R.A., Broder M.W., and Stotzky G.1991. Effects of genetically engineered microorganisms on nitrogen transformations and nitrogen-transforming microbial population in soil. *Applied and Environmental Microbiology* 57: 3213-3219.

Kaneko T., Atlas R.M., and Krichevsky M. 1977. Diversity of bacterial populations in the Beaufort Sea. *Nature* 270: 596-599

Keeler K.H. 1988 Can we guarantee the safety of genetically engineered organisms in the environment? Critical Review in Biotechnology 8: 85-97.

Levin M.A., Seidler R.J. Bourquin A.W., Fowle J.R., and Barkay T. 1987. EPA developing methods to assess environmental release. *Biotechnology* 5: 38-45.

Lindow S.E. 1992. Ice strain of *Pseudomonas syringae* introduced to control ice nucleation active strains on potato, p. 169-174 .In Tjamos E.S. (ed), Biological control of plant diseases. Plenum Press, New York.

Lloyd M., Zar J.H./, and Karr J.R. 1968. On the calculation of informational measures of diversity. *American Midland Naturalist,* 79: 257-272.

Lorenz M.G., Wackernagel W. 1990. Natural genetic transformation of *Pseudomonas stutzeri* by sand-absorbed DNA. *Archives of Microbiology* 154: 380-385.

Lorenz M.G. and Wackernagel W. 1993. Bacterial gene transfer in the environment. In: Worhrmann K. and Tomiuk J. (ed.) Transgenic Organisms: risk assessment of deliberate release. 43-64.

Luckinbill L.S. 1978. r and K selection in experimental population of *Escherichia coli. Science* 202: 1201-1203.

Lynch J.M. and Whipps J.M. 1990. Substrate flow in the rhizosphere. *Plant and Soil* 129: 1-10.

MacArthur R., and Wilson, E. 1967. The theory of island biogeography. Princeton University Press, New Jersey.

Martin J.K. 1975 Comparison of agar media for counts of viable soil bacteria. *Soil Biology and Biochemistry* 7: 401-402.

McDermott T.R. and Kahn M.L. 1992. Cloning and mutagenesis of the *Rhizobium meliloti* ioscitratee dehydrogenase gene. *Journal of Bacteriology* 174: 4790-4797.

Miller A., and Wassel R. 1980. Aspects of diversity measurement for microbial communities. *Applied and Environmental microbiology* 40: 5578-586.

Miller H.J., Henken G., and Van Veen J.A. 1989. Variation and comparison of bacterial populations in the rhizospheres of maize, wheat, and grass cultivars. *Canadian Journal of Microbiology* 35:656-660.

Mueller L.D., and Ayala F.J. 1981 Trade-off between r-selection and K-selection in *Drosophila* populations. *Proceedings of the National Academy of Sciences of the United States of America* 78:1303-1305.

Naseby D.C., and Lunch J.M. 1998 Impact of wild type and genetically modified *Psemdomonas fluorescens* on soil enzyme activities and microbial population structure in the rhizosphere of pea. *Molecular Ecology* 7: 617-625

Oude Elferink S.J.W.H., Boschker H.T.S., and Stams A.J.M. 1998. Identification of sulfate reducers and *Syntrophobacter* sp. in anaerobic granular sludge by fatty-acid biomarkers and 16S rRNA probing. *Geomicrobiology Journal* 15: 3-17.

øvreas L., and Torsvik V. 1998. Microbial diversity and community structure in two different agricultural soil communities. *Microbial Ecology* 36: 303-315.

Panikov N. 1994 Population dynamics of microorganisms with different life strategies. In: *Environmental Gene Release: Models, Experiments and Risk Assessment.* (eds Bazin M.J. and Lunch J.M.) P.47-65 Chapman and Hall, London.

Pianka E. R. (1970). On r-and K-selection. *American Naturalist*. 104, 592-597.

Pianka E. R. (1972). r and K selection or b and d selection? *American Naturalist.* 106, 581-588

Pielou E.C. 1966 Shannon's Formula as a measure of species diversity: its use and misuse. *American Naturalist,* 100: 463-465.

Polz M.F., and Cavanaughhh C.M. 1998 Bias in template-to-product ratios in multiplate PCR. *Applied and Environmental Microbiology* 64:3724-3730.

Reichardt W., Mascarina G., Padre B., and Doll J. 1997. Microbial communities of continuously cropped, irrigated rice fields. *Applied and Environmental microbiology* 63: 233-238.

Ritchie N.J., Schutter M. E., Dick R.P., and Myrold D.D. 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. *Applied and Environmental microbiology* 66: 1668-1675.

Sandt C.H., and Herson D.S. 1991. Mobilization of the genetically engineered plasmid pHSV106 from *Escherichia coli* HB101 (pHSV106) to *Enterbacter cloacae* in drinking water. *Applied and Environmental microbiology* 57: 194-200.

Schepers J.S., Francis, D.D., and Thompson, M.T., 1989 Simultaneous determination of total C, total N and ^{15}N on soil and plant material. *Communications in soil science and plant analysis* 20, 949-959.

Seidler R.J. and Settel J. (eds) 1991 The use and development of environmentally controlled chamber (mesocosms) for evaluating biotechnology products. US EPA/600/9-91/013.

Selbitschka W., Jording D., Mieman S., Schmidt R., Puhler A., Mendum T., and Hirsch P. 1995. Construction and Characterization of a *Rhizobium leguminosarum* biovar *viciae* strain designated to assess horizontal gene transfer in the experiment. *FEMS Microbiology Letters* 128: 255-263.

Short K.A., Seidler R.J., and Olson R.H. 1990. Survival and degradative capacity of *Pseudomonas putida* induced or constitutively expressed plasmid mediated degradation of 2,4-dichlorophenoxyacetate (TFD) in soil. *Canadian Journal of microbiology* 57: 3482-3488.

Smit E, Van Elsas J.D., Van Veen J.A., De Vos W.M. 1991. Plasmid transfer from *Pseudomonas fluorescents* to indigenous bacteria in soil by using bacteriophage R2f for donor counter selection. *Applied and Environmental Microbiology* 57: 3482-3488.

Sorheim R., Torsvik P., Cohen Y., and Ramsing N.B., 1989 Phenotypic divergences between populations of soil bacteria isolated on different media. *Microbial Ecology* 17:181-192.

Suzuki M., and Giovannoni S.J. 1996. Bias cause by template annealing in the amplification mixture of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62: 625-630.

Tate R.L. and Mill A.L., 1983. Cropping and the diversity and function of bacteria in Pahokee Muck. *Soil Biology and Biochemistry* 15: 175-179.

Thomas G.W. 1996. Soil pH and soil acidity. *Methods of soil analysis* Bartels. J.M., Bigham, J.M. (ed.) part 3, p 487-488

Tiedje J.M., Asuming-Brempong S., Nusslein K., Marsh T.L., and Flynn S.J. 1999. Opening the black box of soil microbial diversity. *Applied Soil Ecology* 13: 109-122.

Torsvik V., Salte K., Sorheim R., and Goksoyr J. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Applied and Environmental Microbiology* 56: 776-781

Tsushima S., Hasebe A., Komoto Y., Carter J.P., Miyashita K., Yokoyama K., and Pickup R.W. 1995. Detection of genetically engineered microorganisms in paddy soil using a simple and rapid "nested" polymerase chain reaction methods. *Soil Biology and Biochemistry* 27: 219-227.

Van Winzingerode F., Gobel U.B., and Stackebrandt E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Review* 21: 213-229.

Wych R.D., and Rain, D.W., 1978. Simultaneous measurement of nitrogen fixation estimated by acetylene-ethylene assay and nitrate absorption by soybean. *Plant physiology* 62: 443 - 448.

Zuberer D.A. 1996. Recovery and enumeration of viable bacteria. In: Bartels. J.M., Bigham, J.M. (ed.) *Methods of soil analysis.* part 2. Pp.119-144).

Chapter V

SUMMARY AND CONCLUSION

Legume-rhizobia symbiotic N fixation is a natural process that is not only important for sustaining agricultural production, but also for maintaining the earth's ecosystem and conserving energy. Large-scale legume inoculation has achieved tremendous success in the past several decades. Genetic engineering may further our success providing that we thoroughly understand the conditions required for the establishment of a GMM with minimized environmental risk.

Results from this study indicated that survival and persistence of inoculated *S. meliloti* 104 A14 was enhanced in soils with adequate nutrient supply and high organic matter as well as optimal pH. Presence of host plant favors the establishment and persistence of inoculated strains. Genetic modification of the wild-type strain impaired its ability to thrive in a new environment. This impact, however, was not as prominent when compare with that resulting from the presence of host plants or variation in soil properties.

Results from this study also demonstrated that genetically modified *S. meliloti* strain104A14 promoted bacterial growth in soil, but not the growth of fungi and yeast. However, inoculation of *S. meliloti* resulted in little change in microbial community structure as indicated by EP indices and presences of r/K strategists. Plant growth and health were also not affected significantly by inoculation of GMM. Thus, the observed impact of these GMMs on soil microbial community may be considered trivial under the tested condition.

APPENDIX

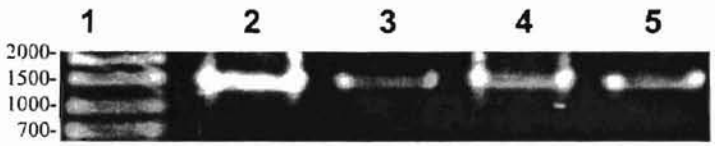


Figure 1. 16S rDNA of bacteria isolated from alfalfa nodules growing in soil inoculated with *S. meliloti*. Lane 1. 50-2000 bp ladder (50ng). The template DNA for lane 2 was from *S. meliloti* 104A14, those of lane 3, 4 and 5 were from bacteria isolate with B, C_+, C- morphology, respectively. Signs +/- indicate corresponding *Nap*D hybridization.

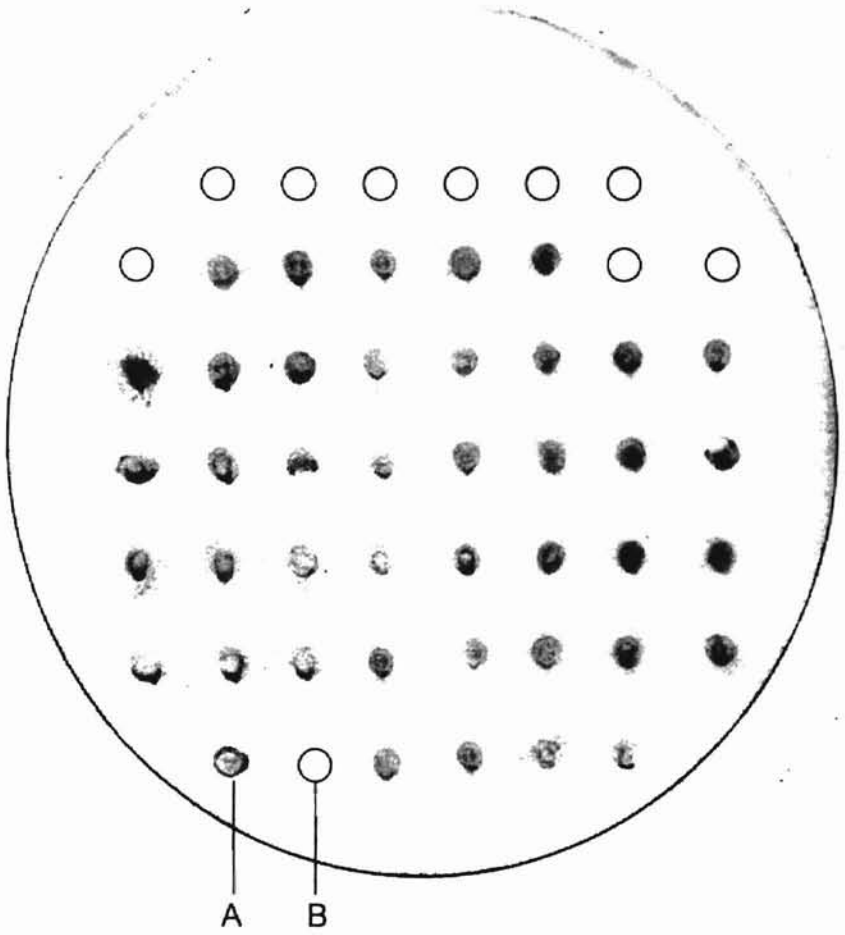


Figure 2. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in sterile soil inoculated with *Sinorhizobium meliloti* NapD mutant by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

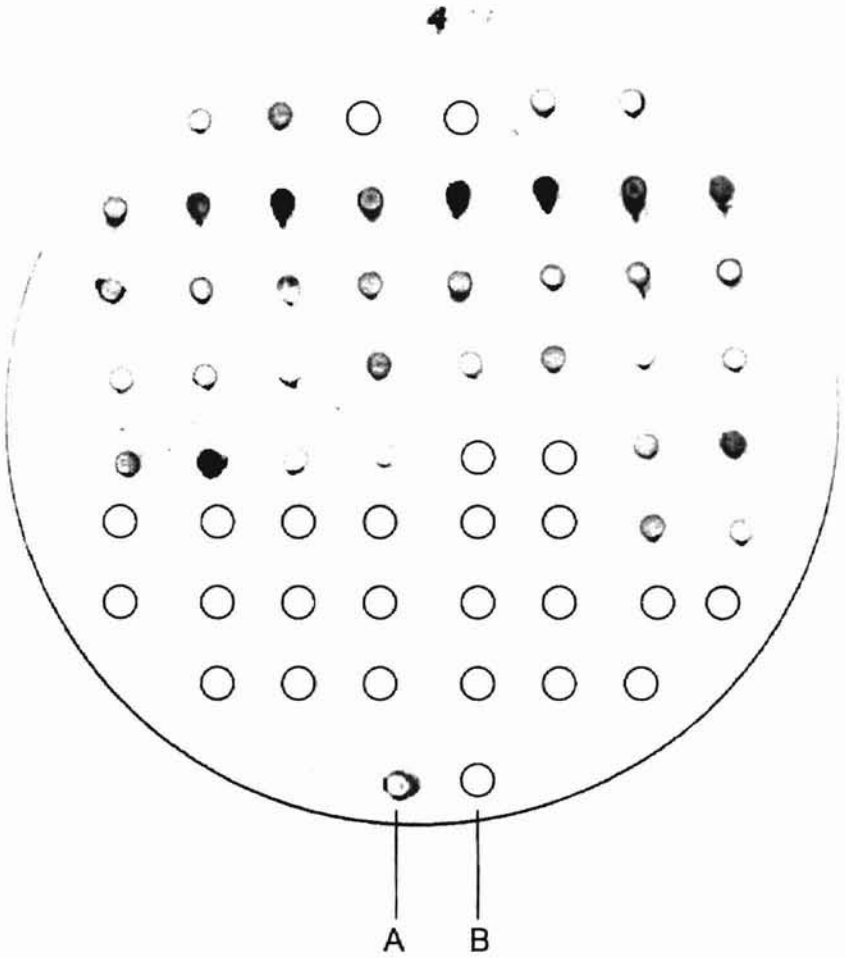


Figure 3. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in sterile soil inoculated with *Sinorhizobium meliloti* NapE mutant and in non-sterile soil without inoculation by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

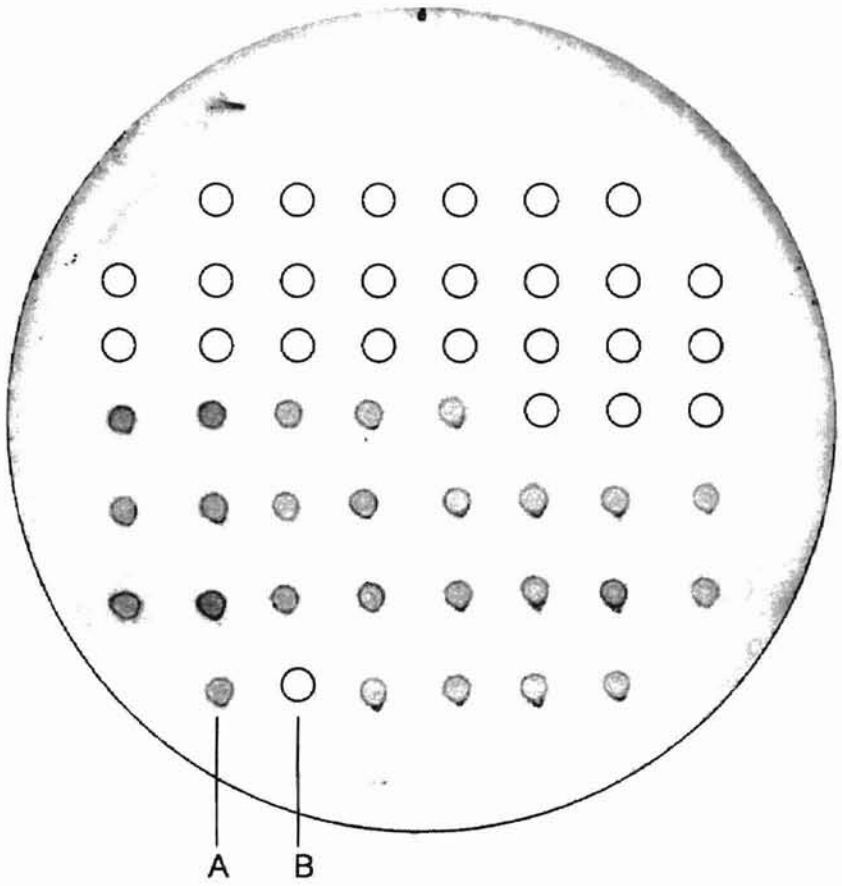


Figure 4. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in non-sterile soil inoculated with wild-type *Sinorhizobium meliloti* by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe . The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

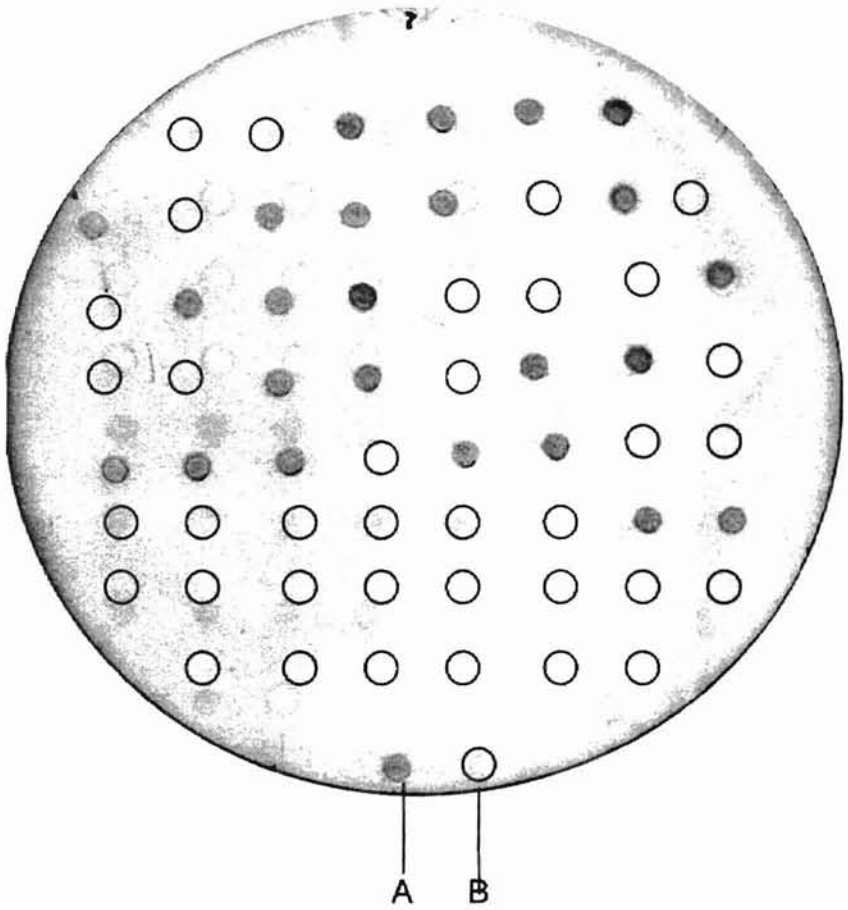


Figure 5. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in non-sterile soil inoculated with *Sinorhizobium meliloti* NapD and in non-sterile soil without inoculation by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

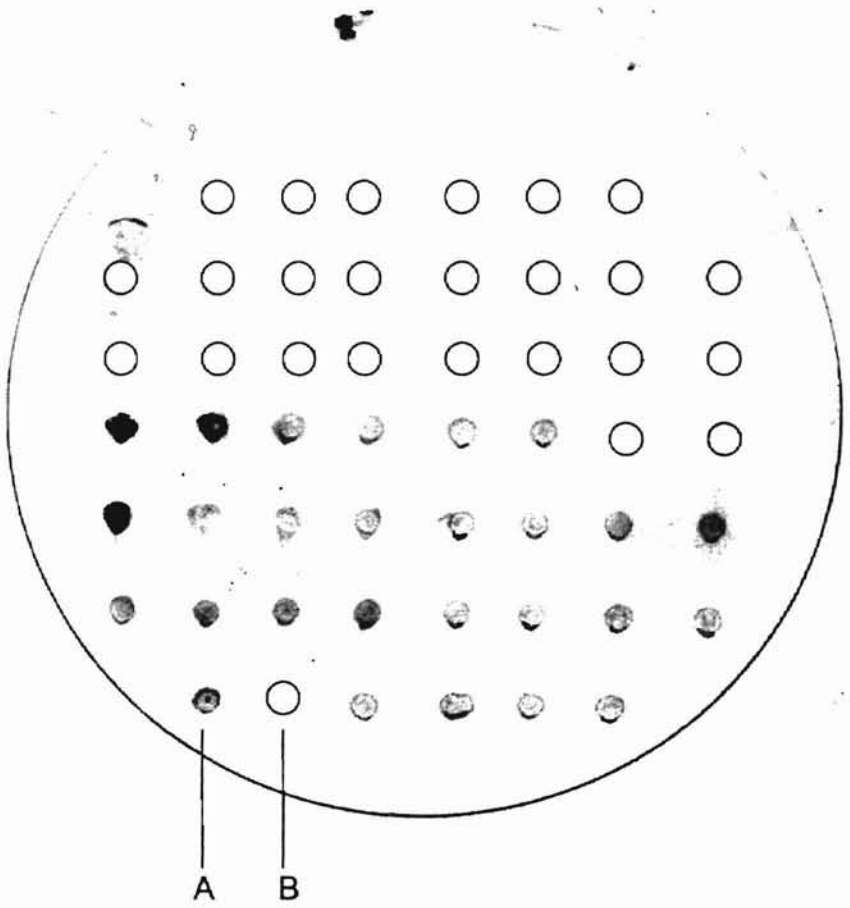


Figure 6. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in non-sterile soil inoculated with *Sinorhizobium meliloti* NapE mutant by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. The open circles indicate bacteria isolate with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

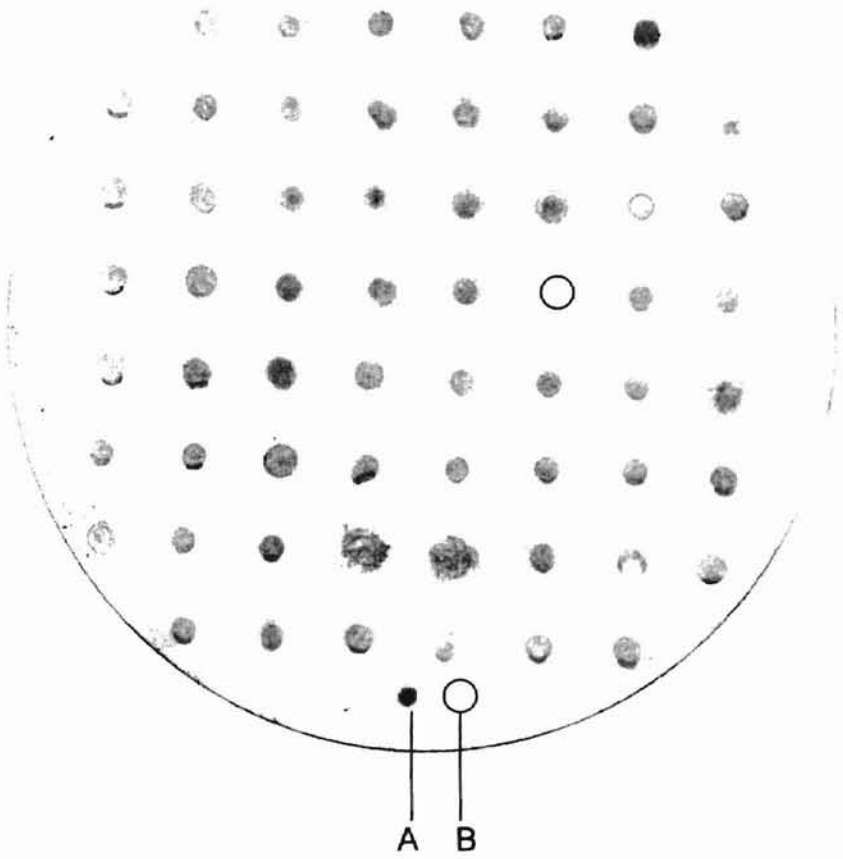


Figure 7. Detection of *Nap*D in bacteria by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. The bacteria were isolated from alfalfa nodules growing in sterile soils that were previously inoculated with wild-type *Sinorhizobium meliloti* or NapD mutant and grew alfalfa for six weeks. Alfalfa seedlings for this experiment were transferred to these soils one month later after harvest. The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as positive control and (B) YMB media as a negative control.

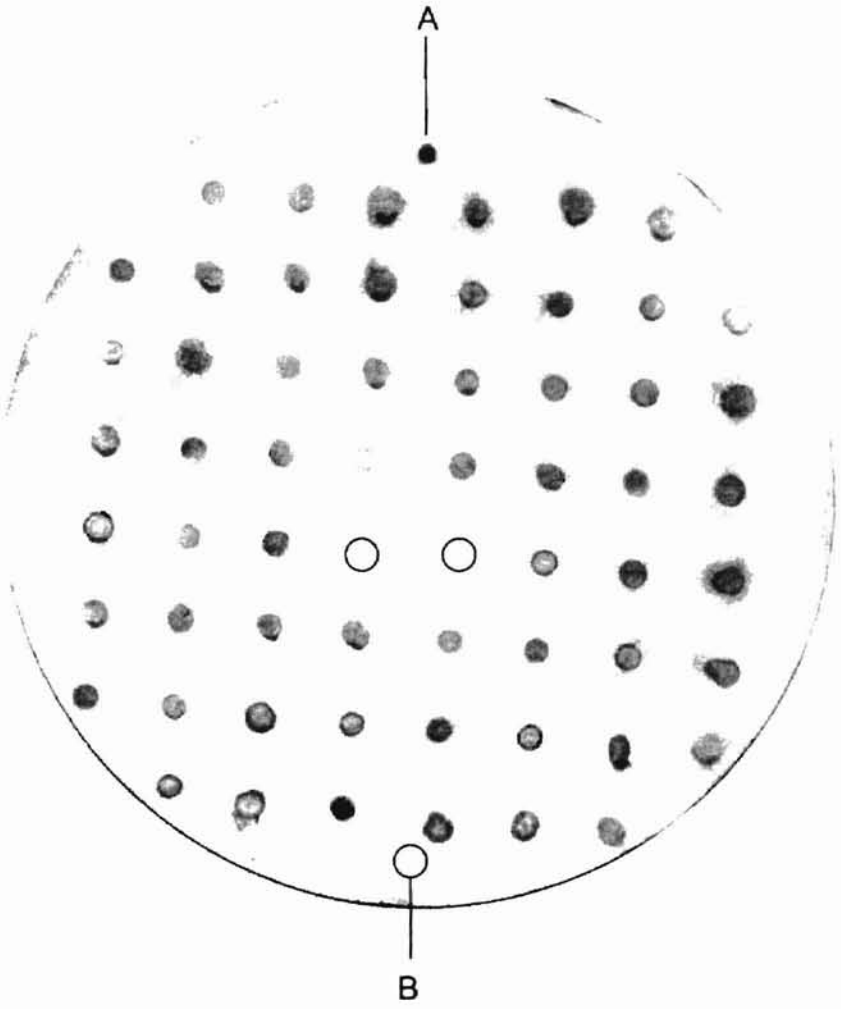


Figure 8. Detection of *Nap*D in bacteria by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. Bacteria were isolated from alfalfa nodules growing in sterile soils that were previously inoculated with *Sinorhizobium meliloti* NapD or NapE mutant and grew alfalfa for six weeks. Alfalfa seedlings for this experiment were transferred to these soils one month later after harvest. The open circles indicate bacteria isolate with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

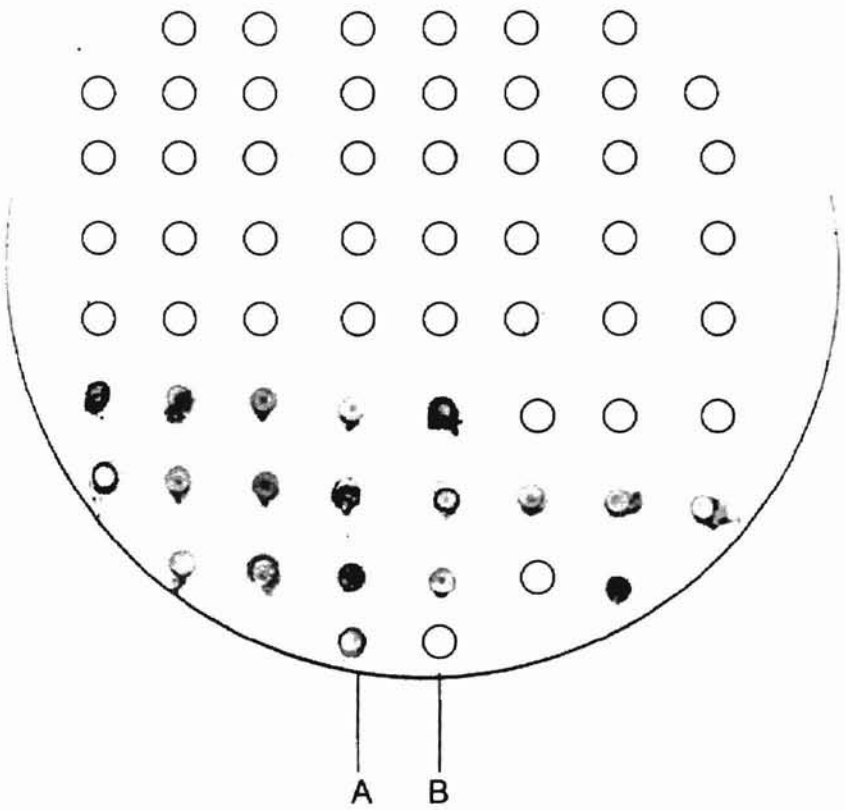


Figure 9. Detection of *Nap*D in bacteria by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. Bacteria were isolated from alfalfa nodules growing in non-sterile soils that previously grew alfalfa for six weeks with or without inoculation of wild-type *S. meliloti*. Alfalfa seedlings for this experiment were transferred to these soils one month later after harvest. The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

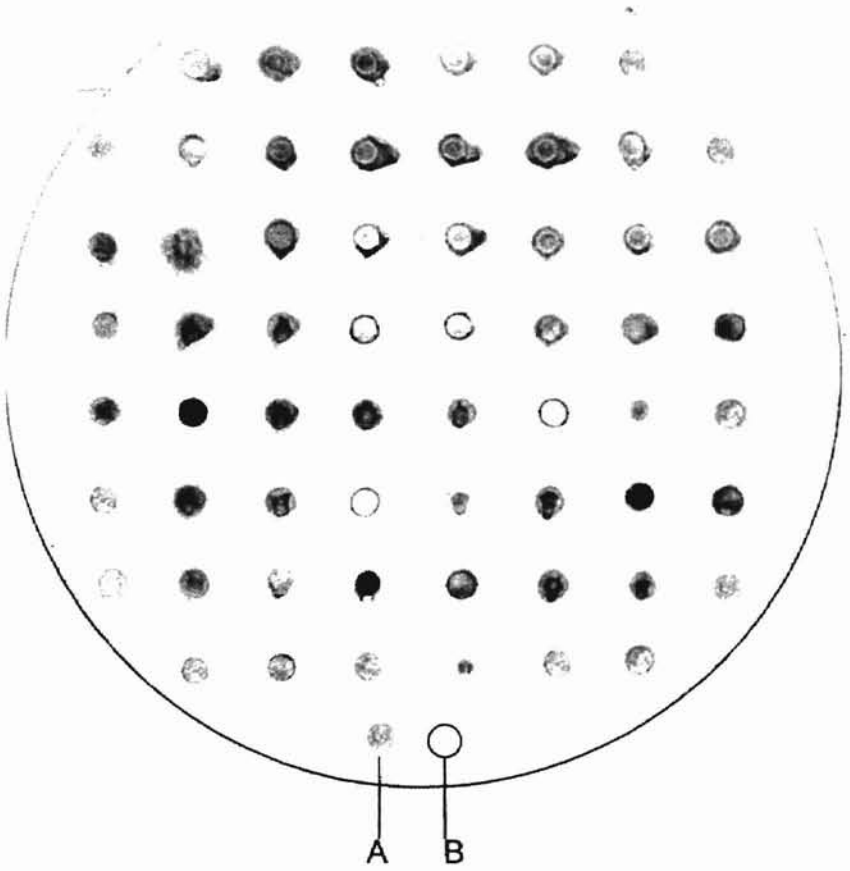


Figure 10. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in non-sterile soil by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. These soils were previously inoculated with wild-type *Sinorhizobium meliloti* or NapD mutant and grew alfalfa for six weeks. Alfalfa seedlings for this experiment were transferred to these soils one month later after harvest . The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

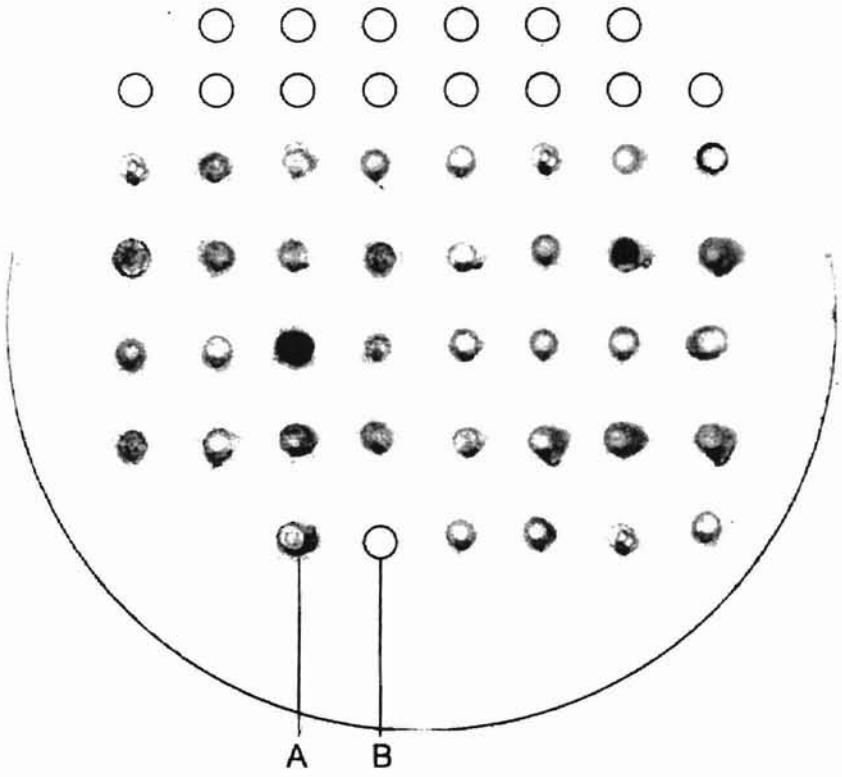


Figure 11. Detection of *Nap*D in bacteria isolated from alfalfa nodules growing in non-sterile soil by colony hybridization using *Nap*D of *S. meliloti* strain 104A14 as a probe. These soils were previously inoculated with *Sinorhizobium meliloti* NapE mutant and grew alfalfa for six weeks. Alfalfa seedlings for this experiment were transferred to these soils one month later after harvest. The open circles indicate bacteria isolates with negative hybridization signals. (A) *S. meliloti* 104A14 was used as a positive control and (B) YMB media as a negative control.

VITA

Huining Da

Candidate for the Degree of

Master of Science

Thesis: SURVIVAL AND PERSISTENCE OF GENETICALLY MODIFIED *SINORHIZOBIUM MELILOTI* STRAIN 104A14 IN SOIL

Major Field: Plant and Soil Sciences

Biographical:

Education: Graduated from Attached High School of Wuhan University, P. R. China, in July, 1985; received Bachelor of Science degree in Pharmacy from Hubei College of Traditional Chinese Medical in July 1989. Completed the requirements for the Master of Science Degree with a major in Plant and Soil Sciences at Oklahoma State University in December 2001.

Experience: Employed as a research associate in Pharmaceutical Research Institute to develop analytical methods, set specifications and perform "Certification of Analysis" tests for releasing new drugs. Worked as a research assistant at University of Hong Kong and was involved in purifying endomorphine from goldfish brain, expressing goldfish growth hormone promoter and estrogen receptor in cell line and goldfish primary cell, and conducting enzyme immunoassay for Gonadotropin-releasing hormone (GnRH). Currently, working as a graduate research assistant at Oklahoma State University to assess survival and persistence of genetically modified *Sinorhizobium meliloti* in the soil environment, to purify an acid phosphatase from *S. meliloti*, and to determine soil microbial diversity and community structure using molecular biology techniques.