การถ่ายโอนชุดยีนที่ควบคุมภาวะพึ่งพาอาศัยซึ่งกันและกันของแบคทีเรียกลุ่ม *Bradyrhizobiaceae* ที่คัดแยกจากดินในประเทศไทย

นางสาวรุจิเรข น้อยเสงี่ยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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SYMBIOTIC GENES TRANSFER OF *BRADYRHIZOBIACEAE* STRAINS ISOLATED FROM SOIL IN THAILAND

Rujirek Noisangiam

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctoral of Philosophy in Biotechnology

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SYMBIOTIC GENES TRANSFER OF *BRADYRHIZOBIACEAE* STRAINS ISOLATED FROM SOIL IN THAILAND

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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รุจิเรข น้อยเสงี่ยม : การถ่ายโอนชุดยีนที่ควบคุมภาวะพึ่งพาอาศัยซึ่งกันและกันของแบคทีเรียกลุ่ม *BRADYRHIZOBIACEAE* ที่คัดแยกจากดินในประเทศไทย (SYMBIOTIC GENES TRANSFER OF *BRADYRHIZOBIACEAE* STRAINS ISOLATED STRAINS ISOLATED FROM SOIL IN THAILAND) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 172 หน้า

แบคทีเรีย *Bradyrhizobium* ที่แยกโดยตรงจากแหล่งดินในประเทศไทย จำแนกออกเป็นกลุ่มของ symbiont และ nonsymbiont โดยนีลวรรณและคณะวิจัยในปีพ.ศ. 2545 งานวิจัยครั้งนี้ได้ใช้วิธี polyphasic ในการจัดจำแนกตัวแทนกลุ่ม nonsymbiont ที่ยังไม่สามารถจัดจำแนกได้อย่างชัดเจนจำนวนสี่สายพันธุ์ การวิเคราะห์ลักษณะทางฟีโนไทพ์พบว่า nonsymbiont สามารถต้านทานโลหะหนักได้ในปริมาณที่สูงมาก และมีลักษณะทางฟีโนไทป์ที่คาบเกี่ยวอยู่ระหว่าง *Bradyrhizobium* และ *Rhodopseudomonas palustris* ในทำนองเดียวกันเมื่อทำการวิเคราะห์โดยวิธี multilocus sequence analysis จากยีน 16S rRNA และ housekeeping genes ได้แก่ *atpD recA* และ *glnII* พบว่า nonsymbiont ทั้งสี่จัดกลุ่มแยกออกมาจากแบคทีเรียกลุ่มอื่นอย่างชัดเจน โดยมีตำแหน่งในแต่ละ phylogenetic tree ที่แตกต่างกันไป โดยรวมแล้วมีความสัมพันธ์ใกล้ชิดกับทั้ง *Bradyrhizobium* และ *Rhs. palustris* ดังนั้นจึงได้เสนอให้เป็นแบคทีเรียในสกุลใหม่ชื่อ *Metalliresistens boonkerdii* ความสัมพันธ์ใกล้ชิดระหว่างแบคทีเรีย *Bradyrhizobium spp. Rhs. palustris* และ nonsymbiont ทั้งสี่สายพันธุ์ ก่อให้เกิดสมมุติฐานเกี่ยวกับบทบาทของการถ่ายโอนในแนวราบของยีนที่ควบคุมภาวะพึ่งพาอาศัยซึ่งกันและกันที่มีต่อวิวัฒนาการและการปรับตัวของแบคทีเรียเหล่านี้ ดังนั้นจึงได้ศึกษาความสามารถในการถ่ายโอน symbiosis island ของ *Mesorhizobium loti* ไปสู่แบคทีเรียกลุ่ม *Bradyrhizobiaceae* พบว่า integrative vector ที่ประกอบไปด้วยส่วนของ *attP* และยีน *intS* สามารถรวมเข้าในโครโมโซมของ *Bradyrhizobium Rhs. palustris* และ nonsymbiont ที่ตำแหน่งยีน *phe*tRNA ความสามารถของเอนไซม์ IntS ในการเร่งกระบวนการ integrative recombination ต่อลำดับดีเอ็นเอเป้าหมายที่มีความแตกต่าง แสดงให้เห็นว่าเอนไซม์ IntS สามารถทำงานได้ในแบคทีเรียเจ้าบ้านที่มีความหลากหลาย และการศึกษาการแสดงออกของโพรโมเตอร์สันนิษฐานของยีน intS ยืนยันว่าเอนไซม์ IntS สามารถทำงานได้ในแบคทีเรียกลุ่ม *Bradyrhizobiaceae* ถึงแม้ว่ามีระดับการแสดงออกต่ำกว่า เมื่อเปรียบเทียบกับ *M. loti* ที่น่าสนใจคือ symbiosis island สามารถรวมเข้าในโครโมโซมของ *B. yuanmingense* S7 อย่างไรก็ตามพบว่าการถ่ายโอน symbiosis island ไม่ได้ช่วยเพิ่มความสามารถในการอยู่ร่วมกันกับพืช *Lotus* และยังเป็นสาเหตุของการสูญเสียความสามารถในการอยู่ร่วมกันกับถั่วเหลืองที่เป็นพืชอาศัยเดิม นอกจากนี้ยังตรวจพบว่ามีการลบทิ้งยีนใน symbiosis island บางส่วนออกไปหลังจากกระบวนการถ่ายโอน เป็นไปได้ว่าการลบทิ้งยีนเหล่านี้เพื่อรักษาความสามารถในการอยู่ร่วมกันแบบพึ่งพาอาศัยซึ่งกันและกันกับพืชอาศัยเดิมเอาไว้ หรือเพื่อกำจัดส่วนที่ไม่เป็นประโยชน์ออกไป การศึกษาครั้งนี้ได้ค้นพบผลกระทบของการถ่ายโอนในแนวราบของยีนที่ควบคุมภาวะพึ่งพาอาศัยซึ่งกันและกันต่อประสิทธิภาพของแบคทีเรียหัวเชื้อ ซึ่งแบคทีเรียเหล่านี้อาจสูญเสียความสามารถในพื้นที่ปลูกซึ่งมีแบคทีเรียที่มียีนควบคุมภาวะพึ่งพา

อาศัยซึ่งกันและกันที่ไม่เสถียร นอกจากนี้ได้วิเคราะห์หาจุดเริ่มต้นของการถอดรหัส ตำแหน่งโพรโมเตอร์ และบริเวณที่จำเป็นต่อโพรโมเตอร์ของยีน *intS* เพื่อที่จะเข้าใจระบบการควบคุมการแสดงออกของยีน *intS* พบว่าจุดเริ่มต้นของการถอดรหัสตั้งอยู่ที่ตำแหน่งเหนือรหัสพันธุกรรมเริ่มต้น ATG 16 นิวคลีโอไทด์ และบริเวณเหนือจุดเริ่มต้นของการถอดรหัส 200 นิวคลีโอไทด์ มีความจำเป็นต่อการถอดรหัส จึงเป็นไปได้ว่าบริเวณนี้เป็นที่ตั้งของตำแหน่งจับของโปรตีนควบคุมการถอดรหัส

สาขาวิชา เทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมืออาจารย์ที่ปรึกษาร่วม_____

RUJIREK NOISANGIAM : SYMBIOTIC GENES TRANSFER OF *BRADYRHIZOBIACEAE* STRAINS ISOLATED FROM SOIL IN THAILAND. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr. rer. nat, 172 PP.

BRADYRHIZOBIACEAE/*MESORHIZOBIUM LOTI*/PHYLOGENETIC ANALYSIS/ SYMBIOSIS ISLAND/MULTILOCUS SEQUENCE ANALYSIS/SITE-SPECIFIC INTEGRATION

Bradyrhizobium strains directly isolated from soybean field soil in Thailand were classified into symbiotic and non-symbiotic groups. Polyphasic study was performed to determine taxonomic positions of four representatives of unidentified nonsymbiotic strains. Phenotypic analyses showed that the nonsymbiotic strains were found to be highly resistant to heavy metals and had characteristics intermediate between *Bradyrhizobium* species and *Rhodopseudomonas palustris*. Moreover, from the multilocus sequence analysis of 16S rRNA gene and housekeeping genes (*atpD*, *recA*, and *glnII*), the nonsymbiotic strains represented an independent phylogenetic lineage that was inconsistently related to the *Bradyrhizobium* species and *Rhs. palustris*. Therefore, the nonsymbiotic strains were proposed as a novel genus and species, named *Metalliresistens boonkerdii* gen. nov., sp. nov. The close relationship among *Bradyrhizobium* species, *Rhs. palustris* and the nonsymbiotic strains led to the hypothesis that horizontal transfer of symbiotic or photosynthetic regulatory perhaps played an important role in evolution and adaptation of these bacteria. Therefore, the

ability of *Mesorhizobium loti* symbiosis island to transfer to *Bradyrhizobiaceae* bacteria was determined in this study. The integrative vector containing *attP* and *intS* was found to integrate into a *phe*tRNA gene in the chromosome of *Bradyrhizobium* strains, *Rhs. palustris* and the nonsymbiotic *Bradyrhizobiaceae* strains. The ability of the *intS* to catalyze the integrative recombination with mismatches of the core sequence suggests that the *intS* has a broad host range. Analysis of integration sites provided a proposal of staggered cleavage sites of the *intS*. Expression analysis of putative *intS* promoter confirmed that *intS* could function in *Bradyrhizobiaceae* bacteria, although the levels of expression were lower than that of *M. loti*. Interestingly, symbiosis island was found to integrate into the chromosome of *B. yuanmingense* strain S7. However, the integration did not provide symbiotic ability on *Lotus* plants and caused symbiotic deficiency on its own soybean host plant. The symbiosis island genes were deleted after integrative recombination in all transconjugants, probably to maintain their symbiotic ability or to eliminate disadvantage parts. This study reveals the impact of horizontal transfer of symbiotic genes on the efficiency of inoculated strains. One thing possible is the inoculated strain may lose the symbiotic potential in field soil containing the bacteria that have unstable symbiotic properties. In addition, transcription start site, promoter region and essential regions for promoter of *intS* were determined to understand the regulatory expression system of the *intS*. The transcription start site was found to locate at 16 nt upstream of the first ATG start codon. The 200-nt region upstream of

the transcription start site were essential for the transcription. Probably, the region is the location of the transcription factor-binding sites.

School of Biotechnology

Academic Year 2010

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CHAPTER I

INTRODUCTION

Members of the genus *Bradyrhizobium* are symbiotic nitrogen-fixing soil bacteria that has the ability to form nodules on the root and/or stem of specific leguminous plants (Jordan and Allen, 1974). The economic legumes nodulated by *Bradyrhizobium,* include soybean, peanut, cowpea and mungbean. In the nodules, the bacteria fix atmospheric nitrogen and provide the host plants with nitrogen in the form of ammonium. The beneficial effect of *Bradyrhizobium* in legume in terms of biological nitrogen fixation is an important aspect of sustainable and environmental friendly food production and long term crop productivity. Consequently, the symbiotic bacteria are of enormous ecological and economic values.

Bradyrhizobium is classified into the family *Bradyrhizobiaceae* with 8 other genera of *Afipia, Agromonas, Blastobacter, Bosea, Oligotropha, Rhodopseudomonas, Nitrobacter,* and *Rhodoblastus*. Sequence analysis of 16S rDNA region has revealed a very close relationship among these bacteria (Saito et al., 1998; Seewaldt et al., 1982; van Berkum et al., 2006b; Willems and Collins, 1992), leading to caution in the taxonomy and systematics of this group of bacteria (Sawada et al., 2003; Vinuesa et al., 1998). Thus, polyphasic approach including phenotypic and genotypic analyses is recommended for identification of these bacteria. Based on 16S rRNA sequence analysis, *Rhs. palustris*, which is a phototrophic purple non-sulfur bacterium, is

closely related to nodule-forming *B. japonicum* 22other rhizobia (Khamis et al., 2003; Martens et al., 2008a; Parker, 2004; Rivas et al., 2009c; Sameshima et al., 2003; Vinuesa et al., 2005c).

In the previous study (Pongsilp et al., 2002), *Bradyrhizobium* isolates were obtained from heavily inoculated soybean field soil and uninoculated soybean free soil in Thailand, using BJSM medium (Tong and Sadowsky, 1994). The *Bradyrhizobium* species were expected to be directly isolated from soil, however nonsymbiotic isolates were found to be a significant component in both fields. From the 16S rRNA gene sequence analysis, a number of nonsymbiotic isolates appeared to belong to family *Bradyrhizobiaceae*. These nonsymbiotic isolates could not be clearly affiliated, since they had 96-97% identity to different genus in *Bradyrhizobiaceae* family including *Bradyrhizobium*, *Rhodopseudomonas* and *Afipia*. Therefore, the polyphasic approach, including analyses of phenotypic and genotypic characteristics, was used to identified the nonsymbiotic isolates. The phenotypic characteristics analyzed in this study included colony and cell morphology, biochemical characteristics, cellular fatty acids composition, G+C content, resistance to antibiotics and heavy metals and photosynthetic pigmentation. In addition, multilocus phylogenetic analysis of individual and combination of 16S rRNA gene, *atpD*, *glnII* and *recA* were used to elucidate taxonomic relationships of the nonsymbiotic isolates. The identification of nonsymbiotic *Bradyrhizobiaceae* isolates would provide the useful information of regarding to the evolutionary history of symbiotic and photosynthetic regulatory systems. The polyphasic study of the nonsymbiotic isolates indicated that they had characteristics intermediate between *Bradyrhizobium* species

and *Rhs. palustris*. It was hypothesized that horizontal transfer of either symbiotic or photosynthetic regulatory may play an important role in the evolution these *Bradyrhizobiaceae* bacteria.

Rhizobia are soil bacteria capable of forming nitrogen-fixing nodules on root or stem of leguminous plants. In several species of rhizobia, genes required for the symbiotic interaction with host plants are on large plasmids. Exceptions in which the symbiosis genes are encoded on the chromosome, termed as symbiosis island, include strains of *Mesorhizobium loti*, *B. japonicum*, and *Azorhizobium caulinodans*. Symbiosis island was first identified in *M. loti* strain ICMP3153 (Sullivan and Ronson, 1998) and then in strain MAFF303099 and R7A (Sullivan et al., 2002). The symbiosis island of *M. loti* has ability to transfer to nonsymbiotic mesorhizobia in the environment and convert them to symbionts of their host legume (Sullivan et al., 1995). Symbiosis island transfer is facilitated by a tyrosine recombinase that belongs to the satellite bacteriophage P4 integrase family (Ramsay et al., 2006). A family of integrase usually targets the 3′ end of tRNA genes for integration (Zhao and Williams, 2002). The genome structure of *B. japonicum* USDA110 is similar to that of *M. loti* in that many of genes for symbiotic nitrogen fixation are clustered on the chromosome (Gottfert et al., 2001; Kundig et al., 1993). Instead of the *phe*tRNA gene in *M. loti*, a *val*tRNA gene was found at one end of the symbiosis island of *B. japonicum* USDA110. Moreover, there is a number of parts of tRNA genes distributed in chromosome of *B. japonicum* USDA110. This is possible that *M. loti* symbiosis island can be transferred to *Bradyrhizobium* species and their related genus, using the tRNA genes as the target sites for the integration. In this study, the transfer ability of *M. loti* symbiosis island to *Bradyrhizobiaceae* bacteria including *Bradyrhizobium* species,

Rhs. *palustris*, and nonsymbiotic isolated was determined. Subsequently, the effects of the transfer on the symbiotic properties of the bacterial recipient were observed. The transfer of symbiosis island provides useful informations relevant to evolution of rhizobia and could have important ecological consequences.

Objectives

1. To characterize the nonsymbiotic isolates by using a polyphasic approach, and to describe the novel isolates for which the new genus was proposed.

2. To determine an ability of *M. loti* integrase for integration into *Bradyrhizobiaceae* strains.

3. To determine an ability of *M. loti* symbiosis island transfer to *Bradyrhizobiaceae* strains.

4. To determine effects of symbiosis island transfer on symbiotic ability of recipient strains.

CHAPTER II

LITERATURE REVIEW

2.1 Significance of rhizobia

The symbiotic relationship between nitrogen-fixing rhizobia and legume plants has been studied for over 100 years as a classic example of mutualistic associations (Rivas et al., 2009a). Rhizobia or sometimes *Rhizobium* is the general name given to a phylogenetically diverse group of soil bacteria that form nitrogen-fixing nodules on root or stem of leguminous plants. These bacteria are environmental heterotrophs with great metabolic plasticity that may survive in many different environments as saprophytes or symbionts of plant hosts. Rhizobia have significant interest because of their ability to form symbiotic associations with a variety leguminous plants. Symbiosis between rhizobia and legumes is the result of millions of years of co-evolution of a complex and exquisitely regulated process, called biological nitrogen fixation, that plays an important role because it offers the ability to convert atmospheric nitrogen into useable form by the plant. In effective nodules, the bacteria fix atmospheric nitrogen into ammonia, which is assimilated by the plant and supports growth, particularly in nutrient deficient soils. In return the rhizobia are supplied with nutrients, and are protected inside the nodule structure. The nitrogen-fixing symbiotic relationship has been exploited in agriculture to enhance crop without the addition of nitrogen fertilizers and represents an inexpensive alternative to

the use of chemical nitrogen fertilizers in the long-term production of foods and crops. Furthermore, currently, the subject of biological nitrogen fixation is of great practical importance because the use of nitrogenous fertilizers has resulted in unacceptable levels of water pollution increasing concentrations of toxic nitrates in drinking water supplies and rivers (Zahran, 1999). Therefore, rhizobia are of global significance, both in terms of agriculture and ecological relationships as an environmental friendly source of nitrogen for plants.

2.2 Taxonomy of rhizobia

Rhizobia are ecologically and economically valuable, thus they have been classified and studied extensively. Taxonomy of rhizobia has been driven by technological advances in criteria, such as morphological, physiological and sequence analysis, used in taxonomy. In 1888, Beijerinck reported isolation of the root-nodule bacteria and established that they were responsible for this process of nitrogen fixation, and these bacteria was named *Bacillus radicicola* (Beijerinck, 1888). At the earliest, names of these bacteria were made after the host plant (Hiltner and Störmer, 1903). Later, the name was changed to *Rhizobium* with originally just one species, *R. leguminosarum* and a classification based on nodulation range with emphasis on host plant was proposed (Frank, 1889). Extensive testing of nodulation of diverse legume hosts by different bacteria in the beginning of the 20th century, led to the establishment of cross-inoculation groups, with rhizobia from one plant in a cross-inoculation group supposed to nodulate all other plants in the group (Willems, 2006). However, this approach was abandoned as an unreliable taxonomic marker, in part

because of aberrant cross-infection among plant groups. In fact, there are about 18,000 species of legumes as well as countless rhizobia (Broughton, 2003) and the host range of both bacteria and plants is widely dispersed in nature. It is not associated with a particular bacterial or plant taxonomic group and is not correlated with the growth habit of the legume (Perret et al., 2000). For examples, a number of genera within the *Phaseoleae* (e.g., *Phaseolus* and *Vigna*) form nodules with about half of all rhizobia presented to them (Michiels et al., 1998) and some individual rhizobia (e.g., *Rhizobium* sp. NGR234) are able to nodulate about 50% of all legumes (Pueppke and Broughton, 1999).

Afterward, in the early 1960s, bacteriologists started using a large diversity of morphological, nutritional and metabolic characters, as well as serology and simple DNA characteristics in numerical taxonomy studies. This demonstrated the relatedness of *Rhizobium* and *Agrobacterium* and led to a clear distinction between the fast and slow growing rhizobia (Willems, 2006). Thus, the genus *Bradyrhizobium* subsequently placed in a separate genus (Jordan, 1982). In the first edition of *Bergey's Manual of Systematic Bacteriology* only two rhizobial genera (*Bradyrhizobium* and *Rhizobium*) with four species were described (Krieg and Holt, 1984). Since then, extensive phenotypic and genotypic variations have been described in rhizobia. Use of PCR tools and sequencing methods has led to description of new, and re-organization of the existing genera. In addition, with the introduction of more genetic characteristics, such as DNA–DNA and DNA–rRNA hybridizations, more diversity was discovered among the rhizobia and their relationships with other groups of bacteria became apparent. This led to a gradual increase in the number of genera.

Currently, most of rhizobia species are in the class Alphaproteobacteria including *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Bradyrhizobium*, *Azorhizobium*, *Ochrobactrum*, *Phyllobacterium*, *Devosia*, and *Methylobacterium* (Chen et al., 1988; de Lajudie et al., 1998; Elkan, 1992; Garrity et al., 2004) There are also three rhizobial species in two families in the Betaproteobacteria, all of which are in the family *Burkholderiaceae* (*Cupriavidus* and *Burkholderia*) and *Oxalobacteraceae* (Garrity et al., 2004; Van Rhijn and Vanderleyden, 1995).

Nowadays, bacterial classification involves techniques to determine both phenotypic and genotypic characteristics. Of the genotypic methods, 16S rRNA gene sequencing and genomic DNA–DNA association serve as gold standards for bacterial species determination (Stackebrandt and Goebel, 1994). However, DNA–DNA hybridization is a technically challenging, labour-intensive and time-consuming method. Also, it is not possible to establish a central database, mainly because the technique provides a non-cumulative, relative DNA relatedness value, but also because of technical non-uniformity and variability between different laboratories and methodologies (Martens et al., 2008b). Compared to former techniques, 16S rRNA gene sequence analysis has demonstrated high resolving power for measuring the degree of relatedness between organisms above the species level (Stackebrandt and Goebel, 1994). However, the 16S rRNA gene sequence was argued that it ofter lacks resolving power at and below the species level. Several studies have reported bacteria that represent different species with identical or nearly identical 16S rRNA gene sequences (Fox et al., 1992; Jaspers and Overmann, 2004; Sullivan et al., 1996). Another limitation of the 16S rRNA gene for identification purposes is its

heterogeneity due to the occurrence of multiple copies of the 16S rRNA gene within single genomes (Acinas et al., 2004; Broughton, 2003).

As more whole-genome sequences become available, various new opportunities to study the genetic relatedness of bacterial strains may be exploited. Several novel approaches, e.g. comparison of gene order, gene content, nucleotide composition and codon usage were described, to assess bacterial relationships based on whole-genome sequences (Coenye et al., 2005). In spite of, the fact that whole-genome sequencing projects are delivering new sequences at a rapidly increasing rate, the limited availability of whole-genome sequences of related strains and taxonomic reference strains currently restricts the use of whole-genome based approaches for broad-spectrum identification and phylogenetic purposes. Therefore, reliable alternatives, which do not require full genome sequences, for the assessment of bacterial relationships are needed. Recently, the analysis of multilocus sequence analysis (MLSA) of housekeeping genes has become a widely applied tool for the investigation of taxonomic relationships (Martens et al., 2008b). The use of information from the comparison and combination of multiple genes can give a global and reliable overview of inter-organismal relationships. In comparison with 16S rRNA genes, the higher degree of sequence divergence of housekeeping genes is superior for identification purposes, since the more-conserved rRNA gene sequences do not always allow species discrimination. Examples of genes that were used as molecular markers to elucidate taxonomic relationships among *Bradyrhizobium* species and other rhizobia included *atpD* (ATP synthase F1, β-subunit), *dnaK* (heat-shock protein Hsp70), *gap* (glyceraldehyde-3-phosphate dehydrogenase), *glnA*

(glutamine synthetase type I), *glnII* (glutamine synthetase type I), *gltA* (citrate synthase I), *gyrB* (DNA gyrase β-subunit), *pnp* (polyribonucleotide nucleotidyltransferase), *recA* (recombinase A), *rpoB* (RNA polymerase β-subunit), *thrC* (threonine synthase), and 23S rRNA gene (Khamis et al., 2003; Martens et al., 2008a; Rivas et al., 2009c; Sameshima et al., 2003; Vinuesa et al., 2005c). The ad hoc committee for re-evaluation of the species definition regarded the sequencing of a minimum of five well-chosen housekeeping genes, universally distributed, present as single copies and located at distinct chromosomal loci, as a method of great promise for prokaryotic systematics (Stackebrandt et al., 2002). MLSA data should be interpreted with caution and should always be compared with data from the 16S rRNA gene so as to minimize the impact of possible recombination events when assessing phylogenetic relationships for taxonomic purposes.

2.3 *Bradyrhizobiaceae*

The family *Bradyrhizobiaceae* was circumscribed on the basis of phylogenetic analysis of 16S rRNA gene sequences belonging to the alpha-2 subgroup of proteobacteria. The family is phenotypically, metabolically, and ecologically diverse. Currently, the family *Bradyrhizobiaceae* consists of 9 genera, which are *Bradyrhizobium*, *Afipia, Agromonas*, *Blastobacter*, *Bosea, Nitrobacter*, *Oligotropha*, *Rhodoblastus*, and *Rhodopseudomonas*.

2.3.1 *Bradyrhizobium*

Members of genus *Bradyrhizobium* are symbiotic nitrogen- fixing soil bacteria that has the ability to form nodules on the root or stem of specific leguminous plants. *Bradyrhizobium* species are Gram-negative bacilli (0.5-0.9 x 1.2-3.0 μm) with a single subpolar or polar flagellum. These bacteria are nonspore-forming and aerobic, and possess a respiratory type of metabolism with oxygen as the terminal electron acceptor. They usually contain granules of poly-β-hydroxybutyrate, which are refractile by phase-contrast microscopy. Colonies are circular, opaque, rarely translucent, white, and convex, and tend to be granular in texture. They are slow growing in contrast to *Rhizobium* species, which are considered fast growing rhizobia. In a liquid media broth, it takes *Bradyrhizobium* species 3-5 days to create a moderate turbidity and 6-8 hours to double in population size. They tend to grow best with pentoses as a carbon source. Colonies often do not exceed 1 mm in diameter within 5-7 days incubation on yeast-mannitol-mineral salts agar. They produce an alkaline reaction in mineral salts medium containing mannitol and several other carbohydrates. Growth on carbohydrate media is usually accompanied by the production of an extracellular polysaccharide slime. *B. japonicum* is the most agriculturally important species because it has the ability to form root nodules on soybeans (*Glycine max*). *B. japonicum* USDA110, which was originally isolated from soybean nodule in Florida, USA in 1957, has been widely used for the purpose of molecular genetics, physiology, and ecology, because of its superior characteristics regarding symbiotic nitrogen fixation. The genome of *B. japonicum* USDA110 is a single chromosome 9,105,828 bp in length. The average G-C content of the genome is 64.1 mol %. Fifty-two

percent of the 8,317 potential protein-coding genes are like genes of known function, 30% of the genes are hypothetical, and 18% have no similarity to any reported genes. In addition, 34% of the genes were like genes in *M. loti* and *S. meliloti*, and 23% of the genes were unique to *B. japonicum* (Kaneko *et al.*). The genome structure of *B. japonicum* USDA110 is similar to that of *M. loti* in that many of the genes for symbiotic nitrogen fixation are clustered on the chromosome.

To date, the genus *Bradyrhizobium* consists of 9 species including *B. japonicum* (type species) nodulating *Glycine* (Jordan, 1982), *B. betae* from the roots of *Beta vulgaris* afflicted with tumor-like deformations (Rivas et al., 2004), *B. canariense* from genistoid legumes from the Canary Islands (Vinuesa et al., 2005a), *B. elkanii* nodulating *Glycine* (Kuykendall et al., 1992), *B. iriomotense* from a Tumor-Like Root of the *Entada koshunensis* from Iriomote Island (Islam et al., 2008), *B. jicamae* and *B. pachyrhizi* from nodules of *Pachyrhizus erosus* (Ramirez-Bahena et al., 2009), *B. liaoningense* nodulating *Glycine* (Xu et al., 1995), and *B. yuanmingense* nodulating *Lespedeza* (Yao et al., 2002). In addition to the species subdivision, a number of serogroups have been described among slow-growing soybean symbionts. Many other slow-growing rhizobia have been isolated from other legume hosts and are commonly referred to as *Bradyrhizobium sp.*, followed by the name of the legume host. A special feature of the *Bradyrhizobium*–legume symbiosis is that some strains of *Bradyrhizobium sp*., such as BTai1 and ORS278 can form stem nodules on some plant species, produce bacteriochlorophyll and perform photosynthesis (Molouba et al., 1999). In addition, photosynthetic *Bradyrhizobium* strains have also been reported as endophytes of African wild rice (Chaintreuil et al., 2000).

2.3.2 *Rhodopseudomonas*

Rhodopseudomonas bacteria are purple nonsulfur phototrophic organisms that can be found many types of marine environments and soils. These bacteria are capable of producing energy through photosynthesis and converting atmospheric carbon dioxide into biomass. The genus *Rhodopseudomonas* consists of 17 species. *Rhs. palustris* is a type species of the genus. *Rhs. palustris* has the potential to be very useful because it can degrade and recycle several different aromatic compounds that make up lignin, the main constituent of wood and the second most abundant polymer on earth. Thus, they are useful in removing these types of waste from the environment. In addition, *Rhs. palustris* converts N_2 into NH_4 and H_2, thus H_2 which thucan be used as a biofuel.

Rhs. palustris is a purple photosynthetic bacterium that is widely distributed in nature as indicated by its isolation from diverse sources, such as swine waste lagoons, earthworm droppings, marine coastal sediments and pond water. It has extraordinary metabolic versatility and grows by any one of the four modes of metabolism that support life: photoautotrophic or photosynthetic, photoheterotrophic, chemoheterotrophic, and chemoautotrophic. *Rhs. palustris* enjoys exceptional flexibility within each of these modes of metabolism. It grows with or without oxygen and uses many alternative forms of inorganic electron donors, carbon and nitrogen (Larimer et al., 2004). This metabolic versatility has raised interest in the research community and make this bacterium suitable for potential use in biotechnological applications. Thus *Rhs. palustris* is a model organism to study how the web of metabolic reactions, operating within the confines of a single cell, adjusts and

reconstructs itself in response to changes in light, carbon, nitrogen and electron sources (Larimer et al., 2004). Members of genus *Rhodopseudomonas* are Gram-negative bacilli with a single subpolar or polar flagellum. Cells show polar growth, budding and asymmetric cell division. They contain internal photosynthetic membranes that appear as lamella underlying and parallel to the cytoplasmic membrane and their photosynthetic pigments include bacteriolchlorophyll a and carotenoids of the sperilloxanthin series (Brenner et al., 2005). Preferred mode of growth is photoheterotrophic under anoxic conditions in the light.

The genome of *Rhs. palustris* CGA009 consists of is a 5,459,213-bp chromosome and a 8,427- bp plasmid, which consist of 4,836 predicted protein-encoding genes. *Rhs. palustris* encodes 63 signal transduction histidine kinases and 79 response regulator receiver domains. This genome sequence is a starting point to use *Rhs. palustris* as a model to explore how organisms integrate metabolic modules in response to environmental influences. Almost 15% of the genome is devoted to transport (Larimer et al., 2004). The average G-C content of the genome is 65.2 mol % and 3 horizontally transferred genomic islands were identified. (Zhang and Zhang, 2004). These genomic islands appear to encode functions that expand the environmental niches accessible to this bacterium.

2.4 Phylogenetic analysis

2.4.1 Principle

Phylogenetics is the science of estimating the evolutionary past, in the case of molecular phylogeny, based on the comparison of DNA or protein sequences. A

phylogeny is the evolutionary history of a group of entities. Given that this can only truly be known in exceptional circumstances, the main aim of phylogeny reconstruction is to describe evolutionary relationships in terms of relative recency of common ancestry. These relationships are represented as a branching diagram, or tree, with branches joined by nodes and leading to terminals at the tips of the tree (Figure 2.1).

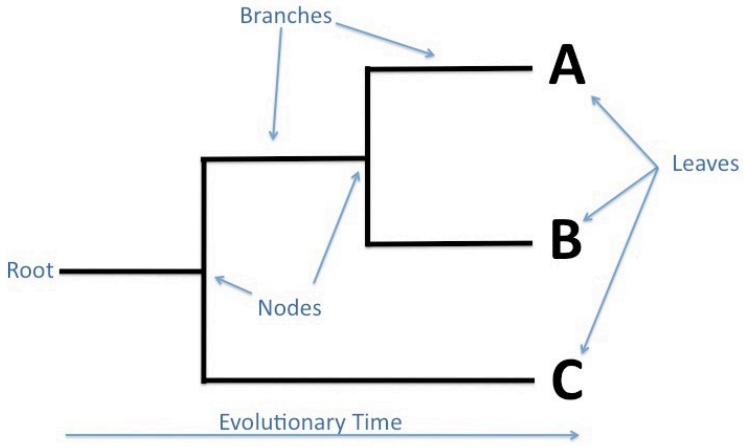

Figure 2.1 The crucial components of a phylogenetic tree are nodes, branches, leaves, and the root. Each leaf represents an organism. Each node, represents the common ancestor of all the organisms that branch out of it. The root represents the common ancestor of every organism on the tree. Evolutionary time is represented along the "x" axis of the tree. On this tree, the common ancestor (node) of organisms A and B is more recent than the common ancestor of A and C. Now lets work backwards and talk about how this tree could have been built.

Figure 2.2 shows three main types of relationship distinguished are monophyly, paraphyly and polyphyly (Baldauf, 2003). Monophyletic and paraphyletic groups have a single evolutionary origin. Monophyletic groups include all the descendants from a single ancestor, as well as that ancestor. If one lineage emerging from a monophyletic group is removed, a paraphyletic group remains. In contrast, polyphyletic groups result from convergent evolution, and the characters that support the group are absent in the most recent common ancestor.

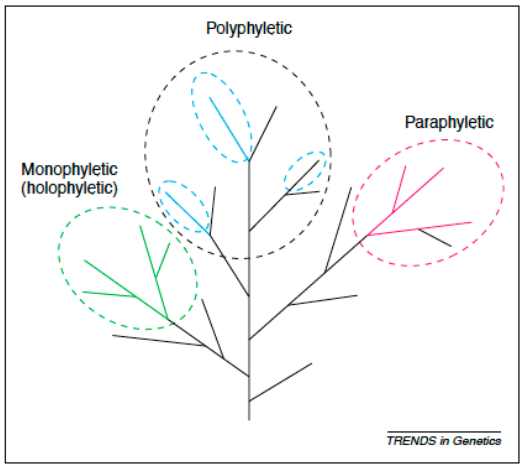

Figure 2.2 Trees are about groups: monophyletic (holophyletic), paraphyletic and polyphyletic. (Source: Baldauf, 2003)

Evolution is about homology, that is the similarity due to common ancestry. Homologues can be orthologues or paralogues (Figure 2.3). Orthologues only duplicate when their host divides, i.e. along with the rest of the genome (Figure 2.3a). They are strictly vertically transmitted (parent to offspring), so their phylogeny traces that of their host lineage (Figure 2.3b). Within a monophyletic gene group, each species is represented by a single orthologue. In contrast, paralogues are members of

multigene families. They arise by gene duplication. Thus, within a gene group each species may be represented by a number of paralogues.

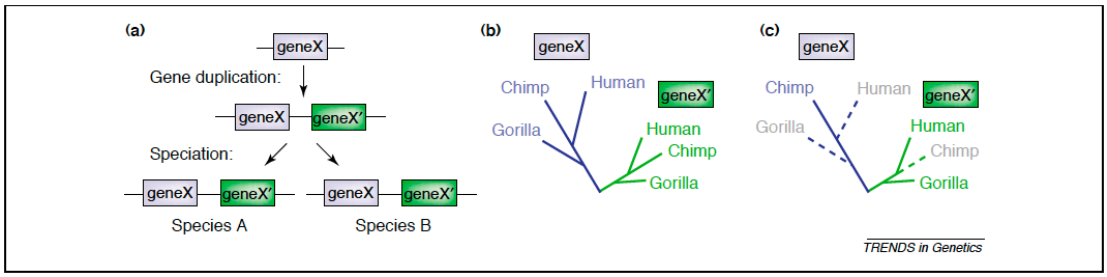

Figure 2.3 Schematic illustration of paralogue and orthologue. (a) Paralogous genes are created by gene duplication events. Gene X is duplicated in a common ancestor to species A and B resulting in two paralogous genes, X and X' . All subsequent species inherit both copies of the gene (unless one or the other is lost somewhere along the way). (b) Phylogenetic analysis of the X/X' gene family gives two parallel phylogenies. All sequences of gene X are orthologues of each other, and all the sequences of gene X' are orthologues of each other. However, X and X' are paralogues. Both the X and X' subtrees show the true relationships among the three species. The subtrees are also each other's natural outgroup, and as a result each subtree is rooted with the other (reciprocally rooting). (c) A tree of the X/X' gene family can be misleading if not all the sequences are included (because of incomplete sampling or gene loss). If the broken branches are missing, then the true species relationships are misrepresented. (Source: Baldauf, 2003)

2.4.2 Phylogenetic reconstruction

A good first step is to survey the literature pertaining to the group of interest. This will inform the choice of species and genes to be included in the analysis and will indicate clades in which relationships are likely to be resolved and statistically supported in the resulting phylogeny. It will also highlight clades that should benefit from greater sampling or more attention in alignments.

Next step is sampling the group of interest. The main sources of data for reconstructing the phylogeny of a gene family includes published sequences of characterized genes, gene databases such as NCBI, EST project databases and unpublished data from colleagues. In addition to searching a number of different databases, sampling is optimized by using the TBLASTX option at the sequence retrieval stage in BLAST searches (Altschul et al., 1997). As opposed to other options, this program translates the nucleotide sequence in all six frames and compares the output against all the translated sequences in the database. Therefore, it maximizes the potential for retrieving sequences similar to the gene of interest.

The number of sequences retrieved from BLAST searches varies depending on the size of the gene family, and what is chosen for inclusion in further analyses will vary accordingly. When sequences are retrieved from BLAST searches they are allocated an e-score, which is an indication of the degree of similarity between the initial sequence used for searches and the sequence retrieved. The closer the e-value is to 0, the higher the degree of similarity between the two sequences. For large gene families there may be a clear cut-off point between the e-scores of the group of interest and further gene family members. If there is no clear cut-off, the literature can

be used to identify genes in the list of retrieved sequences that, on the basis of their function, are likely to be outside the group of interest. The e-scores of those sequences can then be used as a guide for the cut-off point (Harrison and Langdale, 2006).

After retrieving the sequences, a common practice of phylogenetic reconstruction requires the analysis of a multiple alignment of the sequences. The quality of the alignment may have an enormous impact on the final phylogenetic tree; especially, when the compared sequences are very divergent and of different length (Dereeper et al., 2008). Alignment quality may have as much impact on phylogenetic reconstruction as the phylogenetic methods used. Not only the alignment algorithm, but also the method used to deal with the most problematic alignment regions, may have a critical effect on the final tree. Therefore, after generation of a multiple alignment, problematic regions should be removed, either manually or using automatic methods, in order to improve phylogenetic performance (Talavera and Castresana, 2007).

Once data are aligned, there are many different types of phylogenetic analysis that can be implemented. The type of analysis used will be determined by compromise between the length of computational time and the degree of rigor required. The main techniques are distance, parsimony and likelihood (including Bayesian analysis). There are many alternative programs that perform the same functions and are equally valid to use (see web resource list at http:// evolution.genetics.washington.edu/phylip/software.html).

Distance methods (neighbour joining (NJ), distance and minimum evolution)calculate pairwise distances between sequences and group sequences that

are most similar. This approach has potential for computational simplicity and speed. However, distance methods do not allow an analysis of which characters contribute to particular groupings. As with other methods, the outcome may depend on the order in which entities are added to the starting tree, but because only one tree is outputted it is not possible to examine conflicting tree topologies. Although distance methods are often useful for making an initial tree, they should be used for final trees with caution. Instead, parsimony and likelihood are preferred because they have the potential to rigorously explore the relationship between the tree and the entities included. Parsimony and likelihood use different criteria to choose the best trees. In these analyses the branches of a starting tree are rearranged to form the tree that minimizes the number of character state changes (parsimony) or the tree that best fits the data (likelihood).

Parsimony assumes that shared characters in different entities result from common descent. Groups are built on the basis of such shared characters, and the simplest explanation for the evolution of characters is taken to be the correct, or most parsimonious one. With multiple characters, different groupings may be equally plausible, or equally parsimonious, and therefore multiple trees are generated. In such cases, a strict consensus tree should be derived that includes only topologies that are not contradicted in any of the initial trees. If the strict consensus tree is unresolved there is no congruence between initial trees, and thus it is likely that the data used to build the tree are phylogenetically uninformative. A majority rule consensus tree shows nodes that are consistent in half to all of the most parsimonious trees and the percentage of trees in which a given topology exists is shown on the branches.

However, since by definition all most parsimonious trees are considered equally good, if any one contradicts the others the node in question should collapse. Hence majority rule trees are not informative about phylogeny.

In contrast to parsimony, maximum likelihood analyses compute the probability that a data set fits a tree derived from that data set, given a specified model of sequence evolution. A good first step is to compare the data against a set of models of sequence evolution and choose the one that best describes the observed pattern of sequence variation. A programs that can be used is Modeltest (Posada and Crandall, 1998). Alternatively a user-specified model may be chosen. This model of sequence evolution is then used in the likelihood analysis. The analysis starts with a specified tree derived from the input dataset and swaps the branches on the starting tree until the tree with the highest likelihood score (i.e. the best probability of fitting the data) is gained. This score is a function both of the tree topology and the branch lengths (number of character state changes). Likelihood analysis therefore allows an explicit examination of the assumptions made about sequence evolution. Likelihood methods are the most computationally demanding techniques for phylogenetic analysis.

2.4.3 Multilocus sequence analysis (MLSA)

Multilocus sequence analysis (MLSA) represents the novel standard in microbial molecular systematics. In this context, MLSA is implemented in a relatively straightforward way, consisting essentially in the concatenation of several sequence partitions for the same set of organisms, resulting in a supermatrix which is used to infer a phylogeny by means of distance-matrix or optimality criterion-based methods.

This approach is expected to have an increased resolving power due to the large number of characters analyzed, and a lower sensitivity to the impact of conflicting signals (i.e. phylogenetic incongruence) that result from eventual horizontal gene transfer events. The strategies used to deal with multiple partitions can be grouped in three broad categories: the total evidence, separate analysis and combination approaches.

Although the current classification of bacteria is based in the 16S rRNA gene that has allowed the description of various species of rhizobia, it has limitations to differentiate among close species and for this purpose several metabolic genes (housekeeping) have been proposed in several groups of bacteria (Maiden, 2006; Martinez-Salazar et al., 2009; Rivas et al., 2009a). In rhizobia, the two first genes analyzed were *recA* and *atpD* (Gaunt et al., 2001) and currently they have been sequenced in many rhizobial species showing their usefulness in differentiation of species whose 16S rRNA genes are nearly identical. Currently, the MLSA based in the analysis of several housekeeping genes have been applied phylogenetic analyses of concrete groups of rhizobia as *Ensifer* (Martens et al., 2008b; Martens et al., 2007; van Berkum et al., 2006a) and *Bradyrhizobium* (Rivas et al., 2009b; Vinuesa et al., 2008). For instance, these studies indicate that housekeeping genes sequencing is superior for the assessment of genetic relatedness between *Ensifer* species and support the suggestion that *Ensifer xinjiangensis* is a later heterotypic synonym of *Ensifer fredii* (Martens et al., 2008b). The ad hoc committee for re-evaluation of the species definition suggested that species should be identified phenotypic and genomic methods and a promising approach is the determination of a minimum of

housekeeping genes (Stackebrandt et al., 2002). It was suggested that analysis of less than five suitable housekeeping genes might be sufficient for a reliable classification (Zeigler, 2003). For this reason, to describe a new rhizobial species, the analysis of at least 2 housekeeping genes have been included commonly to know the closest related species.

Besides the housekeeping genes also named "core" genes, some "auxiliary" or "accessory" genes involved in the legume symbiosis are commonly included in species description of rhizobia (Silva et al., 2005; Vinuesa et al., 2005b). In addition, symbiotic genes such as *nodD, nodA*, *nodC* and *nifH* were also used for classification of rhizobia (Estrella et al., 2009; Laguerre et al., 2001; Laranjo et al., 2008; Silva et al., 2005; Steenkamp et al., 2008; Stepkowski et al., 2007). Nevertheless, the symbiotic genes are not useful in taxonomy because due to their ability to be transferred in nature (Finan, 2002; Mergaert et al., 1997) from plasmids to islands (Nakatsukasa et al., 2008), from bacteria to plants (Broothaerts et al., 2005) and among bacteria(Rogel et al., 2001; Sullivan and Ronson, 1998). Therefore the analysis of symbiotic genes is overall useful to identify non-rhizobial species able to nodulate legumes and to carry out biogeography studies of legume endosymbionts.

2.5 Genes controlling nodulation

Rhizobial infection of the plant requires the products of the nodulation genes, which encode for the production of a lipochitin nodulation signal. This Nod signal, upon recognition by the plant, induces organogenesis leading to nodule formation. The bacteria invade the root through root hairs and penetrate inside a plant-produced

infection thread. Upon endocytosis into an infected cortical cell, the bacteria are enclosed in a membrane-bound symbiosome where they differentiate into bacteroids that are capable of fixing N_2 into a form that the plant can utilize. In return, the bacteroids are supplied with an environment rich in carbon as an energy source.

Rhizobial genes that play an important role in nodule formation are called nodulation (*nod*) genes (Heidstra and Bisseling, 1996). Some of *nod* genes appear to be interchangeable for nodulation function between different species of rhizobia and have been designated as "common nod genes". On the other hand, some *nod* genes are involved in the nodulation of a particular host and are referred to as host-specific *nod* genes (Van Rhijn and Vanderleyden, 1995). The common *nod* gene region is a cluster of *nod* genes that are structurally and functionally conserved in many *Rhizobium* and *Bradyrhizobium* strains. The common *nodYABC* genes also are involved in nodule morphogenesis, encode functions that stimulate cell division without direct contact between the bacteria, and stimulated plant cells. In response to the release by host plants of appropriate inducers, rhizobia synthesize and secrete a family of lipochitooligo-saccharides (LCOs) called Nod factors. The *nodYABC* genes also encode for the production of the backbone structure of the Nod factor which affect roots and roots hairs. The first step in Nod factor assembly is performed by an N-acetylglucosaminyl-transferase encoded by *nodC*. Chain elongation by NodC takes place at the nonreducing terminus. Then the deacetylase NodB removes the N-acetyl moiety from the nonreducing terminus of the N-acetylglucosamine oligosaccharides. Finally, an acyltransferase encoded by *nodA* links the acyl chain to the acetyl-free C-2 carbon of the nonreducing end of the oligosaccharide. Although not essential, NodI

and NodJ seem to be involved in the export of Nod factors (Fernández-López et al., 1996; Spaink et al., 1995; Vázquez et al., 1993). However, *nodA* and *nodC* are also components of host-specific nodulation (Perret et al., 2000). NodA varies in its specificity for different fatty acid substrates, thus contributing to the host range. NodC is also a determinant of the length of the Nod factor backbone and thus of host specificity (Kamst et al., 1999; Kamst et al., 1995).

Host specific *nod* genes, varying in different rhizobial species, are involved in the modification of the fatty acyl chain or the addition of strain specific substitutions that are important in determining host specificity (Carlson et al., 1995). In addition, particular decorations contribute to Nod factor stability, for example sulfated Nod factors of *S. meliloti* are more resistant to degradation than the corresponding non-sulfated molecules (Staehelin et al., 1994). Host-specific nod genes are involved in the addition of extra groups to the core lipo-oligosaccharides. Modified factors permit nodulation of specific plants. For example, the *nodH* and *nodPQ* genes of *R. meliloti* are involved in the sulfation of the oligosaccharide signals (Roche et al., 1991). In vitro studies of NodL, which shares homology with O-acetyltransferases, showed that NodL 6-Oacetylates the non-reducing glucosaminyl residue (Bloemberg et al., 1994). Both of *nodE* and *nodF* are involved in synthesis of the lipid chain (Demont et al., 1993), while *nodZ* and *nolO* play a role in fucosylation of *B. japonicum* nodulation signals (Luka et al., 1993; Stacey et al., 1994). The *nodSU* genes of *Rhizobium sp.* NGR234 form an operon, the activity of which is required for nodulation of *Leucaena leucocephala* (Lewin et al., 1990). *nodSU* are also present in *R. fredii* USDA257, *B. japonicum*, and *A. Caledonians* (Jabbouri et al., 1995).

2.6 Mechanism of symbiotic gene transfer

2.6.1 Symbiotic genes

In different species of rhizobia, genes required for symbiotic interactions with host plants are present on one or more megaplasmids known as symbiotic plasmids (pSym) , such as pNGR234a (536 kb) in *Rhizobium sp.* NGR234, pSymA (1.35 mb in *Encipher meliloti* 1021, pRL10 (488.13 kb) in *R. leguminosarum* biovar *viciae* 3841, and p42d (371.25 kb) in *R. etli* CFN42. In contrast, *Bradyrhizobium*, *Azorhizobium*, and *Mesorhizobium* species carry most of the symbiotic information on the chromosome as genomic islands, termed as "symbiosis island".

2.6.2 Transfer of symbiotic plasmids

Self-transmissible pSyms have been described in different rhizobia including *R. leguminosarum* (Brewin et al., 1980; Johnston et al., 1982)*, E. meliloti* (Mercado-Blanco and Olivares, 1993) and *Rhizobium* sp. NGR234 (He et al., 2003). The transfer of these symbiotic plasmids is regulated by a quorum-sensing mechanism (Lithgow et al., 2000; Wilkinson et al., 2002). Sequence analysis of various rhizobial symbiotic regions revealed the presence of transfer-related genes and sequences homologous to the *luxI-luxR* type of quorum-sensing regulators (Barnett et al., 2001; Gonz·lez et al., 2003; Marketon and Gonzalez, 2002). Nevertheless, the conditions under which they are functional remain to be elucidated.

Rhizobium sp. strain NGR234, for example, is unique among the rhizobia for its unusual ability to nodulate over 100 different legume species and at least one non-legume. Sequencing of the symbiotic plasmid pNGR234a revealed a cluster of

genes encoding plasmid replication (*rep*) and conjugal transfer (*tra*) functions similar to those encoded by the *rep* and *tra* genes from the tumor-inducing (Ti) plasmids of *A. tumefaciens* (Freiberg et al., 1997). These genes included the quorum-sensing regulators, *traI*, *traR*, and *traM* and all of them are orthologues of the *A. tumefaciens* quorum-sensing regulators. In *A. tumefaciens*, TraI, a LuxI-type protein, catalyzes synthesis of the acylated homoserine lactone (acyl-HSL) N-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8- HSL). TraR binds 3-oxo-C8-HSL and activates expression of Ti plasmid *tra* and *rep* genes, increasing conjugation and copy number at high population densities. TraM prevents this activation under noninducing conditions. Although the pNGR234a TraR, TraI, and TraM appear to function similarly to their *A. tumefaciens* counterparts, there are some interesting differences between the two systems. First, The transfer of the Ti plasmid normally occurs at a frequency of 10^{-2}, whereas the transfer of pNGR234a occurs at a frequency of 10^{-9} (He et al., 2003). This is also in contrast to the transfer frequency (10^{-2}) seen for *R. leguminosarum* and *R. etli*. It was speculated that an environmental signal may be required to induce higher levels of plasmid transfer of the pNGR234a. The other difference between the *A. tumefaciens* and NGR234 tra systems were that one of the the pNGR234a *tra* operons (*traAFB* operon) is not activated by TraR, and conjugal efficiency is not affected by TraR and 3-oxo-C8-HSL (He et al., 2003). Furthermore, the *trbE* coding sequence on pNGR234 is split into two separate reading frames, *trbE1* and *trbE2*. These observations thus provide a possible explanation for the low transfer frequency of pNGR234a.

The mechanism of the symbiotic-plasmid transfer of *R. etli* strain CFN42 was described (Brom et al., 2004). *R. etli* CFN42, contains 6 plasmids, designated p42a to p42f, whose sizes range from 185 to 643 kb. Plasmid p42d has been identified as the symbiotic plasmid (pSym), because it carries most of the information required for nodulation and nitrogen fixation. From analysis of the plasmids of *R. etli* CFN42 for transfer functions, only p42a is self-transmissible and the transfer is regulated by quorum sensing. The transfer region (*oriT*, *tra*, and *trb* genes) of p42a is highly similar to the transfer region of the *A. tumefaciens* Ti plasmid (Tun-Garrido et al., 2003). On the other hand, this plasmid carries *traA* and *traCDG* genes (Gonz·lez et al., 2003). The p42d was found to be able to perform self-transfer under special conditions that include artificial overexpression of a small open reading frame, named *yp028*, located downstream of the putative relaxase gene *traA*. In addition, the presence of multiple copies of this *mob* region in CFN42 also promoted conjugal transfer of p42d (Perez-Mendoza et al., 2004). However, conjugative transfer of pSym was fully dependent on the presence of p42a in a normal laboratory environment (Brom et al., 2004). The transfer of p42d required its cointegration with p42a and the cointegration may be achieved either through homologous recombination among large reiterated sequences or through IntA-mediated site-specific recombination between the attachment sites. A proposed model for the mechanism responsible for this cointegration event is shown in Figure 2.4.

Example of p42d conjugative transfer, when cointegration is the result of site-specific recombination, resolution through the same system regenerates the wild-type plasmids (S), but if the cointegrate is resolved through homologous recombination

among other sequences (H1, H2, or H3), RpSyms* are generated (Figure 2.4A). Cointegrates formed and resolved through homologous recombination between 2 repeated sequences regenerate the wildtype plasmids (H1) but give rise to RpSyms* when they are resolved through homologous recombination among different repeats (H2) or through site-specific recombination (S) (Figure 2.4B).

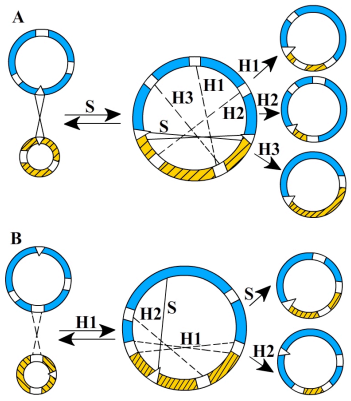

Figure 2.4 Model for the mechanism of p42d (pSym) conjugative transfer. The pSym is indicated by solid lines, and p42a is indicated by cross hatched lines. Triangles represent the *att* sequences, and open rectangles represent large sequences shared by both plasmids. Solid lines and arrows labeled with the letter S indicate site-specific recombination among attachment sequences. Dashed lines and arrows labeled with the letter H followed by a number indicate homologous recombination among large repeated sequences. (Source: Brom et al., 2004)

2.6.3 Transfer of symbiosis island

The symbiosis island was first identified in *M. loti* ICMP3153 as a transmittable DNA segment of approximately 500 kb in length containing a cluster of symbiotic genes (Sullivan and Ronson, 1998). Map of *M. loti* R7A symbiosis island is presented in Figure 2.5. It has ability to transfer to nonsymbiotic mesorhizobia in the environment and convert them to symbionts (Sullivan et al., 2002). Characterization of the island showed that this region was inserted into a *phe*tRNA gene in the genome with a 17-bp duplication of the 3' terminal portion of the gene. A 611-kb DNA region with lower GC contents flanked by a complete and a partial *phe*tRNA genes with a 17-bp duplication was also reported as a putative symbiosis island in *M. loti* MAFF303099 (Kaneko et al., 2002). In *B. japonicum*, on the other hand, a symbiotic region is 681 kb in length and a *val*tRNA gene, instead of the phe-tRNA gene in *M. loti*, was found at one end of this region, but no trace of terminal duplication was detected in the other end of this region (Gottfert et al., 2001). However, a 45-bp segment of the 3 terminal portion of the *val*tRNA gene was found apart from the intact *val*tRNA gene, and a putative integrase gene was found next to this tRNA gene segment, as in *M. loti*. According to Lee et al. (Lee et al., 2008), a symbiotic region of *A. caulinodans* is 87.6 kb in length, that is smaller than that of *M. loti* and *B. japonicum.* This region is flanked by a *gly*tRNA gene in the genome with a duplication of the 3' terminal portion of the gene. In addition, this region is interspersed by more than 18 transposases and 3 integrases, suggesting a complex history of horizontal gene transfer events. The region contains the three *nod* loci and also genes related to chemotaxis, amino acid uptake, and a putative type-IV secretion system.

Figure 2.5 Map of *M. loti* R7A symbiosis island. Coordinates are given in kilobases, starting from the first base pair downstream of the *phe*tRNA gene. Putative genes are colored according to functional class, while gene fragments are colored black. (Source: Sullivan et al., 2002)

The discovery and characterization of the *M. loti* symbiosis island represents an attractive case history, in which initial findings from a field experiment have logically progressed to the report (Sullivan and Ronson, 1998; Sullivan et al., 2002). This work impacts on diverse areas of microbiology, including the rhizobium-legume symbiosis, microbial ecology, and microbial evolution. The studies had their origin in 1986, when *Lotus corniculatus* seeds, coated with a single *M. loti* inoculant strain, were planted in a remote field site in New Zealand. No indigenous rhizobia capable of forming root nodules on *Lotus* were present in the soil, and uninoculated plants died of nitrogen starvation. Seven years later, strains isolated from nodulated *Lotus* plants at the site were found to be genetically diverse but share the same chromosomally located symbiotic DNA (Sullivan et al., 1995). It was subsequently shown that these isolated *M. loti* strains arose by transfer of a symbiosis island from the original inoculant strain to nonsymbiotic mesorhizobia present in the soil. The symbiosis island was found to integrate into the *phe*tRNA gene of the recipients in a process mediated by a P4-type integrase encoded at the left end of the island (Sullivan and Ronson, 1998) (Figure 2.6).

Later, the symbiosis island of *M. loti* R7A was termed ICE*Ml*SymR7A (Ramsay et al., 2006) in accordance with the nomenclature proposed that a subset of mobile genetic elements including conjugative transposons, integrative plasmids and mobile genomic islands should be grouped under a new classification termed Integrative and Conjugative Elements or ICEs (Burrus et al., 2002). Like other ICEs, ICE*Ml*SymR7A was found to excise from the genome to form a circular intermediate prior to conjugative transfer, and efficient excision and conjugative transfer require

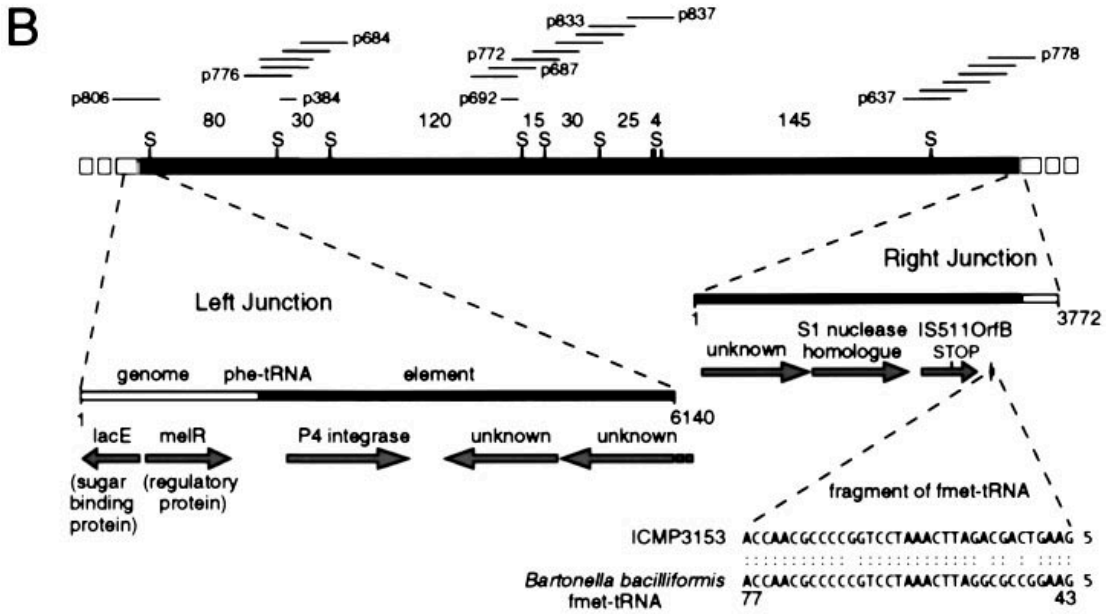

Figure 2.6 Physical map of element and location of features identified by sequencing of ICMP3153 DNA. Sizes of *Spe*I (S) fragments are shown in kilobases. There is 1.6 kb between the left junction of the element and the first *Spe*I site and 52 kb between the last *Spe*I site and the right junction. The sizes of the sequenced regions from ICMP3153 at the left junction (GenBank accession no. AF049242) and right junction (AF049243) are shown in base pairs. The region upstream of the element showed homology to the *Escherichia coli* melibiose regulatory gene *melR* (U14003), and the sugar-binding protein gene *lacE* from *Agrobacterium radiobacter* (X66596). The sequence at the right end homologous to fmet-tRNA is shown in comparison to sequence from *Bartonella bacilliformis* (L10238). (Source: Sullivan et al., 1999)

the ICE*Ml*SymR7A-encoded recombination directionality factor RdfS as well as IntS (Figure 2.7). Genes encoding homologues of RdfS are present in a conserved gene cluster together with genes encoding homologues of TraF (the TrbC protease that is essential for mating pore formation) and the ICE*Ml*SymR7A relaxase RlxS. The *rdfS* and *rlxS* genes are conserved across a diverse range of α- and β-proteobacteria and identify a large family of genomic islands with a common transfer mechanism (Ramsay et al., 2006). In addition, the ICE*Ml*SymR7A contains a *trb* cluster whose products are required for conjugative transfer (Cook et al., 1997; Suzuki et al., 2009).

The excision of ICE*Ml*SymR7A is regulated by quorum sensing with population density as a key factor (Ramsay et al., 2009). ICE*Ml*SymR7A encodes a putative LuxR-family transcriptional regulator TraR and two putative AHL synthases TraI1 and TraI2, which show homology to the TraR and TraI proteins of *A. tumefaciens* that regulate plasmid transfer via quorum sensing (Fuqua and Winans, 1994; Hwang et al., 1994; Piper et al., 1993). Expressions of *tra* and *trb* operons are controlled by a second signaling system involving the transcriptional activator, TraR and the cell-density sensing molecule, 3-oxo-C8- HSL (Ramsay et al., 2009).

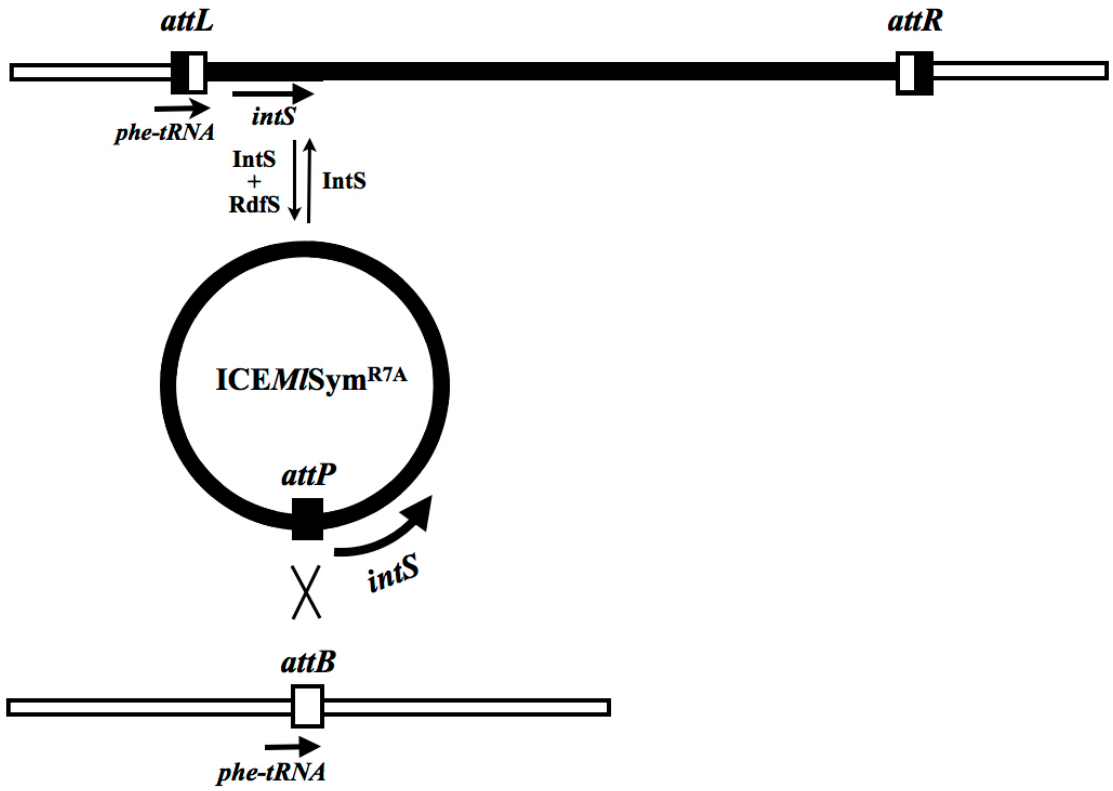

Figure 2.7 Model for ICE*Ml*SymR7A excision and integration. ICE*Ml*SymR7A DNA is shown as a black bar or circle. Chromosomal DNA is shown by white bars. Attachment sites are illustrated by black or white boxes representing their composite structure. Genes are shown by thick arrows. Maps not to scale. (Source: Ramsay et al, 2006).

CHAPTER III

CHARACTERIZATIONS OF NONSYMBIOTIC AND SYMBIOTIC SOYBEAN *BRADYRHIZOBIACEAE* STRAINS

3.1 Background

Rhizobia are nitrogen-fixing bacteria that form root nodules on legume plants. All currently known rhizobia are in the phylum Proteobacteria. Most of rhizobia species are in the class Alphaproteobacteria, which include *Rhizobium*, *Mesorhizobium*, *Ensifer*, *Bradyrhizobium*, *Azorhizobium*, *Phyllobacterium*, *Ochrobactrum*, *Devosia*, and *Methylobacterium* (Chen et al., 1988; de Lajudie et al., 1998; Elkan, 1992; Garrity et al., 2004). There are also three rhizobial species in two families in the Betaproteobacteria, all of which are in the family *Burkholderiaceae* and *Oxalobacteraceae* (Garrity et al., 2004; Van Rhijn and Vanderleyden, 1995). Methods have been developed to allow the direct isolation of rhizobia from field soil. Therefore, occurrences of nonsymbiotic rhizobia have been observed in many studies. For example, nonsymbiotic strains of *R. leguminosarum, R. etli* and *M. loti* have been reported as a significant component of rhizobial populations in the environment (Laguerre et al., 2001; Sawada et al., 2003; Segovia et al., 1991; Soberon-Chavez and Najera, 1989; Sullivan et al., 1996). These nonsymbiotic bacteria failed to nodulate legume plants due to lacking of symbiotic genes. It has been believed the

nonsymbiotic bacteria exist as saprophytes in field soil and adapt themselves by acquiring symbiotic genes from inoculant rhizobia when a legume host is present (Rao and Cooper, 1994; Sullivan et al., 1995).

The family *Bradyrhizobiaceae* consists of 9 genera, which are *Afipia*, *Bosea*, *Agromonas*, *Bradyrhizobium*, *Blastobacter*, *Nitrobacter*, *Oligotropha*, *Rhodoblastus,* and *Rhodopseudomonas*. Sequence analysis of 16S rDNA region has revealed a very close relationship among these bacteria (Saito et al., 1998; Seewaldt et al., 1982; van Berkum et al., 2006b; Willems and Collins, 1992), leading to caution in the taxonomy of this group of bacteria (Sawada et al., 2003; Vinuesa et al., 1998). In case of the genus *Bradyrhizobium*, for example, *B. elkanii* is clearly a separate species from *B. japonicum* and *B. liaoningense* based on 16S rDNA similarity. Moreover, members of *B. japonicum* are phylogenetically closer to other nonsymbiotic genera (*Afipia, Nitrobacter* and *Rhodopseudomonas*) than to *B. elkanii* (Willems et al., 2001). *Rhodopseudomonas palustris* is a phototrophic purple non-sulfur bacterium. Based on 16S rRNA sequence analysis, *Rhs. palustris* is phylogenetically close to nodule-forming nonphototrophic *B. japonicum* (Woese et al., 1984; Wong et al., 1994), as well as stem-nodulating phototrophic *Bradyrhizobium sp*. strain BTai1 and ORS2378 (Eaglesham et al., 1990; Fleischman et al., 1995; Inui et al., 2000; Wong et al., 1994; Young et al., 1991). The fact that other genera (*Afipia, Agromonas, Blastobacter, Nitrobacter* and *Rhodopseudomonas*) are phylogenetically closer to *B. japonicum* than to *B. elkanii* leaves the current classification of *Bradyrhizobium* looking rather unsatisfactory. It has been argued that 16S rRNA gene sequence is insufficient to ensure discrimination of bacterial species. Therefore, phenotypic studies should be

taken up and extended in a comprehensive way. In addition, analysis of combination of several housekeeping genes, such as *atpD*, *recA*, *dnaK*, *glnII*, *gyrB* and *rpoB*, have been used to elucidate taxonomic relationships among *Bradyrhizobium* species and other rhizobia (Khamis et al., 2003; Rivas et al., 2009c; Sameshima et al., 2003; Vinuesa et al., 2005c).

In our previous study (Pongsilp et al., 2002), 130 *Bradyrhizobium* isolates were obtained from heavily inoculated soybean field soil and uninoculated soybean free soil in Thailand. The *Bradyrhizobium* species were expected to be directly isolated from soil by using BJSM medium (Tong and Sadowsky, 1994). The BJSM medium is based on the resistance of *B. japonicum* and *B. elkanii* strains to more than 40 μg of the metal ions Zn^{2+} and Co^{2+} per ml. The isolates were classified into symbiotic and nonsymbiotic groups by inoculation assay on soybean (*Glycine max*), mungbean (*Vigna radiata*), and cowpea (*V. unguiculata*). The nonsymbiotic isolates failed to hybridize to *nod* and *nif* gene probes, indicating that they lacked the symbiotic genes. Analysis of 16S rRNA gene sequence showed that a number of nonsymbiotic isolates appeared to belong to the family *Bradyrhizobiaceae*. However, they could not be clearly affiliated, since they had 96-97% identity to different genus in *Bradyrhizobiaceae* family including *Bradyrhizobium*, *Rhodopseudomonas* and *Afipia*.

3.2 Objective

To characterize the nonsymbiotic isolates by using a polyphasic approach, and to describe the novel isolates for which the new genus was proposed.

3.3 Materials and methods

3.3.1 Strains and culture conditions

Four representatives of the unclassified nonsymbiotic (CMI1, KKI20, CMI23 and KKI28) isolates were chosen to study and renamed as NS1, NS20, NS23 and NS28 (NS referred to the nonsymbiotic strains.), respectively. The nonsymbiotic isolated strains were obtained from heavily inoculated soybean field soil in Chiangmai province (NS1 and NS23) and in Khonkaen province (NS20 and NS28) (Pongsilp et al., 2002). *B. japonicum* USDA110, *B. elkanii* USDA94, and *Rhs. palustris* DSM 123^T were received from Department of Biotechnology, Osaka University, Japan; Graduate School of Life Sciences, Tohoku University, Japan; and German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, respectively. *Bradyrhizobium* strains were grown in YM medium at 28°C (Somasegaran and Hoben, 1994). *Rhs. palustris* were grown in van Niel's medium at 28-30°C (van Niel, 1944) or in succinate basal medium (Pfennig and Truper, 1971). Analysis of growth characteristics, photosynthetic pigmentation, antibiotic resistance and heavy metal resistance of the nonsymbiotic isolates were carried out on succinate basal medium to compare with *Rhs. palustris* DSM 123^T and on YM medium to compare with *B. japonicum* USDA110. Utilization of carbon substrates was analyzed using the basal medium (Pfennig and Truper, 1971) supplemented with a single organic substance in a final concentration of 1% (w/v).

3.3.2 Physiological analyses

3.3.2.1 Colony and cell morphology

Colony and cell morphology were examined during the exponential growth phase. Gram's staining reactions were observed under light microscopy (Olympus SZ-2LGC, Olympus, Japan). Transmission electron microscopy (TEM) was done on 5-day culture of the nonsymbiotic isolates. The bacterial cells were harvested by centrifugation and fixed in oxaloacetate solution. The cells were stained in 4% osmium tetroxide in 0.2 M cacodylate buffer, dehydrated in a series of increasing concentration of ethanol, and embedded in resin. Ultra-thin sections were cut using an ultramicrotome. Bacterial structure was observed using transmission electron microscopes JEM 1200 EX (JEOL, Japan Electron Optics Laboratory Co., Ltd).

3.3.2.2 Determination of G+C content

DNA was purified according to the procedure of Cashion (Cashion et al., 1977) and the G+C content was carried out at the Deutsche Sammlung von Mikroorganisem und Zellkulturen (DSMZ), Braunschweig, Germany by HPLC (Mesbah et al., 1989).

3.3.2.3 Analysis of cellular fatty acids

Analysis of cellular fatty acid composition was carried out at the DSMZ according to the standard procedure (Kuykendall et al., 1988; Miller, 1982). Fatty acid components of the isolated strains were compared to those of other reference strains with microbial identification system (Sasser, 1990).

3.3.2.4 Determination of biochemical characteristics

Bacterial cells were grown for 3 days in 5-ml liquid medium. Enzymatic reactions and substrate utilization were determined by using the API NE20 (bioMe´rieux, France), according to the manufacturer's instructions. Utilization of additional carbon substrates was analyzed using the basal medium supplemented with a single organic substance in a final concentration of 1% (w/v). Oxidase activity was determined from the oxidation of tetramethyl-p-phenylenediamine (Bott et al., 1990). Oxidase reagent (1% N,N,N',N'-tetramethyl-p-phenylenediamine) was dropped on a piece of filter paper in a petri dish. Bacterial colonies were smeared across the wet paper with a toothpick. A positive reaction was scored from a dark purple color that develops in 10 seconds. The organism that remained colorless or turned purple after 10 seconds was scored as negative reaction. Catalase activity was determined by 3% H_2O_2. A small amount of a fresh bacterial colony was placed on a clean glass slide. Solution of 3% hydrogen peroxide was dropped on the colony. Bubble production was identified as a positive reaction. pH range was tested by incubating the cultures in test tubes containing 5 ml broth medium with pH values ranging from 4.0 to 10.0 at 28ºC. Bacterial growth at different NaCl concentrations was examined on agar medium ranging from 0.5% to 2.0% (w/v) at 28ºC. All biochemical tests were also performed for reference strains including *B. japonicum* USDA110 and *Rhs. palustris* DSM 123^T.

3.3.2.5 Observation of photosynthetic pigment

Nonsymbiotic isolates and *Rhs. palustris* were cultured and incubated at 30°C under the following 4 illumination systems: cells were (1)

incubated aerobically in the dark; (2) incubated anaerobically in the dark; (3) incubated aerobically in 16-h/8-h light-dark cycle; and (4) incubated anaerobically in the 16-h/8-h light-dark cycle. Bacterial chlorophyll was also detected from broth culture grown at 30°C for 5 days on a 16-h–8-h light-dark cycle. Cell pellet was extract under dark with cold acetone-methanol (7:2 [v/v]) for 30 min (Lorquin et al., 1997). Absorbance of the supernatant was observed at a wavelength range from 350 to 800 nm.

3.3.2.6 Determination of heavy-metal resistance

Resistance to heavy metals was estimated visually by minimal inhibitory concentration (MIC) method. The metals used were added as $ZnCl_2$, MoO_3, $CoCl_2 \cdot 6H_2O$, $CuCl_2$, $CdCl_2 \cdot 2H_2O$, $PbCl_2$, and $HgCl_2$. Stock solutions of each metal ion were prepared at 10 mg/ml in distilled water, except for Pb^{2+}, which was prepared as a 5 mg/ml stock solution. All metal-stock solutions were filter sterilized before being added to the sterilized media. Late log-phase cultures of the 4 isolated strains, *B. japonicum* USDA110, *B. elkanii* USDA94, and *Rhs. palustris* DSM 123^T were dropped onto the surface of metal-added agar medium. In addition, results were verified by plate dilution method. The cultures were serially diluted in 0.85% NaCl, and dilutions were spread onto the metal-added agar medium. The agar plates without the heavy metals were used as control. The experiments were carried out in triplicate. All plates were incubated at 28°C for 7-10 days. The lowest concentration of the heavy metals at which no growth occurred was considered as MIC. MICs were presented as micro grams of metal ions per milliliter.

3.3.2.7 Determination of antibiotic resistance

Resistance to antibiotics was estimated visually by minimal inhibitory concentration (MIC) method. The antibiotics used were added as neomycin, kanamycin, gentamycin, rifampicin, tetracycline, streptomycin, and spectinomycin. All antibiotic-stock solutions were filter sterilized before being added to the sterilized media. Late log-phase cultures of the 4 isolated strains, *B. japonicum* USDA110, *B. elkanii* USDA94, and *Rhs. palustris* DSM 123^{T} were dropped onto the surface of metal-added agar medium. In addition, results were verified by plate dilution method. The cultures were serially diluted in 0.85% NaCl, and dilutions were spread onto the metal-added agar medium. The agar plates without the antibiotic were used as control. The experiments were carried out in triplicate. All plates were incubated at 28°C for 7-10 days. The lowest concentration of the antibiotics at which no growth occurred was considered as MIC. MICs were presented as micro grams of antibiotic per milliliter.

3.3.3 Determination of symbiotic ability

3.3.3.1 Seedling preparation

Soybean (*Glycine max* cv. SJ5) and siratro (*Macroptilium atropurpureum*) were be used as host plants. Soybean seeds were surface sterilized by rapid immersion in 70% ethanol, followed by incubation in 3% hydrogen peroxide for 5 min. Sterilized seeds were washed with sterilized water, then soaked in sterilized water overnight at 4°C, and germinated on 0.8% water agar at room temperature for 2 days.

Siratro seeds were surface sterilized by soaking in concentrated sulfuric acid for 10 min. The excess acid was drained off and seeds were exhaustively washed with sterile water (Somasegaran and Hoben, 1994). Seeds were germinated in the petri dish containing moist tissue paper and incubated at room temperature for 2 days.

3.3.3.2 Plant nodulation test

Nodulation assay of selected isolates was carried out in plastic growth pouches, by the standard method (Somasegaran and Hoben, 1994). Cells were cultured to early stationary phase. Germinated seeds were individually planted into sterilized pouch, and each seed was inoculated with 10^7 cells. Plants were watered with N-free nutrient solution (Somasegaran and Hoben, 1994). Plants were grown under controlled environmental conditions of 25ºC on a 12/12 h day/night cycle. After 6 weeks, plants were harvested and the nodule number was scored.

3.3.4 Detection of root hair deformation

Experiments dealing with the effects of different bacterial species or strains on root hair deformation were conducted in test tubes (25×150 mm). Soybean seedlings (2 days old) were transferred from water agar plates to test tubes containing 12 ml of Hoagland's solution (Hotter and Scott, 1991) agar slant (12%). Late-log-phase bacteria were inoculated onto 48-h-old seedlings at a rate of 10^7 cells per tube. Seedlings were allowed to grow for 7 days after inoculation. Root hair curling was

observed everyday for a week. Each root was moved carefully from the agar to sterile water and then mounted for microscopic observation.

3.3.5 Detection of nodulation genes

Primers used to amplify the nodulation genes were designed by using Primer Design Assistant; PDA (National Health Research Institutes, Taiwan). 19 to 25 bp-long primers that target a nearly full sequence of each nodulation genes were selected. Designed primers were verified and optimized by amplifying the target genes from genomic DNA of *B. japonicum* strain USDA110 as a positive control. All oligonucleotide primers were manufactured by Invitrogen.

PCR amplification was performed in 50 µl reaction mixtures containing 20 pmol of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 U of Go Taq Flexi DNA Polymerase (Promega, Germany), 1X GoTaq Flexi DNA Polymerase buffer, and 1 µl of template DNA. For amplifications of *nolA*, *nodY*, *nodA*, *nodC*, *nodS*, *nodU*, *nodI*, *nodJ*, *nodMN*, *nodO*, *nodZ*, *nodU* and *nolB*, the thermal cycler was programmed as follows: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. For amplification of *nolYZ*, *nolY*, *nodD1, nodD2*, *nodB*, *nolU* and *nolV*, the thermal cycler was programmed as follows: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were purified with Promega's Wizard PCR-Prep columns (Promega, Germany). Purified PCR products were sequenced using the same primers as for PCR.

3.3.6 Extraction of genomic DNA

DNA was extracted from bacterial cultures using an alkaline-lysis and phenol/chloroform extraction method. Bacterial cells (5 ml) were harvested by centrifugation at 10,000 rpm for 1 min, washed twice in 0.5 ml TEN buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.1 M NaCl, pH 8.0) and resuspended in 0.5 ml TEN buffer. Then 200 µl of 20% sucrose in TEN buffer, 100 µl of 10% SDS and 20 µl of 2.0 mg/ml lysozyme were added. The mixture was mixed by vortex and incubated for 30 min one 37°C. After complete lysis, 75 µl of 5M NaCl was added and mixed by inversion. This solution was extracted with an equal volume of phenol/chloroform/ isoamyl alcohol (25:24:1). Most of proteins and cell debris was removed from the mixture by centrifugation at 13,000 rpm for 10 min. The aqueous phase was transferred to a fresh tube and extracted once with 500 µl chloroform, then centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube. Genomic DNA was precipitated by adding of 0.6 volume of cold isopropanol and 0.1 volume of 3M sodium acetate and then leaving at -20°C for 30 min. Then the DNA was recovered by centrifugation for at 13,000 rpm for 10 min. DNA pellet was washed once with 500 µl 70% ethanol and recovered by centrifugation at 13,000 rpm for 5 minutes. DNA pellet was dried, resuspended in 50 µl of TE buffer, and stored at -20°C.

3.3.7 Amplification and sequencing of photosynthetic genes (*pufLM* and *bchY*)

The fragment of *pufLM* gene encoding the photosynthesis reaction center was amplified with primers *pufL*_F (5'-CTKTTCGACTTCTGGGTSGG-3') and

*pufM*_R (5'-CCATSGTCCAGCGCCAGAA-3') and the *pufM* fragment was amplified with primers *pufM*_F (59-TACGGSAACCTGTWCTAC-39) and *pufM*_R described above (Béjà et al., 2002). In addition, the fragment of *bchY* gene encoding the protochlorophyllide oxidoreductase, which involved in bacteriochlorophyll biosynthesis, was amplified with primers *bchY*_F (5'-CCNCARACNATGTGYCCNG CNTTYGG-3') and *bchY*_R (5'-GGRTCNRCNGGRAANATYTCNCC-3') (Yutin et al., 2009). The PCR amplifications of *pufLM* and *bchY* were performed in 50 µl reaction mixtures, as described for the 16S rRNA gene. The thermal cycler was programmed as follows: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 49°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min, except the annealing temperature at 50°C for the amplification of the *bchY* gene. The purified PCR products were sequenced using the same primers as for PCR.

3.3.8 Amplification and sequencing of 16S rRNA gene

Nearly full-length sequence of 16S rRNA gene was amplified from universal primer set 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane et al., 1985). PCR amplification was performed in 50 µl reaction mixtures containing 20 pmol of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 U of Go Taq Flexi DNA Polymerase (Promega, Germany), 1X GoTaq Flexi DNA Polymerase buffer, and 1 µl of template DNA. The thermal cycler was programmed as follows: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension

at 72°C for 1 min 50 s; and a final extension at 72°C for 10 min. PCR products were purified with Promega's Wizard PCR-Prep columns (Promega, Germany). Purified PCR products were sequenced using the same primers as for PCR.

3.3.9 Amplification and sequencing of 16S rRNA gene *atpD, glnII*, and *recA*

Partial sequences of *atpD*, *glnII* and *recA* were obtained from primers as described by (Vinuesa et al., 2005c). Fragments of *atpD* were amplified with primer *atpD*255F (5'-GCTSGGCCGCATCMTSAACGTC-3') and *atpD*782R (5'-GCCGAC ACTTCMGAACCNGCCTG-3'). Fragments of the *glnII* were amplified with primers *glnII*12F (5'-YAAGCTCGAGTACATYTGGCT-3') and *glnII*689R (5'-TGCATGCCS GAGCCGTTCCA-3'). Fragments of *recA* were amplified with primers *recA*41F (5'-TTCGGCAAGGGMTCGRTSATG-3') and *recA*640R (5'-ACOUSTICRCCGATCTT CATGC-3'). PCR amplification was performed in 50 µl reaction mixtures containing 15 pmol of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.5 U of Go Taq Flexi DNA Polymerase (Promega, Germany), 1X GoTaq Flexi DNA Polymerase buffer, and 1 µl of template DNA. The thermal cycler was programmed as follows: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were purified with Promega's Wizard PCR-Prep columns (Promega, Germany). Purified PCR products were sequenced using the same primers as for PCR.

3.3.10 Phylogenetic analysis

The sequences of 16S rDNA, *atpD*, *glnII* and *recA* generated in this study were subjected to the algorithm BLASTN and TBLASTX to identify the most similar sequences available in the database. Resulting sequences with high e-scores were selected. Two or three sequences with similar e-score were chosen as representatives of each bacterial species. Other DNA sequences of relative strains and outgroup were obtained from the GenBank database. *M. loti* was used as outgroup in the analysis. The selected sequences were displayed in FASTA format and file was saved as a text file (.txt). Multiple alignments were performed with MUSCLE from phylogeny.fr (Dereeper et al., 2008). Where necessary, the alignments were manually edited. Unaligned regions and gaps were excluded from the analyses. In total 1381, 452, 558, and 476 nucleotide positions were used for the phylogenetic analyses of 16S rDNA, *atpD*, *glnII* and *recA*, respectively. Phylogenetic trees were reconstructed by the distance neighbor-joining method (Kahindi et al., 1997). Neighbor-joining analyses were performed using the Kimura-2 correction and confidence levels were estimated for 1,000 replicates with the MEGA 4.1 package (Kumar et al., 2008). In comparison, phylogenetic trees were also reconstructed by maximum likelihood with the PhyML (Guindon and Gascuel, 2003). Phylogenetic trees were manipulated and visualized by using Dendroscope V2.4 program (Huson et al., 2007).

3.4 Results and discussion

3.4.1 Morphological characteristics

Microscopic observations during the exponential phase showed that four nonsymbiotic strains (NS1, NS20, NS23, and NS28) were Gram-negative rod-shaped and non-spore forming motile bacteria. Transmission electron microscopy showed that the strains were of 1.3-2.2 μm in length and 0.3-0.6 μm in width and they possessed many large bright granules (Figure 3.1), possibly PHA, inside the cells.

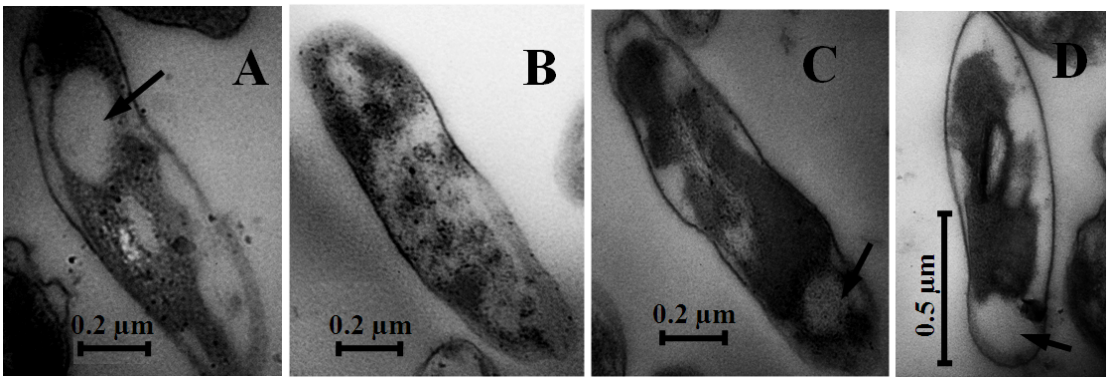

Figure 3.1 Transmission electron micrographs of the non-symbiotic strains, NS1 (A) TEM; magnification ×20 k, NS20 (B) TEM; magnification ×20 k, NS23 (C) TEM; magnification ×12.5 k, and NS28 (D) TEM; magnification ×16 k. They are typically 0.3-0.4 μm wide and 1.3-1.6 μm long. All cells possess bright granules (indicated by arrow), possibly PHA, inside the cells.

3.4.2 Colony and growth characteristics

Colonies on succinate basal medium and van Niel's medium were creamy-white, convex, and opaque. Growth rates of the isolated strains were different relative to each media. When grown in YM at 28ºC, the strains were slow-growing, forming approximately 0.3-0.5 mm diameter colonies within 7-10 days. After incubating on YM plates containing bromothymol blue (BTB), the plate culture turned blue indicating that they produced an alkaline reaction (Somasegaran and Hoben, 1994). On succinate basal medium and van Niel's medium, they grew faster and formed approximately 0.5-1.0 mm diameter colonies within 4-7 days. The optimum growth temperature under aerobic conditions in the dark was about 25-32ºC. No growth was observed at 15ºC and at 37ºC. The pH range was 6.5-8.0 and no growth was detected at pH 6.0 and 10.0. Growth was inhibited in medium containing 2.0% NaCl (w/v). The isolated strains were cytochrome oxidase and catalase positive. Autotrophic growth was not observed on a completely inorganic medium containing either sodium thiosulfate or sodium sulfite.

3.4.3 Heavy metal and antibiotic resistance

The resistance to heavy metals and antibiotics of the 4 isolated strains were determined. *B. japonicum* and *Rhs. palustris* used to compare in this study due to their close relation based on the partial 16S rRNA gene sequence similarity (Pongsilp et al., 2002). The minimal inhibitory concentrations (MIC) to 7 heavy metals and 7 antibiotics are shown in Table 3.1 and 3.2, respectively. MICs were almost identical in the 4 nonsymbiotic isolates. All nonsymbiotic isolates were more resistant to all

tested heavy metals than *B. japonicum* and *Rhs. palustris*. They showed significant resistance to high concentration of molybdenum, copper, zinc, and cobalt; and also had high resistance to cadmium, mercury, and lead. In addition, they showed highly resistance to multiple antibiotics. It is surprising that these strains were very resistant to high concentrations of streptomycin, neomycin, spectinomycin, and tetracycline. The nonsymbiotic isolates had relatively good growth even when tetracycline reached 200 μg/ml and streptomycin reached 2,000 μg/ml. On the other hand, all of the nonsymbiotic isolates were sensitive to rifampicin with MICs less than 10 μg/ml. These results indicated that these isolated strains were highly tolerant to heavy metals and antibiotics.

Table 3.1 Resistance to heavy metals.

Strains	**MIC (μg/ml)**						
	Mo	**Zn**	**Cd**	**Hg**	**Co**	**Pb**	**Cu**
NS1	>5000	>4000	>3000	800	>4000	>3000	>5000
NS20	>5000	>4000	>3000	800	>4000	>3000	>5000
NS23	>5000	>4000	>3000	800	>4000	>3000	>5000
NS28	5000	3000	2000	300	>4000	3000	5000
B. japonicum USDA110	3000	500	100	<10	200	800	200
B. elkanii USDA94	2000	500	200	<10	400	300	200
Rhs. palustris DSMZ 123^T	1000	800	100	50	1000	500	200

Table 3.2 Resistance to antibiotics.

Strains	MIC (µg/ml)						
	Gm	**Nm**	**Km**	**Rf**	**Strep**	**Spec**	**Tet**
NS1	500	500	300	<10	2500	500	100
NS20	500	300	300	<10	2500	500	250
NS23	500	500	300	<10	2500	500	100
NS28	500	300	300	<10	2500	500	250
B. japonicum USDA110	100	100	50	100	200	50	100
B. elkanii USDA94	100	100	50	50	100	300	250
Rhs. palustris DSMZ123T	200	nd	150	nd	100	nd	nd

Gm, gentamycin; Nm, neomycin; Km, kanamycin; Rf, rifampicin; Tet, tetracycline; Strep, Streptomycin; Spec, spectinomycin; nd, not determined.

3.4.4 Root hair curling

Response of the nonsymbiotic isolates on soybean root was determined by observation of root hair deformation. The changes in morphology of the soybean were clearly observed on the root inoculated with *B. japonicum* strains USDA110 and *B. yuanmingense* strains S7 (Pongsilp et al., 2002). After a day, slightly curled root hairs and swelling of root hair tips could be seen but occurred rarely (Figure 3.1). After 2 and 3 days, slightly curled root hairs and swelling of root hair tips occurred frequently and, after 4-7 days, the root curling was spread over, on the root inoculated with *B. japonicum* USDA110 (Figure 3.2) and *B. yuanmingense* S7 (Figure 3.3). On the soybean root inoculated with the nonsymbiotic isolated NS1 and NS23, none of root hair curling or swelling was observed after 2 days. However, some of abnormal shapes such as swelling tips and wavy pattern were occurred after 3 days (Figure 3.4).

The detection of the morphological changes of the soybean root inoculated with the nonsymbiotic isolates led to the hypothesis that the nonsymbiotic isolates might harbor some nodulation genes involved plant signaling. Therefore, the appearance of the gene involved nodulation in the nonsymbiotic isolates was investigated.

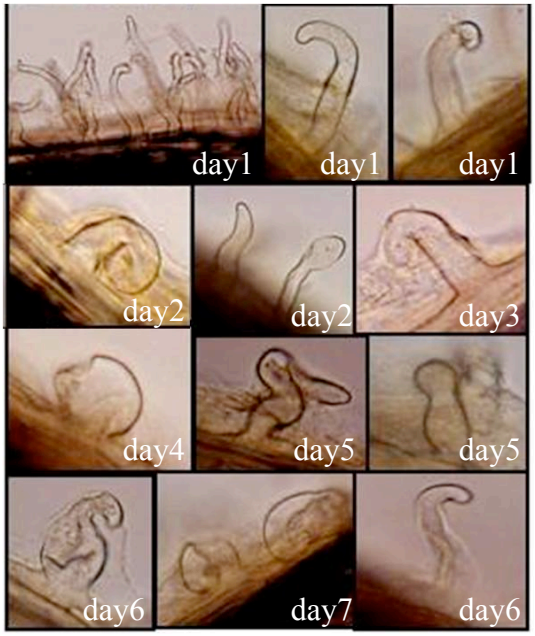

Figure 3.2 Soybean-root hairs at 1-7 days after inoculation with *B. japonicum* strain USDA110.

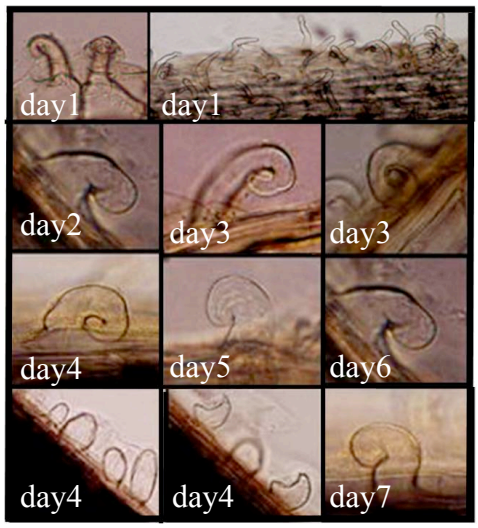

Figure 3.3 Soybean-root hairs at 1-7 days after inoculation with *B. yuanmingense* S7.

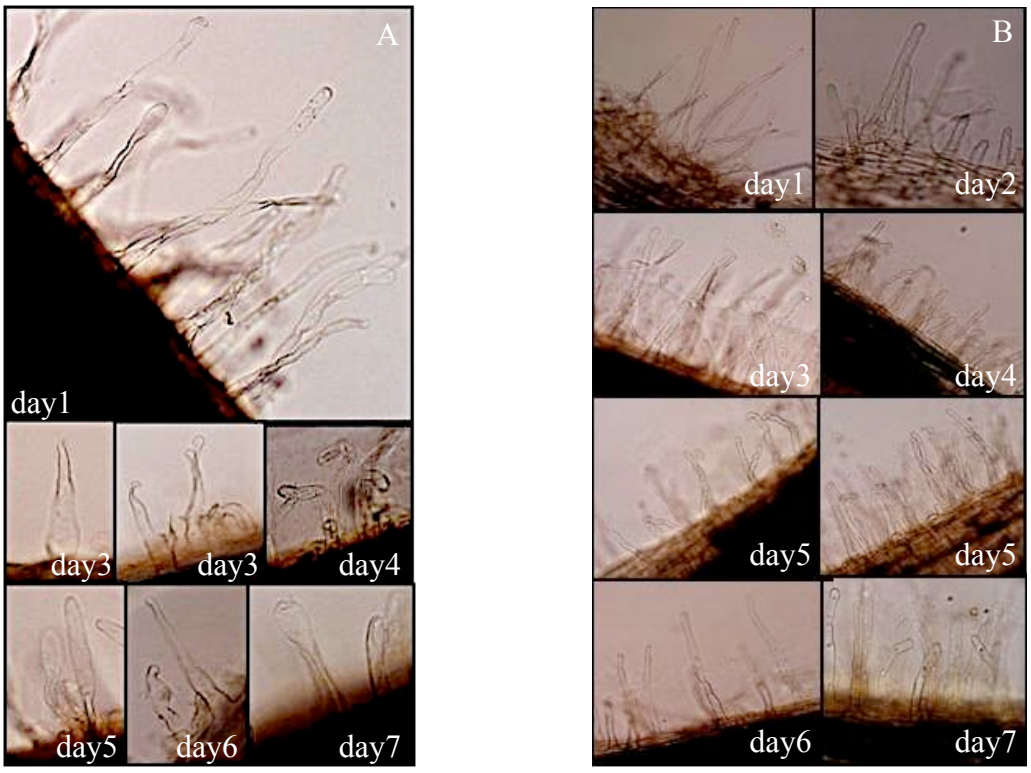

Figure 3.4 Soybean-root hairs at 1-7 days after inoculation with (A) nonsymbiotic isolates NS1, and (B) nonsymbiotic isolate NS23.

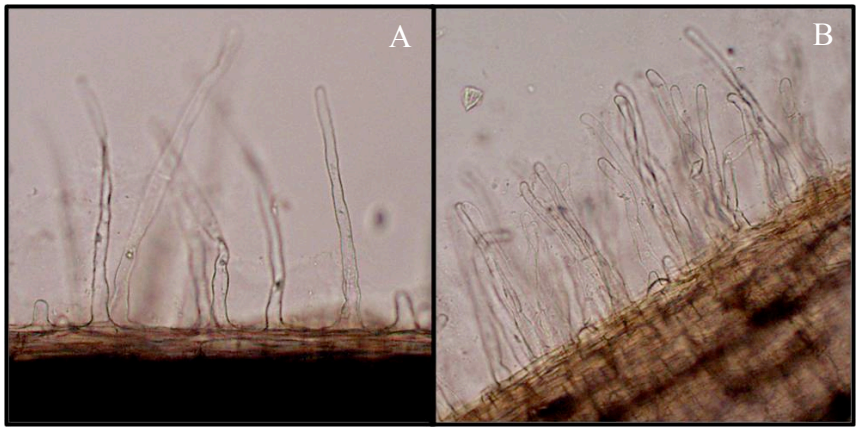

Figure 3.5 Uninoculated soybean-root hairs at (A) 3 days and (B) 7 days after germination.

3.4.5 Detection of nodulation genes

According to the hypothesis that nonsymbiotic isolates would originally be soil bacteria that are absent some symbiotic genes, the appearance of the gene involved nodulation in the nonsymbiotic isolates was investigated. *B. japonicum* strain USDA110 and *B. yuanmingense* strain S7 were chosen as a representatives of the symbiotic strains. A cluster of nodulation genes and coding region of nodulation protein in T3SS were analyzed due to their responsibility for initiation of nodule development and production of Nod factor. All of nodulation genes were observed in *B. japonicum* USDA110, however *B. yuanmingense* S7 contained only 7 nodulation genes, including *nodA*, *nodB*, *nodJ*, *nodS*, *nodU*, and *nolN*, that were identical to those of *B. japonicum* USDA110 (Figure 3.6). In contrast, none of nodulation gene identical to that of *B. japonicum* was detected in the nonsymbiotic isolates. Although the PCR products of some genes were obtained, these products did not correspond to the expected nodulation gene sequences (Table 3.3).

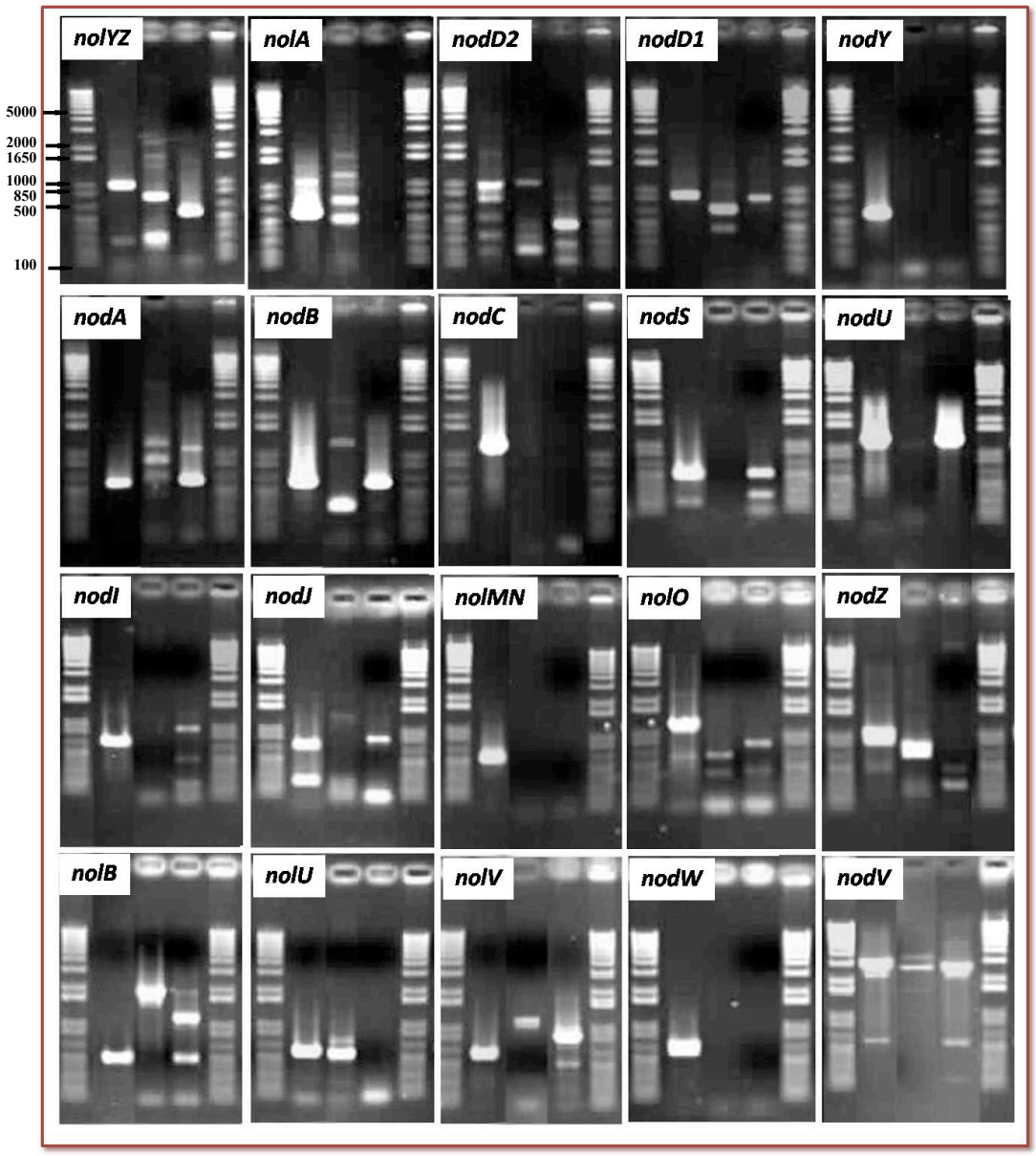

Figure 3.6 PCR analysis of genes involved nodulation for *B. japonicum* USDA110 (lane 2), NS1 (lane 3), and S7 (lane 4). Lane 1 is 1 Kb plus DNA Ladder (Invitrogen).

Table 3.3 Related proteins resulted from BlastX of PCR products obtained from PCR analysis of genes involved nodulation of the nonsymbiotic isolate NS1.

Expected genes and primers used	PCR size (bp)	Related protein and % identity (BlastX)
nodA (*nodA*f28 and *nodA*r627)	800	78 % hypothetical protein blr3159 (*B. japonicum* USDA 110)
		77% conserved hypothetical protein (*Bradyrhizobium sp.* BTAi1)
		73% CoA-binding protein (*Ralstonia metallidurans*)
nolA (*nolA*f38 and *nolA*r637)	700	77% Modification methylase HemK *[Rhs. palustris* BisB18]
		75% hypothetical adenine-specific methylase *[B. japonicum* USDA 110]
		72% modification methylase, HemK family [*Rhs. palustris* BisA53]
		70% Modification methylase HemK [*Bradyrhizobium sp.* BTAi1]
nodD1 (*nodD*1f41 and *nodD*1r940)	800	59% Alkanesulfonate monooxygenase [*Acidovorax avenae subsp. citrul*li AAC00-1]
		61% alkanesulfonate monooxygenase [*Acinetobacter sp*. ADP1]
		60% alkanesulfonate monooxygenase [*B. japonicum* USDA110]
nolYZ (*nolYZ*f8 and *nolYZ*r907)	1500	34% Outer membrane autotransporter barrel [*Dechloromonas aromatica* RCB]
		37% hypothetical protein mll0950 [*M. loti* MAFF303099]
		31%Outer membrane autotransporter barrel [*Bradyrhizobium sp.* BTAi1]
nodZ (*nodZf* 24 and *nodZr*973)	800	58% ABC transporter permease protein [*B. japonicum* USDA 110]
		55% Protein of unknown function DUF140 [*Bradyrhizobium sp.* BTAi1]
		49% putative permease of ABC transporter [*Rhs. palustris* CGA009]

In Table 3.3, for example, based on translational products from BLASTX, the PCR-product sequence of the strain NS1 amplified from *nolA*-primers had 77%, 75%, and 70% similarity to a modification methylase (HemK) of *Rhs. palustris*, adenine-specific methylase of *B. japonicum* USDA110, and HemK of *Bradyrhizobium sp.* BTAi, respectively. In addition, translational products from a sequence amplified from *nodZ* primers had 58%, and 49% similarity to an ABC transporter permease protein of *B. japonicum* USDA110, and *Rhs. palustris* respectively. These results indicated that the non-symbiotic NS1 did not harbor the expected nodulation genes.

Investigating of nodulation genes, none of nodulation genes was detected in the non-symbiotic strains. The symbiotic S7 that was identified as *B. yuanmingense* based on the phylogenetic analyses, it contained only 7 nodulation genes identical to those of *B. japonicum*. These results implied that the non-detected genes might not be significant for soybean-host specificity. A role of soybean-nodulation of the strain S7 might be different to that of *B. japonicum*. A key for the soybean nodulation would not involve whole products of nodulation genes from *B. japonicum* or it would involve other genes responsible for different roles.

In case of nonsymbiotic isolates, they did not harbor any of the nodulation genes although they were genetically related to the members of *Bradyrhizobium.* There is a possibility of symbiotic gene transfer from the symbiotic bacteria to the nonsymbiotic strains in soil since they were found to evolve from a common ancestor. The evidences of symbiotic gene transfer between symbiotic and non-symbiotic rhizobia have been reported (Boucher et al., 2003; Igarashi et al., 2001; Raymond et

al., 2002; Sullivan and Ronson, 1998; Sullivan et al., 2002) suggested that the symbiotic properties of some rhizobia are genetically unstable since the nonsymbiotic isolates, which occur as a significant component of rhizobial populations in environment, acquired symbiotic genes from the *Mesorhizobium*-inoculant strains. In addition, Molouba, et al. (1999) suggested that *Bradyrhizobium* may have evolved from photosynthetic free-living bacteria by the acquisition of symbiotic functions, when they are not exposed to significant levels of light. Consequently, photosynthetic function might be lost during evolution from a free-living existence to a symbiotic one. In this study, an evolutionary link among *Bradyrhizobium*, *Rhodopseudomonas*, and the non-symbiotic isolates suggested a role of the lateral gene transfer of essential regulatory genes on their evolution. The symbiotic gene transfer contributes to the rapid diversification of legume-symbiont populations, if the non-symbiotic bacteria evolve into symbionts able to nodulate their host plants (Rao and Cooper, 1994; Sullivan et al., 1995). However, this can increase of competition of the acquired strain with the inoculated strain. Therefore, the mechanism of the symbiotic gene transfer of *Bradyrhizobium* will be useful for further study in the genetic structure of rhizobial populations, which can be used to assess the fate of released strains and the impact on resident microbial communities.

3.4.6 Physiological and biochemical characteristics

From the analysis of colony and growth characteristic as well as heavy metal resistance, these characteristics were almost identical in the 4 nonsymbiotic strains. Moreover, in molecular level, their 16S rRNA gene sequence were highly

similar to each other (99% identity) and their BOXA1R patterns were identical (Pongsilp et al., 2002). Therefore, the strains NS20 and NS23 were chosen as representatives for further physiological characterization. Members *Bradyrhizobium*, *Rhodopseudomonas* and *Afipia* were focused as reference strains on the comparison of the physiological characteristics, due to of their 16Sr RNA similarity to the isolated strains (Pongsilp et al., 2002). A comparison of physiological characteristics between the isolated strains and the related genera in the family *Bradyrhizobiaceae* was summarized in Table 3.4, showing that the isolated strains NS20 and NS23 are distinct from the other genera. The G+C content of the isolated strains NS20 and NS23 were 62.9 mol % and 62.3 mol %, respectively that were different to that of *Bosea*, *Agromonas* and *Nitrobacter*. The isolated strains NS20 and NS23 were similar to *Afipia*, *Bradyrhizobium*, *Rhodoblastus, Rhodopseudomonas* and *Nitrobacter* in cell size, Gram-negative reaction, rod shape, motility and G+C content. On the other hand, the isolated strains and *B. japonicum* reproduce by binary fission thus they were distinguished from the other budding genera by reproduction process. The strains NS20 and NS23 could be distinguished from *Rhs. palustris* by characteristic of energy consumption. *Rhs. palustris* is a facultative phototrophic bacterium which can grow with or without oxygen, moreover, it is able to utilize benzoate, produce photosynthesis pigment and use thiosulfate as a source of electron donors under anaerobic with light. On the other hand, the strains NS20 and NS23 are aerobic heterotrophic bacteria that are not able to produce photosynthetic pigment and to grow in media containing benzoate and inorganic compounds.

Table 3.4 Characteristics of the nonsymbiotic strains and related species in the family *Bradyrhizobiaceae.*

Characteristics	**NS20**	**NS23**	***Bradyrhizobium*[A] ****	***Rhs. palustris*[AB] *****	***Afipia*[A] ******	***Bosea thiooxidans*[A] *******	***Blastobacter*[A]**	***Agromonas*[A]**	***Nitrobacter*[A] ********	***Oligotropha*[A]**	***Rhodoblastus*[A] *****
Cell size (µm)	0.4-0.8 × 1.2-2.0	0.4-0.8 × 1.2-2.2	0.5-0.9 × 1.2-3.0	0.6-0.9 × 1.2-2.0	0.5-1.0 × 1.2-3.0	0.85 × 1.4-1.6	0.5-1.0 × 1.0-4.5	0.6-1.0 × 2.0-7.0	0.5-0.9 × 1.0-2.0	0.4-0.7 × 1.0-3.0	0.8-1.0 × 1.0-6.0
Reproduction	binary fission	binary fission	binary fission	budding	binary fission and budding	nd	budding	budding	binary fission and budding	nd	budding
Motile	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
Pigmentation	-	-	-	+	-	-	-	-	-	-	+
Nutrient broth growth	+	+	-	+	+	nd	nd	nd	nd	nd	nd
Growth in NaCl at:											
0.5%	+	+	+	+	+	nd	nd	+	nd	nd	+
1.0%	+	+	+	+	+	nd	nd	+	nd	nd	+
2.0%	-	-	-	+	+	nd	nd	+	nd	nd	-
Nitrate reduction	-*	-*	-	-	-	-	+	-	+	+	+
Urease	+*	+*	+	-	+	-	+	+	+	+	-
Indole production	-*	-*	-	-	-	-	nd	nd	nd	nd	nd
Hydrolysis of:											
Gelatin	-*	-*	+	-	-	-	nd	-	nd	nd	nd
Esculin	-*	-*	+	-	-	nd	nd	nd	nd	nd	nd
Growth on:											
D- glucose	-*	-*	+/-	-	-	+	+	+	-	nd	-
L- arabinose	+*	+*	+	-	nd	+	nd	nd	-	nd	nd
D- mannose	-*	-*	+	-	nd	nd	nd	+	-	nd	nd
D- mannitol	+*	+*	+	+	-	-	+	nd	-	nd	-
N- acetylglucosamine	-*	-*	-	-	-	nd	nd	nd	-	nd	nd
D- maltose	-*	-*	-	-	nd	nd	-	-	-	nd	nd
Potassium gluconate	+	+	+	-	nd	+	nd	nd	-	nd	nd
Adipic acid	+*	+*	-	+	nd	nd	nd	nd	nd	nd	nd
Malic acid	+*	+*	+	+	nd	+	nd	+	nd	+	+
Trisodium citrate	-*	-*	-	+	nd	+	-	+	nd	nd	-
Phenylacetic acid	-*	-*	-	-	nd	+	-	nd	nd	nd	nd
Benzoate	-	-	-	+	nd	-	nd	-	nd	nd	-
Glutamate	+	+	+	+	nd	+	+	nd	nd	nd	-
Succinate	+	+	+	+	nd	+	nd	+	nd	nd	+
Thiosulfate (1mM)	-	-	-	+	nd	+	nd	nd	nd	nd	+/-
G + C content (mol%)	62.9	62.3	61-65	64.8-66.3	61.5-69	65-69	59-69	65.1-66	59-62	62.5-63.1	62.2-66.8
Optimum temperature (°C)	25-32	25-32	25-30	nd	nd	30-32	25-27	nd	28-30	nd	25-30
Optimum pH	6.5-8.0	6.5-8.0	nd	nd	nd	7.5-8.5	6.8-7.8	nd	7.5-8.0	nd	5.5-6.0

+, positive; -, negative; nd, not determined, A, Brannier (1995); B, van Niel (1994); *, the results obtained from the API 20NE; **,grown on mineral salt medium [Jordan, 1982]; ***grown on mineral salt medium [Pfennig, 1971]; ****, grown on buffered charcoal-yeast extract (BCYE) agar at 30°C [Brenner, 1991]; *****, grown on Glutamate-yeast extract-mineral salt (GYM) medium [Das, 1996]; ****** grown on basal mineral salt medium [Ehrich, 1995]

The presence of genes encoding for enzymes of reaction center complex (*pufL* and *pufM*), and protochlorophyllide oxidoreductase (*bchY*) was detected in *Rhs. palustris* DSM 123^T by PCR amplification, whereas these genes were not detected in the nonsymbiotic isolates. Although, in *puf* PCR amplification experiments, a number of PCR fragments were amplified from the nonsymbiotic isolates at lower annealing temperatures (data not shown), these fragments did not correspond to the *puf* gene sequences. Similarly, *Rhodoblastus* was distinguished from the strains NS20 and NS23 by ability of photosynthesis and growing at the lower pH. In addition, the strains NS20 and NS23 were negative for the reduction of nitrate, thus the inability of nitrate reduction was of use in differentiation the isolated strains from *Blastobacter*, *Nitrobacter* and *Oligotropha*. The biochemical characteristics of the strains NS20 and NS23 was similar to that of *B. japonicum* excepts for the growth on nutrient broth, utilization of adipic acid as sole carbon source, and inability to hydrolyze gelatin and esculin. In addition, the inability of growing on media containing 2% NaCl was used to differentiate the strains NS20 and NS23 from *Afipia* and *Agromonas* and inability of growing on sole carbon of trisodium citrate was used to differentiate the strains NS20 and NS23 from *Afipia* and *Agromonas.*

3.4.7 Fatty acid compositions

The cellular fatty acid components of the strains NS20 and NS23 were similar to those of all recognized genera in the family *Bradyrhizobiaceae*, being predominantly composed of $C_{18:1}$ ω7c fatty acid (Table 3.5).

Table 3.5 Fatty acid composition of the nonsymbiotic strains and related species in the family *Bradyrhizobiaceae.*

Fatty acid	NS20	NS23	*B. japonicum* A*	*Rhs. palustris* AB **	*Afipia*[AC] ***	*Bosea thiooxidans*[A] ****	*Agromonas*[A]	*Nitrobacter*[A] *****	*Rhodoblastus*[A] **
12 : 0 3-OH	-	-	0.04	-	-	-	-	-	-
14 : 0	0.90	1.27	0.01	-	-	-	-	< 5%	0.35 - 0.45
14 : 0 3-OH	-	-	-	0.4	-	-	-	-	
15 : 0	0.51	0.34	0.04	-	-	2.33	-	-	0 - 0.06
15 : 0 3-OH	-	-		-	-	0.49		-	-
16 : 0	15.09	18.67	11.97	15.4	3 - 23	7.58	9 - 14	6 - 15	8.68 - 11.36
16 : 0ω5c	-	-	1.06	-	-		-	-	
16 : 0 iso 3-OH	-	-	-	0.6	-	3.25	-	< 5%	0.79 - 3.08
11 methyl 16:0	-	-	-	-	-	-	-	6 - 15	-
16 : 1ω5c	-	-	-	-			-	< 5%	0.17 - 0.31
16 : 1ω7c	-	-	-	-	1 - 17	3.7	-	< 5%	45.0 - 46.78
16 : 1ω9c	-	-	-	8.7	-		-	< 5%	-
17 : 0	-	-	0.19	-	-	4.82	-	-	0 - 0.23
17 : 1ω8c	-	-	0.48	-	-	3.56	-	-	-
17 : 1ω6c	-	-	0.15	-	-		-	-	-
17 : 0 CYCLO	7.56	3.60	-	-	2 - 25	2.08	-	-	-
17 : 0 3-OH	-	-		-	-	0.9	-	-	0.15 - 0.45
18 : 0	0.35	0.55	0.62	4.2	-	0.67	-	< 5%	0.63 - 0.87
18 : 1ω7c	55.88	60.30	-	-	15 - 65	61.55	75	> 60%	35.02 - 42.16
18 : 1ω9c	-			-	-	-	-	< 5%	-
18 : 1ω11c	-	-	-	70.0	-	-	-	-	-
18 : 1ω13c	-	-	-	0.5	-	-	-	-	-
11 methyl 18 : 1 ω7c	4.89	3.32	1.91	-	15 - 25[A]	-	-	-	-
18 : 1ω5c	-	-	0.02	-	-	-	-	-	-
19 : 0 CYCLO ω8c	7.63	2.86	1.16	-	-	8.35	-	< 5%	-
19:1	-	-	-	-	-	-	6 -18	-	-
C_{Br} 19:1	-	-	-	-	5 -28		-	-	-
10 methyl 19:0	-	-	-	-	-	0.71	-	-	-
20:0	-	-	-	-	-	-	-	-	0.22 - 0.35
20 : 1ω7c	-	-	-	-	-	-	-	-	0.16 - 0.19
Summed feature 3	7.18	9.08	1.06	-	-	-	-	-	-
Summed feature 7	-	-	81.22	-	-	-	-	-	-

A, Brannier (1995); B, van Niel (1994); C, found in *A. felis*, *A. genospecies*, and *A. massilienis*; Summed Feature 3 identified as 16:1 ω7c/15 iso 2OH; Summed Feature 7 identified as 18:1 ω7*cis*/ ω9*trans*/ ω12*trans*, 18:1 ω7*cis*/ ω 9*cis*/ ω 12*trans*; *, grown on AIE agar with 0.1% gluconate at 28°C [Kuykendal, 1988]; **, grown on succinate-mineral medium at 30°C [Pfennig, 1991]; *** grown on hear tinfusion agar supplemented with 5% rabbit blood and on buffered charcoal-yeast extract (BCYE) agar plates at 30°C [Brenner, 1991]; ****, grown on Glutamate-yeast extract-mineral salt (GYM) medium [8]; ***** grown on basal mineral salt medium [Ehrich, 1995]

The $C_{18:1}$ fatty acid is typical for bacterial species of the family *Bradyrhizobiaceae* (Garrity et al., 2004; van Berkum et al., 2006c). The genera in the family *Bradyrhizobiaceae* including *Afipia*, *Bradyrhizobium*, *Agromonas*, *Bosea*, *Nitrobacter* and *Rhodobacter* shared relatively large amounts of $C_{18:1}$ ω7c fatty acids (Table 3.5). However, unlike the fatty acid compositions of *Rhs. palustris*, $C_{18:1}$ω11c was a major fatty acid and trace amounts of $C_{16:1}$ ω9c and $C_{18:1}$ ω11c were found. The isolated strains differed from *B. japonicum* by containing a significantly lesser concentration of $C_{18:1}$ω7c and differed from *Rhodoblastus* by containing a higher concentration of $C_{18:1}$ω7c. Likewise, the isolated strains were differentiated from *Afipia* and *Bosea* by a lack of $C_{16:1}$ω7c and from *Agromonas* by a lack of $C_{19:1}$. Obviously, the isolated strains NS20 and NS23 possessed $C_{16:1}$ω7c 15 iso 2-OH that was of use for differentiation the both strains from the other genera in the family *Bradyrhizobiaceae*. After comparison to microbial identification system (MIDI) database, the fatty acid profiles of the strains NS20 and NS23 were unable to obtain a match on a microbial identification system (MIDI). Clustering based on the PCA of the fatty acid content showed that the strains NS20 and NS23 were distinct to *Rhs. palustris* and *Rhodoblastus* but were relatively close to *B. japonicum* and *Agromonas* (Figure 3.7). The physiological characteristics and cellular fatty acid profile together with genotypic traits (16S rRNA gene sequence and BOXA1R pattern (Pongsilp et al., 2002)) suggest that the strains NS20 and NS23 belong to the same species and they were proposed as a novel genus of the family *Bradyrhizobiaceae*.

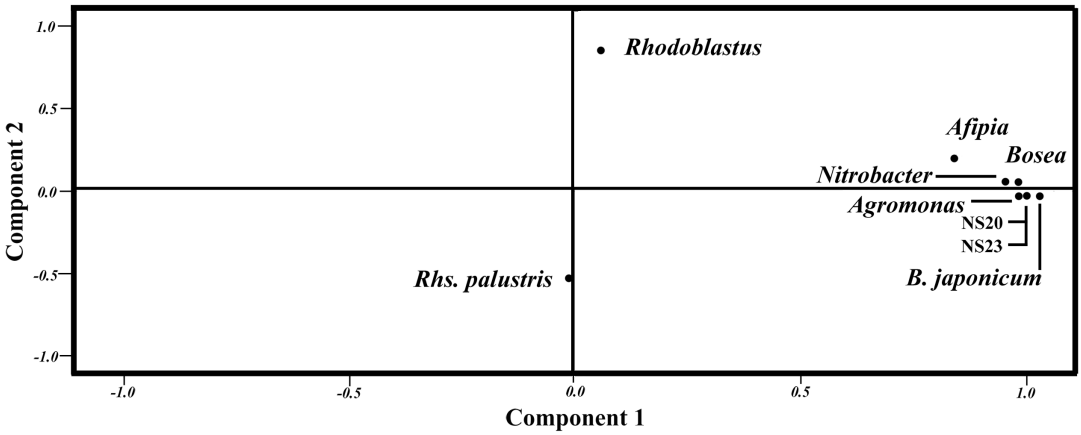

Figure 3.7 Principal component analysis (PCA) of the cellular fatty acid content of the nonsymbiotic strains and related genera in family *Bradyrhizobiaceae*. The analysis was performed by using the SPSS version 13, using a varimax rotation with Kaiser normalization.

3.4.8 16S rRNA sequence

Based on 16S rRNA gene sequence similarity, the nonsymbiotic isolated strains NS1, NS20, NS23 and NS28 had a high level of sequence similarity to each other (99% identity over 1313 bp of unambiguously aligned sequence), despite their differences in geographic origin as described above. The 16S rRNA gene sequence analysis showed that the isolated strains were closely related to bacterial members of the family *Bradyrhizobiaceae*; such as *Afipia, Rhodopseudomonas*, *Bradyrhizobium* and a number of uncultured bacterial clones or unidentified bacteria obtained from different geographic environments. The 4 isolated strains showed the highest level of sequence identity (99%) to uncultured marine bacterium clone KG_A3_120m68, which was obtained from a nature iron-fertilized phytoplankton bloom in the Southern Ocean (West et al., 2008). They also shared 97% to 98% identity with members of

the genus *Afipia*, *Rhodopseudomonas*, and *Bradyrhizobium*, as well as a number of uncultured bacterial clones and unidentified bacteria obtained from different environments. For example, they showed 98% identity to uncultured *Bradyrhizobium sp*. clone 81-24 obtained from various surfaces of Mars Odyssey spacecraft and floors of its encapsulation facility (La Duc et al., 2003) and 97% identity to heavy metal tolerant bacteria strain SK50-23 isolated from nonpolluted garden soil in Japan (Bao et al., 2006). The strain SK50-23, which is one that is highly similar to the 4 isolated strains (97% identity), showed a narrow range of metal tolerance. For example, the strain SK50-23 exhibited a high MIC value for thallium (Tl), zinc (Zn) and nickel (Ni) (Bao et al., 2006). Similarly, the isolated strains were found to be extremely tolerant to heavy metals and antibiotics (Table 3.2 and 3.3). The isolated strains were obtained from the purpose of isolation of *Bradyrhizobium*, based on the resistance of *B. japonicum* and *B. elkanii* strains to high concentration of the heavy metal ions Zn^{2+} and Co^{2+}. The discoveries of contamination of the nonsymbiotic isolated strains, which are highly tolerant to heavy metals, indicated that the BJSM medium is not selective for symbiotic *Bradyrhizobium* strains. In addition, we suggest the caution in the use of the BJSM for directly isolation of *Bradyrhizobium* from soil or other environmental samples.

3.4.9 Phylogenetic analysis of 16S rDNA, *atpD*, *glnII* and *recA*

We found that both neighbor-joining and maximum likelihood methods produced similar trees, thus only the neighbor-joining trees were presented in this paper. The maximum likelihood trees were shown in appendix B. In 16S rDNA tree,

the isolated strains were grouped into a huge cluster with the various members of *Bradyrhizobiaceae* (Figure 3.8). In this cluster, a lineage of the isolated strains was separated from the group of *Rhodopseudomonas* with the bootstrap support of 76% and they formed a group with various uncultured bacteria obtained from different environments as described above. In the family *Bradyrhizobiaceae*, limited taxonomic information can be deduced from 16S rDNA sequences, because there is a high degree of conservation among species and sequence similarity is relatively high (Garrity et al., 2006; Rivas et al., 2009b). The analysis of several housekeeping genes could be useful to improve the reliability of the phylogenies (Stackebrandt et al., 2002; Wolde-meskel et al., 2004). For this reason, the analysis of housekeeping loci was expected to elucidate the taxonomic position of the isolated strains in this study.

16S rRNA gene

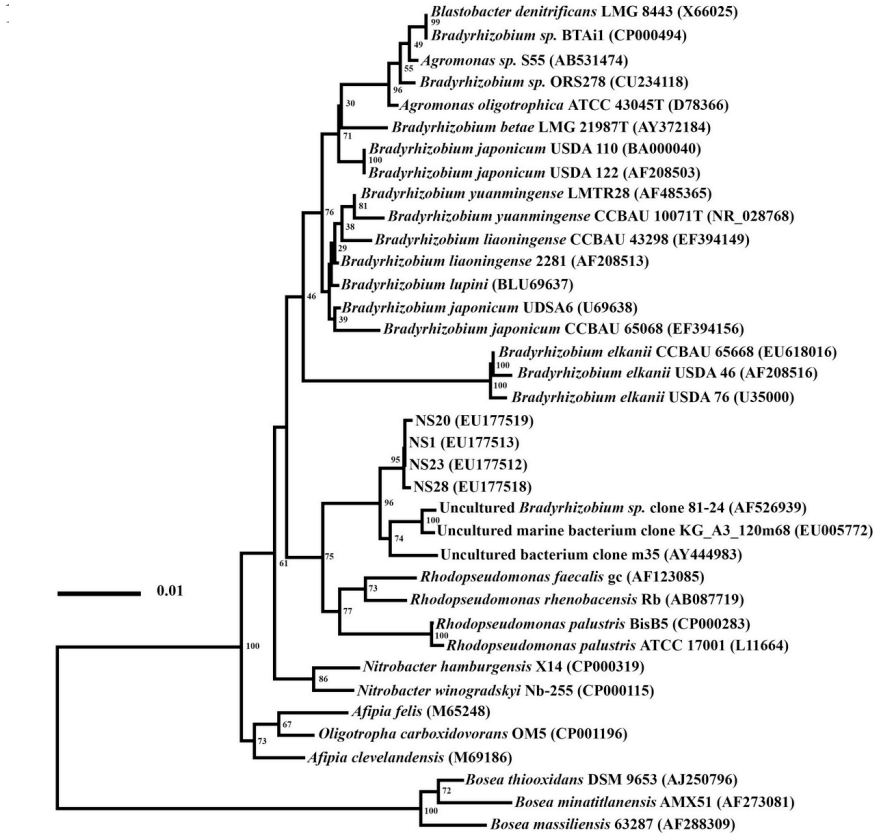

Figure 3.8 Neighbor-joining tree based on sequences of *16S rRNA* gene, showing the classification of the nonsymbiotic isolates from soil in Thailand. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide position. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. NS refers to nonsymbiotic isolate from Thai soil.

On the basis of *atpD* sequence similarity, levels of sequence similarity between the isolated strains and the other relative species were very low (less than 91%). They showed *atpD* sequence identities of 88-91% to *Rhs. palustris*, and 89% to *B. liaoningense* and *B. japonicum*. However, the alignment result of the *atpD* showed a presence of a major deletion (12 nucleotides) that was a major different position between the *Bradyrhizobium* members and the other compared strains. From this reason, the 4 isolated strains were distinguished from the member of *Bradyrhizobi*um with 78% bootstrap support (Figure 3.9). In addition, the tree showed that *atpD* of the isolated strains and *Bradyrhizobium* members evolved from a common ancestor and shared an individual history with *Rhs. palustris*. It was possible that the isolated strains and *Bradyrhizobium* were descendants of *Rhs. palustris*. In different species, the energy-consumption processes might occur in different ways for living in their own environments. For example, the energy provided for the symbiosis process in *Bradyrhizobium* and the photosynthesis processes in *Rhs. palustris* is different. In a case of the photosynthetic *Bradyrhizobium* strain BTai1 and ORS278, they formed a group with *Bradyrhizobium* species and also with *Blastobacter denitrificans*, which is a nitrogen-fixing symbiont of *Aeschynomene indica* (van Berkum and Eardly, 2002). The results indicated that the photosynthetic stem-nodulating *Bradyrhizobium* strains were closely related to root-nodulating *Bradyrhizobium* rather than the photosynthetic bacteria *Rhodopseudomonas*, based on the energy-consumption processes.

atpD

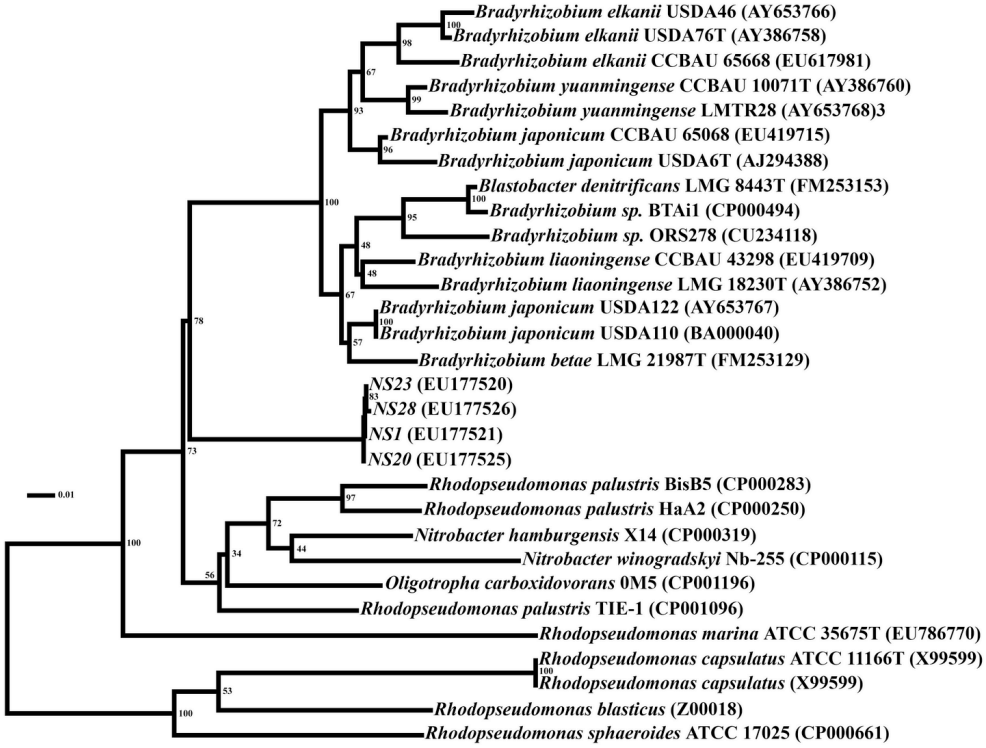

Figure 3.9 Neighbor-joining tree based on sequences of *atpD* gene, showing the classification of the nonsymbiotic isolates from soil in Thailand. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide position. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. NS refers to nonsymbiotic isolate from Thai soil.

The *recA* sequence analysis showed that the 4 isolated strains had sequence identities of 90-91% to *Rhs. palustris*, 87% to *Nitrobacter* and 86-88% to *B. liaoningense, B. yuanmingense*, and *B. elkanii*. For the *glnII* sequence analysis, the 4 isolated strains showed sequence identities of 85% to *Bradyrhizobium* species. From the comparison of the *recA* tree with the *glnII* tree, the results of both trees were in agreement. In both trees, the isolated strains formed a unique branch and they were placed on an unclassified lineage distinct to *Bradyrhizobium* cluster with 100% bootstrap support (Figure 3.10 and 3.11). These results indicated the isolated strains were distinguished from the members of the genus *Bradyrhizobium* and *Rhs. palustris* as well as from the other genera including *Blastobacter*, *Nitrobacter* and *Oligotropha.* The different placement of the isolated strains in individual gene tree analyses may be due to different evolutionary histories of the genes used for analysis. Product of *recA* gene is a multifunctional enzyme that functions in DNA repair system. Therefore, sequence of *recA* is very well conserved among eubacterial species (Eisen, 1995; Karlin et al., 1995; Roca and Cox, 1997).

recA

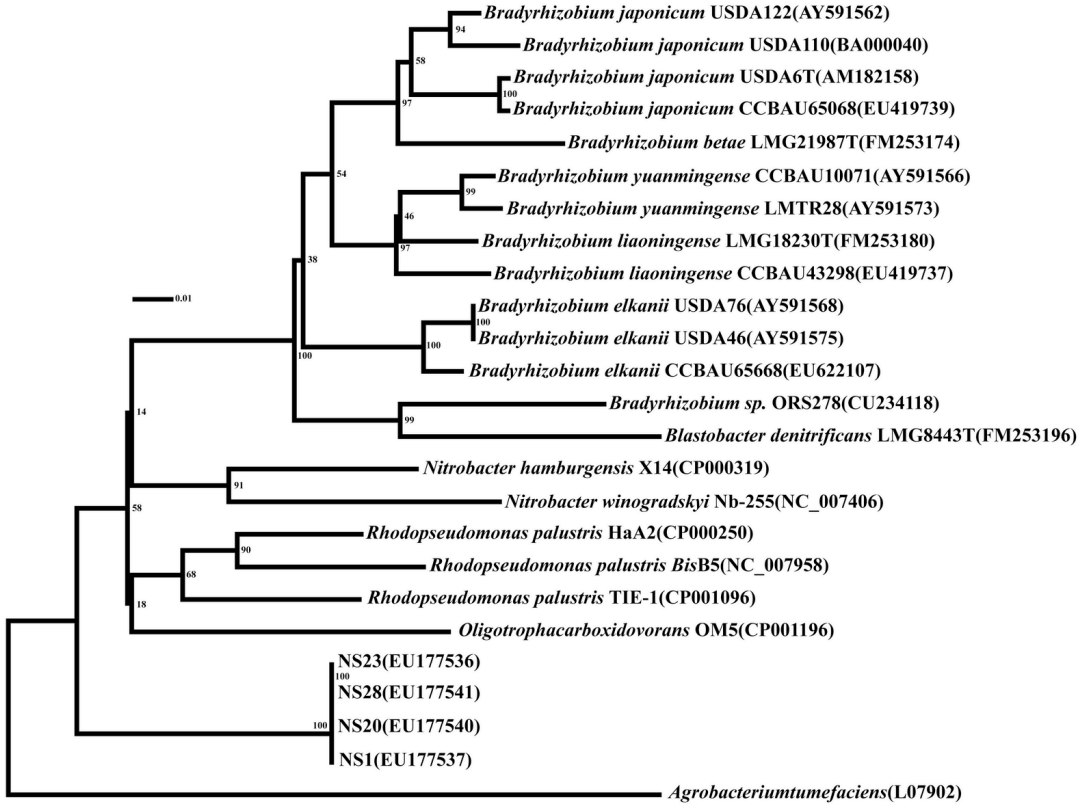

Figure 3.10 Neighbor-joining tree based on sequences of *recA* gene, showing the classification of the nonsymbiotic isolates from soil in Thailand. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide position. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. NS refers to nonsymbiotic isolate from Thai soil.

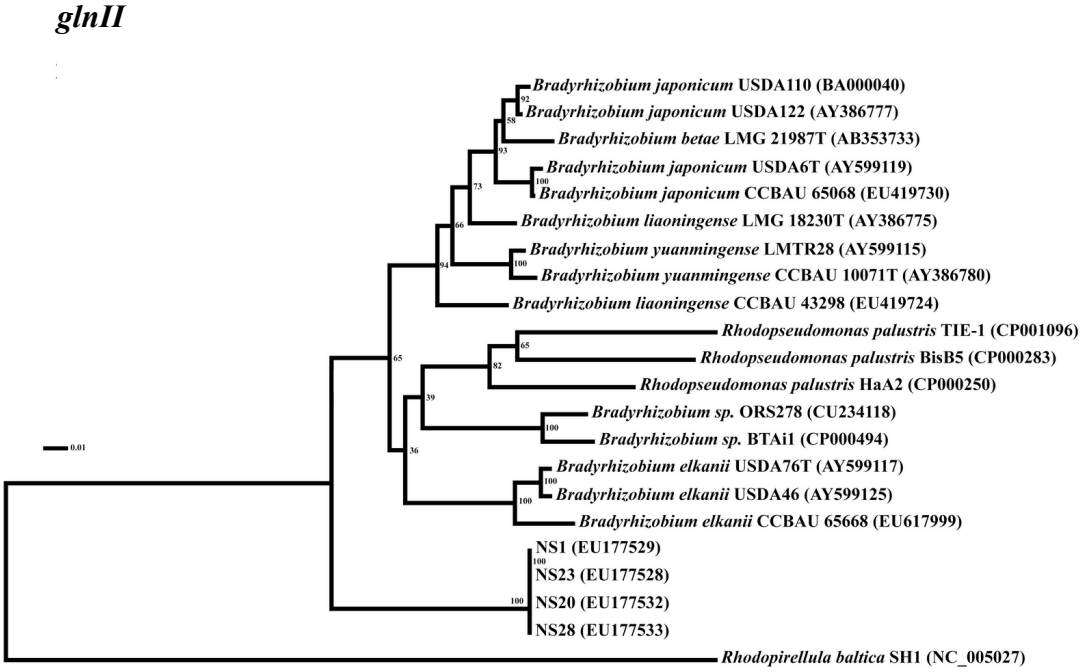

Figure 3.11 Neighbor-joining tree based on sequences of *glnII* gene, showing the classification of the nonsymbiotic isolates from soil in Thailand. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide position. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. NS refers to nonsymbiotic isolate from Thai soil.

High confidence identification was given by phylogenetic reconstructions based on the combination of 16S rDNA, *recA*, *atpD* and *glnII*. The combination of these genes showed that the 4 isolated strains formed a unique branch supported by a bootstrap value of 100% (Figure 3.12). The results of the classification in all trees were congruent to each other. Groupings by analysis of multilocus combination supported that the isolated strains share a common ancestor with *Bradyrhizobium* and *Rhs. palustris* (Figure 3.11). The phylogenetic relation based on the sequence-combining method was limited by quality and quantity of gene-sequences located on available database. More sequences of reference strains would provide a better resolved species groups. In phylogenetic trees based on individual housekeeping genes (*atpD*, *glnII* and *recA*), the level of sequence similarity between the isolated strains and the related species appeared to be relatively low for being members of the same genus. It was supported by the BOXA1R fingerprint-based dendrogram showing that the nonsymbiotic isolates fell into a single major cluster and clearly distinguished to cluster *Bradyrhizobium* (Pongsilp et al., 2002). In addition, the strains NS20 and NS23 had 99 % and 100% sequence similarity to each other, based on the sequences of the 16S rRNA gene and the housekeeping genes (*atpD*, *recA* and *glnII*), respectively. Taken together with the phenotypic characteristics, therefore, Th. e strain NS23 was proposed as a new genus and it was deposited at the NITE Biological Research Center (NBRC) and BIOTECH Culture Collection (BCC).

Combination of 16S rRNA gene, *atpD*, *recA* and *glnII*

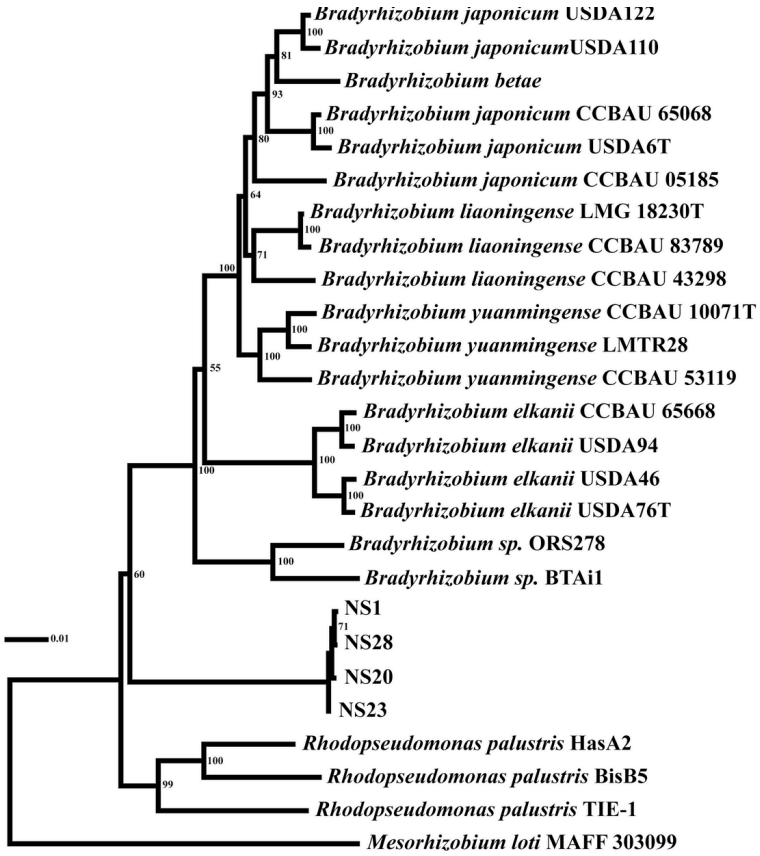

Figure 3.12 Neighbor-joining tree based on sequences of combination of 16S rRNA gene, *atpD*, *recA*, and *glnII* showing classification of the nonsymbiotic isolates from soil in Thailand. Bootstrap value is expressed as percentages of 1,000 replications. The bar represents 1 estimated the substitution per 100 nucleotide position. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. NS refers to nonsymbiotic isolate from Thai soil.

3.5 Conclusion

In conclusion, the data of multilocus sequence analysis, confirmed by physiological and biochemical characteristics, support the suggestion that nonsymbiotic isolates are a novel genus in family *Bradyrhizobiaceae*. The symbiotic and photosynthetic abilities as well as antibiotic and heavy metal tolerance are key features for distinguish the nonsymbiotic isolates to the other genera in this family. The evolutionary link among *Bradyrhizobium* (photosynthetic and nonphotosynthetic *Bradyrhizobium* strains), *Rhodopseudomonas*, and the non-symbiotic strains suggest a role of the lateral gene transfer of photosynthetic and symbiotic regulatory genes on their evolution. Therefore, the transfer of the special functions among these bacteria would be interesting for the future study. We propose the name *Metalliresistens boonkerdii* sp. nov. as the type species of the novel genus. The type strain is strain NS23 (= NBRC 106595^T = BCC 40155^T), isolated from soybean field soil in Chiangmai province, Thailand.

3.6 Description of *Metalliresistens* gen. nov.

Metalliresistens (Me. tal. li. re. si´stens, L. n. metallum, metal; L. part. adj. resistens, enduring, resisting; N. L. macs. n. (N. L. part. adj. used as a substantive) *Metalliresistens*, a bacterium resisting to metals.

Metalliresistens is aerobic, Gram-negative, and non-spore-forming bacterium. Cells are slightly curved rods and and appear as single cells. Cells are motile and multiply by binary fission. Oxidase and catalase positive. Colonies are creamy-white, convex, and opaque on solid agar media. The organism is highly resistant to

molybdenum, copper, zinc, and cobalt; and resistant to streptomycin, neomycin, spectinomycin, and tetracycline. Major fatty acid components are $C_{18:1}$ ω7c, $C_{16:0}$, $C_{16:1}$ ω7c/15 iso 2OH, $C_{17:0}$ CYCLO, $C_{18:0}$, 11 methyl $C_{18:1}$ ω7c, and $C_{19:0}$ CYCLO ω8c. The G+C content of the genomic DNA is 62.3-62.9 mol%. Phylogenetically, it is a novel member of the family *Bradyrhizobiaceae.* The type species of the genus is *Metalliresistens boonkerdii*.

3.7 Description of *Metalliresistens boonkerdii* gen. nov. sp. nov.

M. boonkerdii (boon' kerd. di. i. N. L. gen. n. *boonkerdii*, in honor of Nantakorn Boonkerd, Thai soil microbiologist)

M. boonkerdii is aerobic, Gram-negative, and non-spore-forming bacterium. Cells are slightly curved rods, 1.3-2.2 μm in length, 0.3-0.6 μm in width, and as single cells. Cells are motile and multiply by binary fission. Oxidase and catalase positive. Colonies are creamy-white, convex, and opaque. On succinate basal medium and van Niel's medium colonies of 0.5-1.0 mm in diameter are formed at 30ºC after 4 days. On YM medium, colonies of 0.3-0.5 mm in diameter are formed at 28ºC after 7 days. On succinate basal medium, growth occurs between 25-32 ºC at pH 7.0, but growth does not occur at 15 ºC and at 37 ºC. Growth is inhibited in the presence of 2% NaCl (w/v). A major cellular fatty acid is $C_{18:1}$ω7c following with $C_{16:1}$ ω7c, $C_{17:0}$ CYCLO, $C_{19:0}$ CYCLO ω8c and $C_{16:1}$ ω7c/15 iso 2OH. L-arabinose, D-mannitol, potassium gluconate, adipic acid, malic acid and glutamate are assimilated. D-glucose, D-mannose, N-acetylglucosamine, D-maltose, m-tartrate, trisodium citrate, phenylacetic acid, benzoate and fructose are not assimilated. Urease

and protease are positive. Enzymatic reactions of β-glucosidase, arginine dihydrolase, and β-galactosidase; gelatin and esculin hydrolysis; and nitrate reduction are negative. The organism is highly resistant to molybdenum, copper, zinc, and cobalt; and resistant to streptomycin, neomycin, spectinomycin, and tetracycline. The G+C content of the genomic DNA is 62.3 mol%. The type strain is NS23 (= NBRC 106595^T = BCC 40155^T) isolated from heavily inoculated soybean field soil in Chiangmai, Thailand. The GenBank accession numbers for the sequence of 16S rRNA gene, *atpD*, *glnII* and *recA* sequence of the type strain are EU177512, EU177520, EU177528 and EU177536, respectively.

CHAPTER IV

SITE-SPECIFIC INTEGRATION AND TRANSFER OF *MESORHIZOBIUM LOTI* SYMBIOSIS ISLAND TO *BRADYRHIZOBIACEAE* STRAINS

4.1 Background

Acquisition or loss of specific set of genes from other species through the process of horizontal gene transfer (HGT) is an important process in the diversification and adaptation of most bacterial species. HGT is defined as the transfer of genetic information via mobile genetic elements such as plasmids, phages, transposons and genomic islands (GEIs). GEIs are large movable regions of bacterial chromosome that have distinct base composition compared to the whole genome (Doublet et al., 2008). The GEIs provide various functions for bacterial adaptation, such as additional metabolic activities and antibiotic resistance (Hacker and Kaper, 2000). GEIs also provide a selective advantage to the recipient bacteria within a particular environment, termed “fitness islands” (Juhas et al., 2007). Several types of fitness islands have been described, including pathogenicity islands (PAIs), which contain virulence genes required for host colonization, and symbiosis islands, which contains genes required to form a nitrogen-fixing symbiosis with the legume host (Dobrindt et al., 2004).

Symbiosis island was first identified in *Mesorhizobium loti* strain ICMP3153 (Sullivan and Ronson, 1998) and then in strain MAFF303099 and R7A (Sullivan et al., 2002). *M. loti* is nitrogen-fixing bacterium that able to establish a symbiosis with several *Lotus* species. Symbiosis islands harbor genes essential for nodulation, nitrogen fixation, secretion, metabolism and island transfer. It is transferable to nonsymbiotic *Mesorhizobium* strains and confer them the symbiotic ability with host plants (Sullivan et al., 1995). The symbiosis island of *M. loti* strain R7A was grouped under a classification termed integrative and conjugative elements or ICEs due to its ability of excision, self conjugative transfer, and integration into the chromosome of the recipient bacterium (Burrus et al., 2002). Thus, the *M. loti* R7A symbiosis island was termed ICE*Ml*Sym^{R7A} (Ramsay et al., 2006). Site specific integration of the ICE*Ml*Sym^{R7A} is catalyzed by an integrase of the tyrosine recombinase family, *intS,* which is the first ORF located at the left end of the symbiosis island (Esposito and Scocca, 1997; Sullivan and Ronson, 1998). The ICE*Ml*Sym^{R7A} integrates into a *phe*tRNA gene in the chromosome with a 17-bp duplication of the 3' terminal part of the gene (Sullivan et al., 2002).

Phage integrase catalyzes site-specific recombination between a short common core sequence present in both phage (*attP*) and bacterial host (*attB*) genomes. After recognition of these two sequences, the integrase bind to the *attB*, at which they cleave the DNA backbone. The recognition site for cleaving is so-called staggered cleavage site. A circular phage DNA element integrates into a bacterial host chromosome through single-crossover. The integrated element is flanked by two hybrid sites, *attL* and *attR*, each consisting of half *attP* sequence and half *attB*

sequence (Groth and Calos, 2004). Integration site specificity is determined by the integrase enzyme typically encoded within elements of several types: temperate bacteriophages, integrative plasmids, pathogenicity islands and conjugative and mobilizable elements (Williams, 2002). A family of integrase usually targets the 3′ end of tRNA genes for integration (Zhao and Williams, 2002). In similar to phage integrase, ICE*Ml*Sym^{R7A} integrates into a *phe*tRNA gene and the integration occurs by specific recombination between 17-bp core sequence of circular-form ICE*Ml*Sym^{R7A} (*attP*) and of the nonsymbiotic *Mesorhizobium* strains (*attB*). The role of *intS* and *attP* on symbiosis island transfer was confirmed by Ramsay et al. (Ramsay et al., 2006). They revealed that a mini-island, comprising a suicide plasmid containing just the *attP* region and *intS*, had ability to integrate into the chromosome of the nonsymbiotic *Mesorhizobium* sp. strain CJ4 at the *phe*tRNA locus. This indicates that whether *intS* and *attP* were sufficient to direct integration of ICE*Ml*Sym^{R7A}. In addition, excision and transfer of ICE*Ml*Sym^{R7A} require a recombination directionality factor encoded by *rdfS* and might be regulated by quorum sensing mediated by *traR* and *traI2* genes on the island.

Symbiosis island was also determined in *B. japonicum* USDA110 which has an ability to form root nitrogen fixing nodules on soybeans (Kaneko et al., 2002). The genome structure of *B. japonicum* USDA110 is similar to that of *M. loti* in that many of the genes for symbiotic nitrogen fixation are clustered on the chromosome (Gottfert et al., 2001; Kundig et al., 1993). Instead of the *phe*tRNA gene in *M. loti*, a *val*tRNA gene was found at one end of the symbiosis island of *B. japonicum* USDA110. Moreover, duplication of parts of tRNA genes at 14 locations was found in

B. japonicum USDA110 chromosome and several copies of tRNA gene were found in and around the symbiosis island. These tRNA genes are possible the target sites for a particular integrative system.

Based on 16S rRNA sequence analysis, *B. japonicum* is phylogenetically closer to *B. yuamingense*, *B. liaoningense*, and other nonsymbiotic genera (*Afipia, Nitrobacter* and *Rhodopseudomonas*) (Willems et al., 2001). *Rhs. palustris* is phylogenetically close to nodule-forming nonphototrophic *B. japonicum* (Woese et al., 1984; Wong et al., 1994), as well as the stem-nodulating phototrophic *Bradyrhizobium sp*. strain BTai1 and ORS2378 (Eaglesham et al., 1990; Fleischman et al., 1995; Inui et al., 2000; Wong et al., 1994; Young et al., 1991). Horizontal transfer of either symbiotic or photosynthetic regulatory may play an important role in evolution and adaptation to the provided environment of these *Bradyrhizobiaceae* bacteria. Molouba, et al. (Molouba et al., 1999) suggested that *Bradyrhizobium* may have evolved from photosynthetic free-living bacteria by acquisition of symbiotic functions, when they are not exposed to significant levels of light. Consequently, photosynthetic function might be lost during evolution from free-living existence to a symbiotic one. Therefore, the transfer ability of *M. loti* symbiosis to *Bradyrhizobiaceae* bacteria was determined in this study. The aim of this study is to determine an ability of *M. loti* integrase for integration of symbiosis island into the chromosome of *Bradyrhizobium* strains, as well as of *Rhs. palustris* and nonsymbiotic *Bradyrhizobiaceae* strains (Pongsilp et al., 2002).

4.2 Objectives

1. To determine the ability of *M. loti* integrase for integration into *Bradyrhizobiaceae* strains.

2. To determine the ability of *M. loti* symbiosis island transfer to *Bradyrhizobiaceae* strains.

3. To determine effects of symbiosis island transfer on symbiotic ability of *Bradyrhizobiaceae* recipient strains.

4.3 Materials and methods

4.3.1 Bacterial strains, plasmids, and media

Bacterial strains and plasmids used are listed in Tables 4.1. *Escherichia coli* strains were cultured at 37°C in LB medium (Sambrook et al., 1989). *Bradyrhizobium* strains including isolated strains were cultured in YM media (Somasegaran and Hoben, 1994). *Rhodopseudomonas palustris* DSMZ 123^T was cultured in van Niel's complex medium (van Niel, 1944). *Mesorhizobium* strains were cultured in TY (Somasegaran and Hoben, 1994) or G/RDM media (Ronson et al., 1987) containing 10 mM glucose. Media were supplemented with the following antibiotics when appropriate: for *E. coli* 50 mg/ml kanamycin, 15 mg/ml tetracycline and 25 mg/ml gentamycin; for *Bradyrhizobium* and the isolated strains 300 mg/ml neomycin and 250 mg/ml tetracycline, for *Rhs. palustris* 300 mg/ml neomycin and 100 mg/ml tetracycline.

Table 4.1 Strains and plasmids used.

Strains/plasmids	Relevant characteristics	Reference or source
M.loti		
R7A	Field reisolate of ICMP 3153	Sullivan et al. (1995)
R7AΔ*rpoN*	Δ*rpoN*::*nptII* marker exchange mutant, Nm^R	Sullivan personal communication
R7AZ	R7AΔ*rpoN* containing pJR206, Nm^R and Tc^R	This study
R7ANS	Non-symbiotic derivative of R7A; lacks ICEMlSym^{R7A}	Ramsay et al., 2006
FJX1	R7ANS containing pFJX600, Tc^R	This study
FJX1REV	R7ANS containing pFJXR, Tc^R	This study
B. japonicum		
USDA110	Soybean symbiont	This study
FJX2	USDA110 containing pFJX600, Tc^R	This study
B. elkanii		
USDA94	Soybean symbiont	This study
Bradyrhizobium isolates		
S7	Symbiotic *B. yuanmingense*; soybean field soil isolate	Pongsilp et al. (2002)
S9	Unknown symbiotic species; soybean field soil isolate	Pongsilp et al. (2002)
S7Sym1	S7 derivative; ICEMlSymR7A integrant	This study
S7Sym2	S7 derivative; ICEMlSymR7A integrant	This study
S7Sym3	S7 derivative; ICEMlSymR7A integrant	This study
S7Sym4	S7 derivative; ICEMlSymR7A integrant	This study
FJX3	S7 containing pFJX600, Tc^R	This study
FJX4	S9 containing pFJX600, Tc^R	This study
Non-symbiotic isolates		
NS1	Non-symbiotic *Bradyrhizobiaceae*; soybean field soil isolate	Pongsilp et al. (2002)
NS20	Non-symbiotic *Bradyrhizobiaceae*; soybean field soil isolate	Pongsilp et al. (2002)
NS23	Non-symbiotic *Bradyrhizobiaceae*; soybean field soil isolate	Pongsilp et al. (2002)
NS28	Non-symbiotic *Bradyrhizobiaceae*; soybean field soil isolate	Pongsilp et al. (2002)
FJX4	NS23 containing pFJX600, Tc^R	This study
FJX5	NS28 containing pFJX600, Tc^R	This study
Rhs. palustris		
DSMZ 123T	Photosynthetic bacteria	DSMZ
E. coli		
S17-1	*pro recA* RP4-2(TcS::Mu) (KmS::Tn*7*); Mob+	Simon et al. (1983)
Plasmids		
pBluscript	High copy number ColE1-based phagemid, Ap^R	This study
pJJ607	pPR691 containing *attP* and *intS* amplified from R7A genomic DNA cloned as BamHI fragment	Ramsay et al., 2006
pKnock-Tc	Mobilizable suicide vector; Tc^R	Alexeyev (1999)
pJZ607	pKNOCK-Tc containing *attP-intS* BamHI fragment from pJJ607	This study
pFJX	pFAJ1700 containing promoterless *lacZ* gene from pFUS2; Tc^R	Miller et al., 2007
pFJX600	pFJX containing a promoter region of *M. loti* R7A *intS* ; Tc^R	This study
pFJXR	pFJX containing a promoter region of *M. loti* R7A *intS* in reverse; Tc^R	This study
pJR206	pFAJ1700 containing *traR traI2*	Ramsay et al., 2006

4.3.2 Primers

All primers used in this study are listed in Table 4.2.

Table 4.2 Primers used.

Primer name	Primer sequence (5'-3')	Source/reference
245upstattL_NS	ccgacgacgaggacggcgaatag	This study
198upstattL_Rhs	ccggcaggcccttctgaattccc	This study
UpstattL_Sym	ccgttctgctcctcgcgctg	This study
152DownstattR_NS	gcgcttccgtagccagtagatacc	This study
436DownstattR_Rhs	gggcggaaccgcagcttggttac	This study
220DownstattR_S7	cggcagccgttgacgaatttgttc	This study
332DownStattR_S9	gggacagggtggcgtaggctttg	This study
339DownStattR_USDA110	gcgcggaggatgcgctcaagatac	This study
intSReverse	ccgaaatgcccgacccatttctaag	This study
M. loti specific primers		
nodM370	actgaggtccttgcgcatctcttgg	This study
nodM1569	aaagcgatcatgtggcgcgatgacg	This study
nodD1_89	tcaaccttagtcagccggccatgag	This study
nodD1_888	ctgcaacattatctgccgcatccag	This study
nolL7	gatcacataagagccggagcgaagg	This study
nolL906	gaccaggtaaagcagcagcgtgctc	This study
nodD2_89	tcaaccttagtcagccggccatgag	This study
nodD2_846	gttgtgaagggcgggccattgaacg	This study
nodB13	gatgacagatgggaggtgcagagtg	This study
nodB612	tgcaggaatcagattggacagcgcc	This study
NolO110	aggtcatcgcggccgtagaagag	This study
nolO1971	tgctcccacaaccgtcgcagttc	This study
nodJ31	gccaatgcgtggaactgggttgc	This study
nodJ746	aatggcaacagcgcgtacaggcc	This study
nodI51	gaagcgtaagttgggcccagagg	This study
nodI1028	tcctcgagattggctggacgcag	This study
nodC92	tgcctacaaacgtaccgccgacc	This study
nodC1149	cgcgtatgctttcaacgggagcaag	This study
nodA93	tgagccgaccggagccttcaatg	This study
nodA592	gctctggaccgttccgatcgatc	This study
nodS33	tcgagaaatggccgcagacgacc	This study
nodS593	ggctttcggaagagagccagcag	This study

Table 4.2 (continued).

Primer name	Primer sequence (5'-3')	Source/reference
B. japonicum USDA 110 specific		
nolY_8	cgcagcgtagaactcgggc	This study
nolY_657	cggtaatgccgccacagtc	This study
nolYZ_48	ggtgcgggctggacctttc	This study
nolYZ_947	gttgcgtaggaaagccggc	This study
nolA_38	tagcagcgacagagcgcac	This study
nolA_637	gctgatcctccaatccgcc	This study
nodD1_41	tcgacgccgtgatgacggc	This study
nodD1_940	agcgcctgcgagttggagg	This study
nodA_28	gaaggatcttctgggcgcg	This study
nodA_627	ctcaggcccgttacgatcg	This study
nodB_26	ctgtccgctgcgactacgc	This study
nodB_625	cgcgccgttgtagtgctgg	This study
nodC_195	cgccgaatgtctggagtcg	This study
nodC_1394	cctgagtcatcagccgacc	This study
nodS_22	gacggagatgcggcgtctg	This study
nosS_531	ccggcgtttgattgtgccg	This study
nodU-358	agctacccgcatgtcacgg	This study
nodU-1557	ggtgcagagcagcggaatg	This study
nodI_77	tgtcgttctcggttgcgcg	This study
nodI_826	gcacgctgatttcgtccgg	This study
nodJ_13	tatgcgtcggtcatgccgg	This study
nodJ_712	cgtgtagggcggcattgtc	This study
nolMN_31	tcgcgagtgacctggtctg	This study
nolMN_530	tcccgtgccaacagctgcc	This study
nodZ_24	gccggcaccgcgatttcag	This study
nodZ_973	tgtcgcagcgggcaaggag	This study
SP1_intS5RACE	tcagcttcagagcggctcggg	This study
SP2_ intS5RACE	cggcacgtccctccttcttcagc	This study
SP3_ intS5RACE	gtgcctcagcgagggagatgag	This study
Anchor primer_RACE	gaccacgcgtatcgatgtcgac	5'/3' RACE kit (Roche)
Oligo d(T)-anchor primer	gaccacgcgtatcgatgtcgactttttttttttttttv	5'/3' RACE kit (Roche)
-122	tctcgctccaaacccttggtt	This study
-82	atcaaccatgaagacaaccgt	This study
-69	agacaaccgtgcgtccctgcta	This study
-53	tgctacaacctccggtttgta	This study
476	cttggaggtgcgacgcctctcg	This study
187	gctatggtagtcgtagaggtt	This study
177	tcgtagaggttatggtggctt	This study
71	ttcctaaagtcgatcaacggc	This study
10	tcttctcccctaccacgggtct	This study
0	accacgggtacccgcctta	This study

4.3.3 Bacterial matings

4.3.3.1 Biparental mating

Biparental spot mating for transfer of the integrative vector PJZ608 to the recipient bacteria was carried out as previously described (Ramsay et al., 2006) using *E. coli* strain S17-1 as donor. The *E.coli* S17-1 cells were grown for 16 hr in 5 ml broth of LB medium supplemented with kanamycin. *Bradyrhizobium* cells were grown for 3 days in 5 ml of liquid YM medium. Cells of *Rhs. palustris* and nonsymbiotic strains were grown for 3 days in 5 ml of liquid van Niel's medium for 3 days. 25 µl of S17-1 donor and 25 µl of recipient were dropped and mixed together on dry YM or van Niel's plate without antibiotic. Bacterial spots were dried in a larminar flow hood. After growth overnight at 28°C for 2 days, cells of each spotted were taken from the plate and suspended in 0.5 ml of liquid medium cells. 100 µl of the suspension were then plated on plates with appropriate antibiotics. Alternatively, cells of each spotted the were taken from the plate and directly suspended streaked on plates with tetracycline and gentamycin. Colonies were re-streaked on selection plates to ensure purity and the presence of the antibiotic marker.

Mating for transfer of ICE*Ml*Sym^{R7A} to *Bradyrhizobium* strains was carried out as previously described (Sullivan and Ronson, 1998). *M. loti* strains R7AZ strain harboring pJR206 (Ramsay et al., 2006), which containing *traR* gene of *M. loti* R7A, was used as a donor. *B. japonicum* USDA110, *B. yuanmingense* S7, *Bradyrhizobium sp.* S9, nonsymbiotic *Bradyrhizobiaceae* strains (NS23 and NS28), and *Rhs. palustris* DSMZ 123^{T} were used as recipients. One-milliliter portions from donor and recipient cultures (stationary phase) were filtered onto nitrocellulose filter

and incubated on agar plates for 48 h at 28°C. After incubation, cells were washed off the filters and resuspended in 3 ml of liquid medium. The cell suspension was serially diluted before spreading on plates of selective medium containing neomycin and gentamycin. Transfer frequencies, completed in triplicate, were determined by dividing the number of ICE*Ml*Sym^{R7A} exconjugants by the number of donor cells.

4.3.3.2 Triparental mating

Triparental mating for transfer of *intS-lacZ* reporter pFJXR to the recipient bacteria was carried out using the helper plasmid pRK2013 in HB101 (Ditta et al., 1980) and HB101 donor strains. *E.coli* cells were grown for 16 hr in 5 ml broth of LB medium supplemented with kanamycin. *M. loti* cells were grown for 3 days in 5 ml of liquid TY medium. *Bradyrhizobium* cells were grown for 3 days in 5 ml of liquid YM medium. Cells of *Rhs. palustris* and nonsymbiotic strains were grown for 3 days in 5 ml of liquid van Niel's medium. Mixture of 1 ml donor, 1 ml helper, and 3 ml recipient were pelleted by centrifugation at 10,000 rpm for 1 min. Cell pellets were washed twice with 0.85% NaCl and resuspened in 0.5 ml broth medium. 200 µl of cell suspension was applied onto a membrane filter placed on agar plates and incubated at 28°C for 48 h. After incubation, cells were washed off the filters and resuspended in 1 ml of liquid medium. The cell suspension was serially diluted before spreading on plates of selective medium containing tetracycline and gentamycin.

4.3.4 Preparation of electrocompetent cells

4.3.4.1 Electrocompetent cells of *E. coli*

Competent cells were prepared by the procedure reported by previously (Sheng et al.). Cells were grown in 500 ml SOB broths (without Mg^{2+}) at 37°C with shaking at 200 rpm to an OD550 of 0.7 (never higher than 0.8). Cells were harvested by centrifuging in a pre-chilled JA14 rotor at 7000 rpm for 10 min at 4°C and resuspended in a volume of cold 10% sterile glycerol equal to the original culture volume. Cells were collected by centrifuging at 7000 rpm for 10 min at 4°C. This washing and spinning procedure was then repeated, again using a volume of 10% glycerol equal to the original culture volume. After decanting the supernatant, cells were resuspended in the volume of glycerol remaining in the centrifuge bottles (30 ml).The suspension was transferred to JA20 tubes and centrifuged at 7000 for 10 min at 4°C. After decanting the supernatant, cells were resuspended in 10% glycerol at at a volume of 2 ml/l of original culture. Cells were aliquoted to microcentrifuge tubes (40 µl/tube) and frozen quickly in a dry ice-ethanol bath. Cells were stored until use at -70°C.

4.3.4.2 Electrocompetent cells of *M. loti*

Cells were grown in 200 mL TY broths at 28°C with shaking at 180 rpm to an OD550 of 0.15-0.3. Cells were harvested by centrifuging in a pre-chilled JA14 rotor at 7000 rpm for 10 min at 4°C and resuspended in a volume of cold 10% sterile glycerol equal to the original culture volume. Cells were collected by centrifuging at 7000 rpm for 10 min at 4°C and resuspended in 100 ml cold 10%

glycerol. After decanting the supernatant, cells were resuspended in 4 ml cold 10% glycerol. The suspension was transferred to JA20 tubes and centrifuged at 7000 for 10 min at 4°C. After decanting the supernatant, cells were resuspended in 200 µl 10% glycerol. Cells were aliquoted to microcentrifuge tubes (40 µl/tube) and frozen quickly in a dry ice-ethanol bath. Cells were stored until use at -70°C.

4.3.5 Electroporation

4.3.5.1 Electroporation of *E. coli*

Electroporation was carried out using a Gene Pulser Xcell™ Electroporation System model (Bio-Rad) in 0.1 cm electrocuvettes. Cuvettes were chilled on ice before using. Aliquot (40 µl) of electrocompetent cells in a microcentrifuge tube was thawed on ice. One µl of DNA was added and mixed by pipetting. The DNA and cells mixture was transferred to the cuvette. Electroporation was performed conditions at the following settings: 18 kV/cm, 100 Ω, and 25 µF. Time constants of 4 to 5 ms were reached. After electroporation, 1 ml LB was immediately added to the cuvette and the contents were then transferred to a sterile glass culture bottle. Cells was grown at 37°C for 45 min with shaking at 180 rpm. For each electroporation, 5, 20, 50, 250, 350 µl of cells were spread on LB plates containing appropriate antibiotics.

4.3.5.2 Electroporation of *M. loti*

Electroporation was carried out using a Gene Pulser Xcell™ Electroporation System model (Bio-Rad) in 0.1 cm electrocuvettes. Cuvettes were chilled on ice before using. Aliquot (40 µl) of electrocompetent cells in a

microcentrifuge tube was thawed on ice. One microlitter of DNA was added and mixed by pipetting. The DNA and cell mixture was transferred to the cuvette. Electroporation was performed conditions at the following settings: 18 kV/cm, 200 Ω, and 25 μF. Time constants of 4 to 5 ms were reached. After electroporation, 1 ml TY was immediately added to the cuvette and the contents were then transferred to a sterile glass culture bottle. Cells were grown at 37°C for 45 min with shaking at 180 rpm. For each electroporation, 5, 20, 50, 250, 350 μl of cells were spread on TY plates containing appropriate antibiotics.

4.3.6 Isolation of plasmid DNA from bacteria (*E. coli*)

4.3.6.1 Alkaline Lysis Mini-plasmid Prep

Three milliliter of overnight culture of *E.coli* was collected by centrifugation at 10,000 rpm for 1 min. Pelleted bacterial cells were resuspended in 300 μl Qiagen P1 buffer and mixed by vortex. 300 μl of lysis solution (0.2 M NaOH, 1% SDS) was added and mixed by gentle inverting the tube 6 times. Then, 350 μl of 3M potassium acetate plus 5% formic acid was added, mixed by inverting 6 times. The mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and plasmid DNA was precipitated by adding 560 μl of isopropanol. Then, DNA was pelleted by centrifugation at 13,000 rpm for 10 min. DNA pellet was washed once with 700 μl of 70% ethanol and recovered by centrifugation at 13,000 rpm for 5 minutes. DNA pellet was dried and resuspended in 50 μl of sterile Milli-Q water.

4.3.6.2 Qiaprep Spin Miniprep

Three milliliter of overnight culture of *E.coli* was collected by centrifugation at 10,000 rpm for 1 min. Pelleted bacterial cells were resuspended in 250 µl P1 buffer and mixed thoroughly by vortexing. 300 µl of P2 buffer was added and mixed by gentle inverting the tube 6 times. Then, 350 µl of N3 buffer was added and immediately mixed by gentle inverting 6 times. The mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to the QIAprep column by pipetting. The flowthrough was discarded by centrifugation at 13,000 rpm for 1 min. The QIAprep spin column was washed by adding 500 µl of PB buffer. The flowthrough was discarded by centrifugation at 13,000 rpm for 1 min. To remove residual wash buffer was removed, the flowthrough was discarded by additional centrifugation at 13,000 rpm for 1min. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. Plasmid DNA was eluted by adding 75 µl of EB buffer to the center of QIAprep column, letting the column to stand for 1 min and centrifugation at 13,000 rpm for 30 s.

4.3.6.3 Qiagen midiprep

Cells of *E. coli* were grown in liquid LB medium (50 ml) with appropriate antibiotic for 16 h. Cells were collected by centrifugation at 10,000 rpm for 10 min, in JA20 rotor. Supernatant was poured off and the remaining on cell pellet was removed by pipetting. Cells were resuspended in 5 ml GTE (0.9% glucose, 25 mM Tris pH 8.0, and 10 mM EDTA) and vigorously mixed by vortexing and pipetting up and down until no cell clumps remain. Four milliliter of 4 mg/ml lysozyme was

added, followed by vigorously inverting the tube 6 times and incubating at room temperature for 10 min. Ten milliliter of NaOH/SDS solution (0.2M NaOH and 1% SDS) was added the suspension was then incubated on ice for 10 min. 5 ml of chilled 5M potassium acetate was added and mixed by inverting 6 times. The suspension was incubated on ice for 20 min, followed by centrifugation at 10,000 rpm for 5 min. Supernatant was transferred to fresh JA20 tube by pipetting. 0.6 volume of isopropanol was added and DNA was recovered by centrifugation at 10,000 rpm for 20 min. The pellet was dried and resuspended in 3 ml of TE buffer. 3 ml of 5 M Ammonium acetate was added and the suspension was incubated on ice for 20 min, followed by centrifugation at 10,000 rpm for 15 min. Supernatant was carefully transferred to new tube. 6 ml of isopropanol was added and DNA was recovered by centrifugation at 10,000 rpm for 20 min. The pellet was dried and resuspended in 500 µl of TE buffer. The solution was transferred to 1.5 microcentrifuge tube and extracted twice by adding 500 µl of 50:50 phenol/chloroform, followed by a centrifugation step at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and finally extracted with 500 µL chloroform, followed by a centrifugation step at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and plasmid DNA was precipitated by adding of 500 µl isopropanol and 50 µl 3M sodium acetate. DNA was recovered by centrifugation at 13,000 rpm for 10 min. DNA pellet was washed with 500 µl 70% ethanol and recovered by centrifugation at 13,000 rpm for 5 minutes. DNA pellet was dried and resuspended in 100 µl of TE buffer.

4.3.7 Isolation of genomic DNA

4.3.7.1 DNA extraction using guanidine thiocyanate

Three milliliters of bacterial cells were harvested by centrifugation at 13,000 rpm for 1 min. Cell pellet was resuspended in 500 µl lysis buffer (4M guanidinium thiocyanate, 1 mM 2-mercaptoethanol, 10 mM EDTA, and 10% tween). Taking twice, cell suspension was snap-frozen at -70°C for 5 min and incubated at 65°C for 10 min. The cell suspension was immediately chilled on ice for 5 min. The aqueous phase was extracted once with 500 µl chloroform, followed by a centrifugation step at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and extracted once with 500 µl phenol:chloroform (1:1), followed by a centrifugation step at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and extracted with 500 µl chloroform, followed by a centrifugation step at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and genomic DNA was precipitated by adding of 0.7 volume of isopropanol and 0.1 volume of 3M sodium acetate. DNA was recovered by centrifugation for at 13,000 rpm for 10 min. DNA pellet was washed once with 1 ml 70% ethanol and recovered by centrifugation at 13,000 rpm for 5 minutes. DNA pellet was dried and resuspended in 50 µl of TE buffer.

4.3.7.2 Preparation of DNA template using the PrepMan Ultra reagent

DNA template for PCR was prepared using the PrepMan Ultra reagent (Applied Biosystems), following the protocol of the manufacturer. One milliliter of bacterial cells was harvested by centrifugation at 13,000 rpm for 1 min.

Cell pellet was resuspended in 100 µl PrepMan reagent. Cell suspension was thoroughly mixed by vortex and boiled at 100°C for 10 min in a water bath, followed by a centrifugation step at 13,000 rpm for 5 min. The supernatant was transferred into a new microcentrifuge tube. The supernatant is ready for PCR.

4.3.8 Quantification of DNA

The concentration and purity of DNA was determined using ND-1000 Spectrophotometer (NanoDrop). Analysis of dsDNA was performed using a programmed method as recommended by the manufacturer.

4.3.9 Construction of an integrative vector

For the construction of integrative plasmid PJZ608 (Figure 4.1), a *Bam*H1 fragment of pJJ608 (Ramsay et al., 2006) comprising *attP*, *intS* and any regions required for expression of *intS* was ligated into a suicide plasmid pKNOCK-Tc (Alexeyev, 1999). The regions contained the left end of ICE*Ml*Sym^{R7A} including *intS* preceded by right-terminal 436 bp of the island. Restriction enzyme digestion of the plasmids was carried out in a volume of 60 µl containing 6 µl of Buffer B (10X), 2 µl of *BamH1* (Promega), 500 ng of pKNOCK-Tc, and H_2O up to 60 µl. The mixture was incubated at 37°C for 30 min in a water bath. The linearized plasmids were dephosphorilated using alkaline phosphatase in order to avoid plasmid re-ligation. 1 µl was added into the the *Bam*H1-digestion mixture and incubated 37°C for 30 min. The enzyme was inactivated by heating at 65°C for 10-15 min. DNA was analysed on an agarose gel electrophoresis to ensure complete digestion of DNA. The target DNA

was purified from agarose gel using High-Pure PCR product purification kit (Roche) according to the manufacturer's specifications. Ligation reactions were carried out using T4 DNA ligase according to the manufacturer's specifications. Ligation was performed in 30 µl-reaction mixture using an insert:plasmid ratio of 3:1. The mixture was incubated at 13°C overnight. DNA was precipitated by adding 75 µl of ethanol and 3 µl of 3M sodium acetate and centrifuging at 13,000 rpm for 15 min. DNA pellet was washed once with 70% ethanol, dried, and resuspended in appropriate volume of water. The recombinant plasmid (pJZ608) (Figure 4.1) was subsequently transferred into *E. coli* S17-1 competent cells.

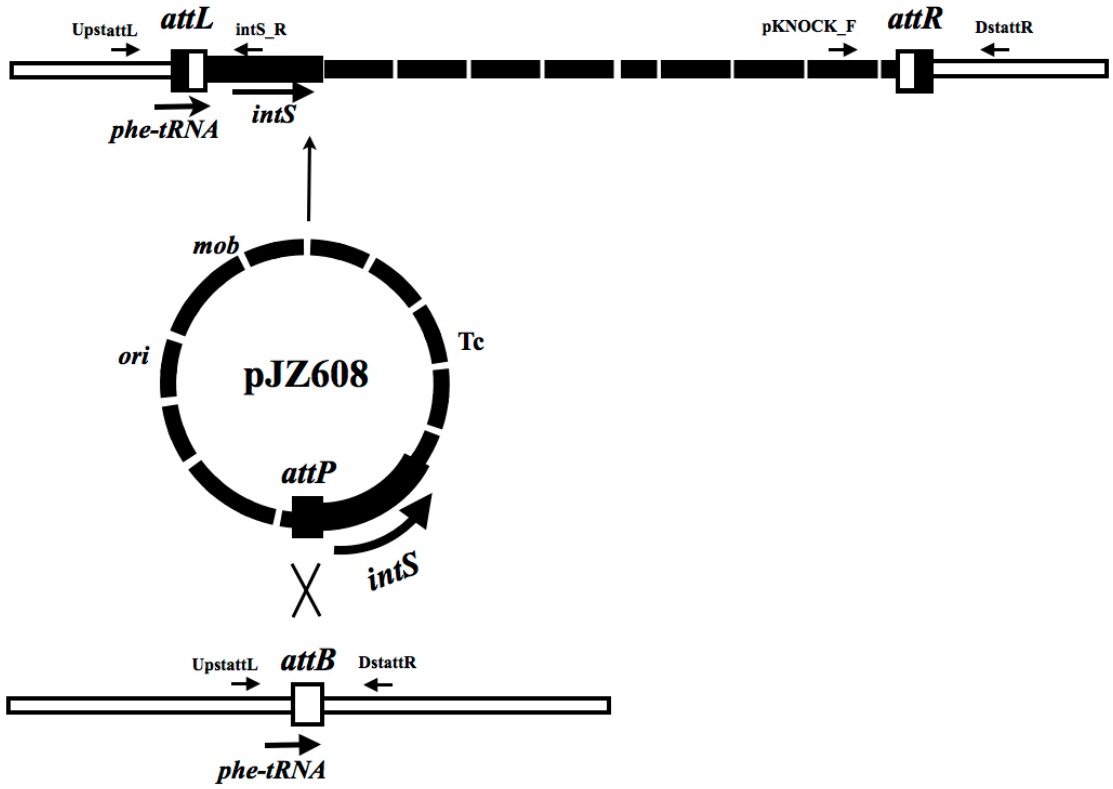

Figure 4.1 Model for integration of integrative vector pJZ608 and location of primers used to detect pJZ608 integration. pJZ608 DNA is shown as a black bar or circle, chromosomal DNA is shown by white bars and vector DNA is hatched. Attachment sites are illustrated by black or white boxes representing their composite structure. Location of primers used for standard PCR are shown by small arrows. Genes are shown by thick arrows. Maps not to scale.

4.3.10 Isolation of pJZ608 integrative events and sequencing of *attL* and *attR*

E. coli S17-1 was used to mobilize the integrative vector pJZ608 into *Bradyrhizobium* strains, *Rhs. palustris*, and the nonsymbiotic *Bradyrhizobiaceae* strains, by biparental spot mating. The chromosomal DNA of each integrant was digested with the restrictive enzyme that did not cleave within the PJZ608 used. *SacI* was chosen since it provided smear bands of chromosomal DNA, indicating that various sizes of DNA fragments were generated. Restriction enzyme digestion was carried out in a volume of 40 µl containing 4 µl of Buffer B (10X), 2 µl of *SacI* (Promega), approximately 1 mg of chromosomal DNA, and H_2O up to 40 µl. The mixture was incubated at 37°C for 30 min in a water bath. Digested DNA was extracted once with phenol/chloroform by adding 160 µl of Milli-Q water and 200 µl of phenol:chloroform, followed by a centrifugation step at 13, 000 rpm for 10 min. The supernatant was transferred to a fresh tube and extracted with 500 µl chloroform, followed by a centrifugation step at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and DNA was precipitated by adding of 400 µl of isopropanol and 4 µl of 3M sodium acetate. DNA was recovered by centrifugation at 13,000 rpm for 15 min. DNA pellet was washed once with 300 µl 70% ethanol and recovered by centrifugation at 13,000 rpm for 5 minutes. DNA pellet was dried and resuspended in 26 µl Milli-Q water, preparing for re-ligation step. Restriction fragments were circularized by re-ligation. Ligation was performed in 30 µl-reaction mixture by adding 3 µl of ligation buffer and 1 µl of T4 DNA ligase into the 26-µl restricted DNA solution. The mixture was incubated at 13°C overnight. Ligated DNA was cleaned and precipitated as described by above. DNA pellet was washed once

with 70% ethanol, dried, and resuspended in 5 µl Milli-Q. The re-ligated DNA (1 µl) was introduced into *E. coli* by electroporation. Tetracycline transformants (Tet^R) were selected to rescue plasmids bearing the integrative vector pJZ608. Plasmid DNA was prepared from resulting colonies of the transformants. The plasmid of each transformant was digested with *SacI* and patterns of digestion were analysed on an agarose gel electrophoresis. In order to determine *attL* and *attR*, the rescue plasmids were sequenced with primers *intS*_R and pKNOCK_F (Table 4.2 and Figure 4.1), respectively.

4.3.11 Analysis of *attB*

The *attB* of different bacterial hosts were determined by PCR and sequencing. In each host strain forward and reverse primers for *attB* PCR were designed from the sequence upstream of *attL* and sequence downstream, respectively (Figure 4.1). The primers, which bind to upstream region of *attL* and downstream region of *attL* of each recipient strains, used for *attB* amplification are listed in Table 4.2. The primer pairs were Upst*attL*_Sym and 339Dst*attR*_USDA110 for *B. japonicum* USDA110, Upst*attL*_Sym and 220Dst*attR*_S7 for the strain S7, Upst*attL*_Sym and 332Dst*attR*_S9 for the strain S9, 245Upst*attL*_NS and 152Dst*attR*_NS for the nonsymbiotic strains, 198Upst*attL_Rhs* and 436Dst*attR*_Rhs for *Rhs. palustris*. PCR products were amplified using the Phusion High-Fidelity PCR kit (Finnzymes). PCR amplification was performed in a 50 µl reaction volume containing 20 pmol of each primer, 200 µM of each dNTP, 1 U of Phusion DNA Polymerase, 1X Phusion HF Buffer and 1 µl of template DNA. The thermal cycler

was programmed as follows: an initial denaturation at 98°C for 1 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. PCR products were purified with High-Pure PCR product purification kit (Roche). Purified PCR products were directly sequenced using the same primers as for PCR.

4.3.12 Construction of i*ntS-lacZ* reporter

For construction of *intS-lacZ* reporter, a 600 bp region containing potential promoter region of *intS* was amplified from R7A genomic DNA by PCR using primers 122UpstattP and 476DSTattP (Table 4.2). This region contained the left end of ICE*Ml*Sym^{R7A} including *intS* preceded by right-terminal 476 bp of the island (122 bp upstream of *attP* to the first 279 bp of *intS)*. The PCR product was cloned as a *Bam*H1 fragment into pFJX adjacent to its promoterless *lacZ* gene, creating pFJX600. pFJX600 was transferred into *M. loti* strain R7ANS and *Bradyrhizobiaceae* strains by conjugation and tetracycline-resistant exconjugants that contained the reporting plasmid were selected. Reverse of the promoter region was cloned into the pFJX for creating pFJXR. The pFJXR was used to introduce to R7ANS for using as a negative control. The *lacZ* reporter construct on pFJX600 was used to monitor *intS* gene expression in *M. loti* strain R7A and *Bradyrhizobiaceae* strains.

4.3.13 β-Galactosidase assay

β-Galactosidase assay were carried out on stationary-phase cultures by the method described previously (Miller, 1972). Stationary phase cells (200 μl) were

pelleted by centrifugation at 13,000 rpm for 1 min. The cell pellet was resuspended in 1 ml Z-buffer. Optical density of the cell suspension was measured at 600 nm in a sterile cuvette. 800 µl of the cell suspension was transferred from the cuvette to a microcentrifuge tube. The cells were lysed with chloroform/SDS by adding 40 µl chloroform and 20 µl SDS (0.1%), followed by mixing and incubating at room temperature for 5 s. After cell lysis, 200 µl of ortho-nitrophenylgalactoside (ONPG) was added and the reaction was incubated at 28°C until yellow color was visible. After sufficient color has developed, the reaction was stopped by adding 0.5 ml of 1M sodium carbonate and the stopping time was recorded. The solution was cleared by centrifugation at 13,000 rpm for 3 min before measuring the optical density at 420 nm. Units of activity were calculated as described by Miller (1972). Miller units (MU) are calculated using the following equation:

$$MU = 1000 \times (ABS420/ABS600) / [volume\ (ml) \times time(min)]$$

4.3.14 PCR and sequencing of ICE*Ml*Sym^{R7A} genes in *B. yuanmingense* S7 exconjugants

ICEMlSymR7A was allowed to transfer to the *Bradyrhizobiaceae* strains by filter mating as described above. For detection of presences of ICE*Ml*Sym^{R7A} in the chromosome of the S7-exconjugants, *nptII* gene and several ICE*Ml*Sym^{R7A}-specific genes were detected by PCR amplification and sequencing. *M. loti* R7A and *B. yuanmingense* strain S7 were used as positive and negative control, respectively. The ICE*Ml*Sym^{R7A} genes and the primers used in this study are listed in Table 4.2.

The purified PCR products were sequenced using the same primer as for the PCR amplification.

4.3.15 Analysis of *attL, attR* and *attB* of *B. yuanmingense* S7

Left-integrative junctions of ICE*Ml*Sym^{R7A} integrants were amplified using a reverse primer *intS*_R and S7-specific forward primers Upst*attL*_Sym. The upst*attL*_ Sym was designed from DNA sequence upstream of *attL* in pJZ608-integrants (Figure 4.1). Right integrative junctions of ICE*Ml*Sym^{R7A} integrants were amplified using a forward primer 122Upst*attP* and S7-specific reverse primer 220Dst*attR*_S7. The primer 122Upst*attP* binds the sequence located upstream of circular ICE*Ml*Sym^{R7A} *attP*. The primer 220Dst*attR*_S7 was designed from DNA sequence downstream of *attR* in pJZ608-integrants (Figure 4.1). The purified PCR products were sequenced using the same primer as for the PCR amplification.

4.3.16 Nodulation test on *Lotus*

For preparation of inocula, the strains S7 and ICE*Ml*Sym^{R7A} exconjugants (S7Sym1, S7Sym2, S7Sym3, and S7Sym4) were grown for 72 h and *M. loti* was grown for 48 hr in YM broths. Nodulations of *L. japonicus* and *L. corniculatus* were observed by the agar slant method (Vincent, 1970). The plants were inoculated with 100 ml of broth culture. Uninoculated plants and plants inoculated with strain R7A and S7 were used as controls. Plants were grown under controlled environmental conditions of 70% humidity, 22 to 25ºC during the day, and 14ºC at night on a 16/8 h day/night cycle. After 4 weeks, plants were harvested and detected for the presence

nodules. After 6 weeks, shoot length was measured. No significant difference in shoot weight or shoot length in comparison to uninoculated controls indicated an ineffective (Fix-) symbiosis.

4.3.17 Nodulation test and symbiotic phenotype on *Glycine max*

Inocula of the strains S7, R7A and ICE*Ml*Sym^{R7A} exconjugants were prepared as described above. Seeds of soybean (*Glycine max*) were surface sterilized with 3% hydrogen peroxide and germinated on 0.8% water agar as described (Somasegaran and Hoben, 1994). Symbiosis of *G. max cv.* SJ5 were determined Leonard jars containing vermiculite and inoculated with 1 ml of bacterial inocula, equivalent to 10^8 cells ml^{-1}. Hoagland's solution (Hotter and Scott, 1991) was added to each jar as required. Plants were grown under controlled environmental conditions of 25ºC on a 12/12 h day/night cycle. After 6 weeks, plants were harvested and then analyzed for nitrogenase activity. Nitrogenase activity was estimated on the entire plant by measurement of acetylene reducing activity (ARA) as described (Somasegaran and Hoben, 1994). After ARA assay, nodules were detached from the roots and the number of the nodule was scored. The root, shoot, and nodules dry weight were measured after drying at 70°C for 72 h.

4.3.18 RNA isolation, reverse transcriptase-PCR and 5-prime RACE analysis

M. loti G/RDM cultures were grown to an OD600 of 0.4–0.6 and 8 ml culture was then added to 10 ml of boiling lysis buffer [2% SDS, 30 mM NaAc (pH 5.5), 3 mM EDTA], mixed thoroughly and incubated at 100°C for 3 min. Protein,

genomic DNA and other material were then removed from the aqueous phase by thorough mixing with two 16 ml volumes of acidified phenol (65°C), one 16 ml volume of Tris-equilibrated phenol and one 16 ml volume of chloroform. Nucleic acids were then precipitated with 2 vols of ethanol, after which pellets were washed with 10 ml 70% ethanol and suspended in 1 ml of DEPC-treated H2O containing 400 U of Invitrogen RNaseOUT. Samples were then treated with Ambion TURBO DNase and applied to Qiagen RNeasy columns as per manufacturer's instructions. Samples were treated a second time with DNase and applied to a second RNeasy column that produced DNA-free RNA samples. A Roche 5'/3' RACE second-generation kit was used to map the transcriptional start site of intS as described in manufacturer's instructions. The primers SP1_intS5RACE, SP2_intS5RACE and SP3_intS5RACE correspond to specific primers SP1, SP2 and SP3, respectively, as referred to in the kit instructions. A single PCR product was obtained in both the first and second rounds of PCR amplification and so this PCR product was sequenced directly without cloning, using primer SP3_intS5RACE.

4.3.19 Construction of *intS* Promoter-LacZ fusion plasmids

A variable fragments of the *M. loti attP-intS* (Figure 4.2 and Table 4.1) was generated with different primers indicated in Figure 4.9A with *Bam*HI site. Each PCR products was cloned into high copy number plasmid pBluescript II SK(+). These fragments were subcloned into pJX600 resulting of the transcriptional fusions of the *attP-intS*. Each transcriptional fusions were transferred to *M. loti* R7ANS and β-galactosidase assay was carried out as described above.

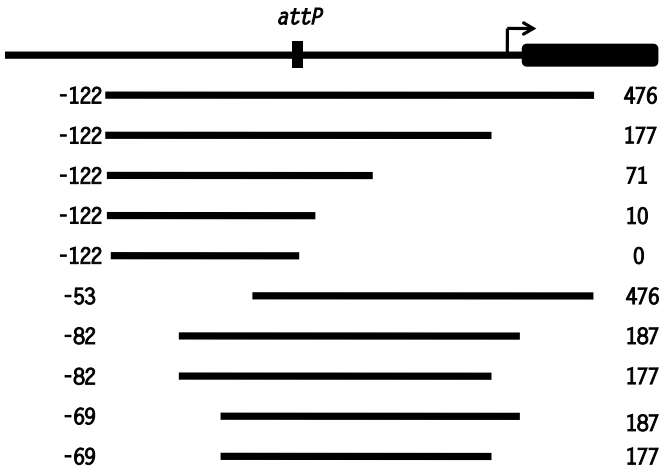

Figure 4.2 *M. loti attP–intS* region used to constructed transcriptional fusion with *lacZ*. The first nucleotide of the 17-bp core sequence (*attP*) is the 1st position.

4.3.20 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the SPSS version 11.5. Data transformation was carried out where necessary before Duncan's multiple range test was used to identify differences between means at $p \leq 0.05$.

4.4 Results

4.4.1 Determination of integration sites of *M. loti intS* in the chromosome of *Bradyrhizobiaceae* strains

pJZ608 was introduced into the *Bradyrhizobiaceae* strains by conjugative transfer from *E. coli* strain S17-1. Since this plasmid was unable to replicate in *Bradyrhizobium* and *Rhs. palustris* (Alexeyev, 1999), thus the tetracycline resistant transconjugants were assumed to arise as a result of *intS*-mediated integration in the chromosome. In order to determine the integration site, pJZ608 was rescued from the transconjugants. A number of exconjugants of each bacterial host were selected for the analysis (Table 4.3) and their genomic DNA was purified from each cultured clone. After digestion of each genomic DNA with *SacI*, an enzyme that does not cleave within pJJ608, the fragments were circularized by self ligation. Each ligation reaction was then independently introduced into *E. coli* and the transformants (Tet^R) were selected to rescue plasmids bearing *attL* and *attR*. To examine whether there are different integration sites in chromosome of the *Bradyrhizobiaceae* exconjugants, at least four plasmids from each of the independent *E. coli* transformations were prepared for sequencing of the integration site. The plasmids were predicted to contain the plasmid pJJ608 and two regions of bacterial host chromosomal DNA extending from the left and right flanking junctions of the integration site. Sequences of *attL* and *attR* were determined by sequencing of left and right integrative junctions. The *attL* and *attR* resulting sequences of each bacterial host were used to design primer pairs for PCR and sequencing of *attB* from their chromosomal DNA. Table 4.3 shows nucleotide sequences of *attB* and of *attL* and *attR*, obtained from the

plasmid chromosome junctions of pJZ608 integrations in different *Bradyrhizobiaceae* strains. Using the NCBI BLAST search program, we determined the chromosomal location wherein the *attB* site resides. We found that the nonsymbiotic isolates, *Bradyrhizobium* strains and *Rhs. palustris* have common-gene annotations, around *phe*tRNA. In their chromosome, the *phe*tRNA locates downstream of gene encoding zinc binding protein and upstream of gene encoding transmembrane protein DUF6.

Table 4.3 Nucleotide sequences of *attB* and of *attL* and *attR*, obtained from plasmid chromosome junctions of pJZ608 integrations.

Strains	***attL***	***attR***	***attB***	***attP*** **(ICEMlSym^{R7A})**	**No. of exconjugant**
B. japonicum USDA110	TCCGCC**C**CTGGGCACCA	TCCGCC**T**CTGGGCACCA	TCCGCC**C**CTGGGCACCA	TCCGCCTCTGGGCACCA	10/12
	TCCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA			2/12
S7	TCCGCC**C**CTGGGCACCA	TCCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA		4/12
	TCCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA			8/12
S9	TCCGCC**C**CTGGGCACCA	TCCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA		4/8
	TCCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA			4/8
NS1	**C**CCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA	CCCGCC**C**CTGGGCACCA		4/4
NS23	**C**CCGCCTCTGGGCACCA	TCCGCCTCTGGGCACCA	CCCGCC**C**CTGGGCACCA		3/6
	CCCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA			3/6
NS28	**C**CCGCCTCTGGGCACCA	TCCGCC**C**CTGGGCACCA	CCCGCC**C**CTGGGCACCA		6/10
	CCCGCCCCTGGGCACCA	TCCGCCTCTGGGCACCA			4/10
Rhs. palustris DSM 123^{T}	**C**CCGCCCCTGGGCACCA	TCCGCCTCTGGGCACCA	CCCGCC**C**CTGGGCACCA		4/4

Bold underlined letters refer the differences between *attP*, *attB*, *attL* and *attR*. The sequences of *attL* and *attR* were determined in independent exconjugants.

Sequence alignment of the attachment sites (*attB*) for *intS* integration in *M. loti*, the *Bradyrhizobiaceae* host strains in this study and the closely related species is shown in Figure 4.3. The alignment result shows that *Bradyrhizobium* species (*B. japonicum*, *B. yuanmingense* S7, and *Bradyrhizobium* sp. S9) had common17-bp core sequence, which was only 1 nucleotide different to that of *M. loti*. In addition, *attB* of the nonsymbiotic isolates and *Rhs. palustris* were identical and were only 1 nucleotide different to that of *M. loti*. Therefore, after *intS* integration into their chromosome, variant *attL* and *attR* sites were formed. These *attL* and *attR* results allow an estimation of the sites where strand exchange occurs during integration by comparing of *attP*, *attB*, *attL* and *attR* sequences. We proposed the putative staggered cleaving sites of the ICE*Ml*Sym^{R7A} *intS* in Figure 4.4. Cleaving by *intS* at the proposed sites, alternative *attL* and *attR* would be formed as shown in Table 4.3 in all bacterial host studied. However, the proposed cleaving site does not fit to the resulting *attL* and *attR* of the nonsymbiotic isolates NS23. It is possible that the point mutation occurred during the recombination event of the core sequence.

M. loti	GCCCAGATAGCTCAGTTGGTAGAGCAGCGGACTGAAAATCCGCGTGTCGGTGGTTCGAATCCGCCTCTGGGCACCA
B. japonicum	GCCCAGGTAGCTCAGTTGGTAGAGCA**T**GCGACTGAAAATCGC**A**GTGTCGGTGGTTCGA**T**TCCGCC**C**CTGGGCACCA
***Bradyrhizobium* sp. BTAi1**	GCCCAGGTAGCTCAGTTGGTAGAGCA**T**GCGACTGAAAATCGC**A**GTGTCGGTGGTTCGA**T**TCCGCC**C**CTGGGCACCA
***Bradyrhizobium* sp. ORS278**	GCCCAGGTAGCTCAGTTGGTAGAGCA**T**GCGACTGAAAATCGC**A**GTGTCGGTGGTTCGA**T**TCCGCC**C**CTGGGCACCA
Rhs. palustris	GCCCAGGTAGCTCAGTTGGTAGAGCA**T**GCGACTGAAAATCGC**A**GTGTCGGTGGTTCGA**TC**CCGCC**C**CTGGGCACCA
N. winogradskti	GCCCAGGTAGCTCAGTTGGTAGAGCA**T**GCGACTGAAAATCGC**A**GTGTCGGTGGTTCGA**TC**CCGCC**C**CTGGGCACCA
S. meliloti	GCCC**G**GATAGCTCAGTTGGTAGAGCA**G**CGGA**T**TGAAAATCCGCGTGTCGGTGGTTC**A**AATCCGCCTC**C**GGGCACCA
***Rhizobium sp.* NGR234**	GCCC**G**GATAGCTCAGTTGGTAGAGCA**G**CGGA**T**TGAAAATCCGCGTGTCGGTGGTTC**A**AATCCGCCTC**C**GGGCACCA
R. etli	GCCCAG**G**TAGCTCAGTTGGTAGAGCA**G**CGGA**T**TGAAAATCCGCGTGTCGGTGGTTCGA**T**TCCGCC**C**CTGGGCACCA

Figure 4.3 Alignment of *phe*tRNA sequences of different species. The 17 nucleotides of the 3' end of the *phe*tRNA are marked in grey. Nucleotides which differ from that of *M. loti* are indicated by underscore boldfaces.

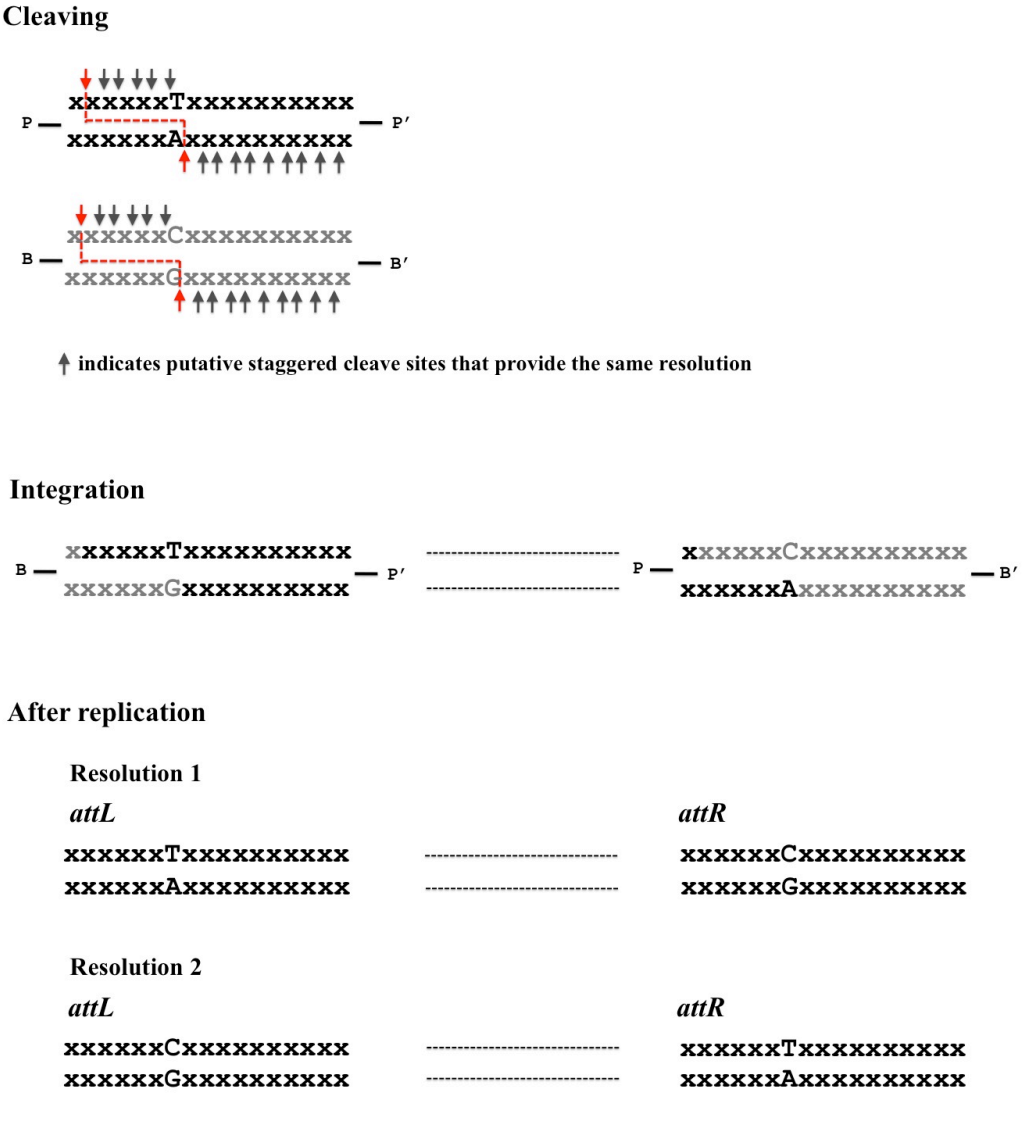

Figure 4.4 Putative staggered cleave sites for *intS* integration at *attP* of integrative vector pJZ608 and *attB* of *B. japonicum* strains USDA110, S7 and S9, *Rhs. palustris* and the nonsymbiotic *Bradyrhizobiaceae* isolates NS1 and NS28. Underlined letters show the differences between *attP*, *attB*, *attL* and *attR*. The arrows indicate the putative staggered cleave sites that provide the same resolution of *attL* and *attR*.

4.4.2 Expression of *intS* promoter

To determine expression of *intS* in *Bradyrhizobiaceae* strains, pJZX comprising potential *intS* promoter region-*lacZ* fusion were introduced to these bacteria. The levels of β-galactosidase expression in different *Bradyrhizobiaceae* species are presented in Figure 4.5. We found that the expression of *intS* increased during exponential growth in all tested strains. The highest level of expression was detected in nonsymbiotic *M. loti* derivative strain FJX1. Interestingly, levels of β-galactosidase were detected in all *Bradyrhizobiaceae* strains tested in this study. This indicates that *intS* promoter appears to be widespread among *Bradyrhizobiaceae* species, even though their levels of expression were significantly lower than that of R7ANS (Figure 4.5). Among the *Bradyrhizobiaceae* species tested, the β-galactosidase expression was highest in photosynthetic bacteria *Rhs. palustris*; intermediate in symbiotic *Bradyrhizobium* strains S7, S9 and USDA110; and lowest in nonsymbiotic isolates NS28 and NS2. These results suggest a bacterial host specificity of the *intS*.

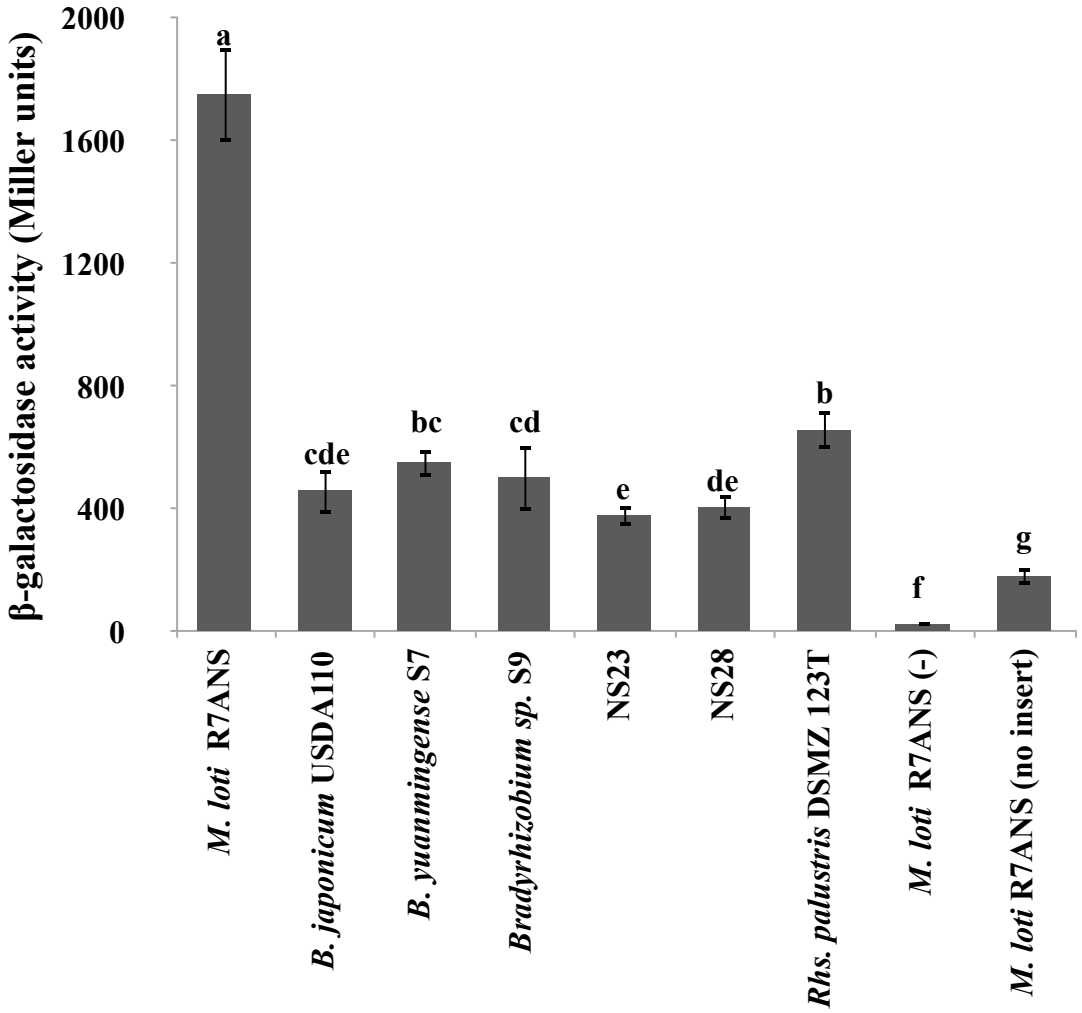

Figure 4.5 Expression of ICEMlSym^{R7A} *intS* promoter region in *M. loti* R7ANS and various *Bradyrhizobiaceae* strains at stationary phase. Activity values were the average of five assays ± SD. *M. loti* R7A carrying pFX, which contains *intS* promoter region in reverse, were used as a negative control. Each bar represents a mean (±SD) of five replicates. Different letters above the bars indicate that means were significantly different, as determined by an Duncan's Multiple Range Test (P = 0.05).

4.4.3 Transfer of *M. loti* symbiosis island (ICE*Ml*Sym^{R7A}) to *B. yuanmingense*

From the previous experiment, we found that pJZ608 could mediate site-specific integration in various *Bradyrhizobiaceae* strains including symbiotic strains (USDA110, S7 and S9), nonsymbiotic strains (NS1, NS23, and NS28) and photosynthetic *Rhs. palustris* strain. To examine whether ICE*Ml*Sym^{R7A} could integrate into chromosome of these strains, ICE*Ml*Sym^{R7A} was transferred by conjugation to the different strains using *M. loti* strain R7AZ strain as a donor. The donor R7AZ harbors pFJR206 containing *traR* gene that regulate plasmid conjugal transfer in response to population density. The presence of pFJR206 resulted in excision of ICE*Ml*Sym^{R7A} in all cells regardless of culture density (Ramsay et al., 2006). Medium containing antibiotics (neomycin and gentamycin) and bromothymol blue was used to select exconjugants that had received ICE*Ml*Sym^{R7A}. Three independent experiments were performed for each mating system. The occurrence of neomycin-resistant colonies indicates recipients which had acquired the symbiosis island from the donor. We found that R7AZ transferred the ICE*Ml*Sym^{R7A} to the isolated *B. yuanmingense* strain S7 at an average frequency of 8.8×10^{-5} (ranged from 1.2×10^{-5} to 2.5×10^{-4}) conjugants per donor. The frequency of the transfer to the strain S7 was lower than that transfer to R7ANS control that was between 8×10^{-5} to 3×10^{-4} conjugants per donor (Ramsay et al., 2006). However, transfer of the ICE*Ml*Sym^{R7A} in other recipient strains was not detected. For each mating experiment, 15 exconjugant colonies were selected and screened by PCR the presence of *nptII* gene. PCR product of the *nptII* was detected in all of exconjugants indicating that the ICE*Ml*Sym^{R7A} integrated into their chromosomes.

Four transconjugants obtained from two independent mating experiments were selected as representatives for analysis of integration site. S7Sym1 and S7Sym2 obtained from the same mating experiment were termed as type 1 transconjugant and S7Sym3 and S7Sym4 obtained from another mating experiment were termed as type 2 transconjugant. Left and right ICE*Ml*Sym^{R7A} integrative junctions were analyzed by PCR and sequencing from primer pairs *intS_R* and Upst*attL*_Sym and 122Upst*attP* and 220DstattR_S7, respectively. From PCR and sequencing, we obtained *attL* with upstream sequence and *attR* with downstream sequence. The obtained sequences confirmed that ICE*Ml*Sym^{R7A} integrated at *phe*tRNA gene as previously observed with the integrative plasmid pJZ608. Staggered cleaving would occur at the same site proposed in Figure 4.4. In addition, we examined the presence of *attB* by PCR, using a primer pair Upst*attL*_Sym and 220Dst*attR*_S7. PCR analysis showed that *attB* was present in the S7 wild type, while it was not detected in the four S7 derivatives (Table 4.4). In addition, sequences of *attL* and *attR* of four transconjugants were similar to that obtained from pJJ608 integration in the strain S7 (Table 4.3). Taken together, these data demonstrated the integration of symbiosis island at the *phe*tRNA gene of *B. yuanmingense* S7.

Four ICE*Ml*Sym^{R7A} transconjugants (S7Sym1, S7Sym2, S7Sym3 and S7Sym4) were examined for ICE*Ml*Sym^{R7A}-specific loci *msi031*, *msi042*, *msi158*, *msi170*, *traR*, *msi195*, *kbl*, *msi280*, *nifB*, *nolK*, and *nodZ*. PCR amplification of these loci were carried out in the transconjugants compared to the positive control *M. loti* donor. The PCR products obtained were confirmed by sequencing. The presences of ICE*Ml*Sym^{R7A}-specific loci in the transconjugants were summarized in Table 4.4.

From PCR amplification and sequencing, five ICE*Ml*Sym^{R7A}-specific loci including *kbl, msi280, nifB (msi347), nolK (msi353),* and *nodZ* (*msi355*) were detected in all transconjugants. The detected genes were located at 346- to 427- kb positions of the symbiosis island. In addition, a part of DNA sequence at the end of the island was also detected from the sequencing of right junction of the integration. These results implied that the sequence from 346- to 502-kb position of the symbiosis island was maintained in the transconjugants after transfer and recombination. No PCR product of *nodB (msi122), nolO (msi125), nodJ (msi126), nodI (msi127), nodC (msi128), nodA (msi129), nods (msi131), msi158, msi170, traR (msi347)* and *msi195* was detected in any of transconjugants. This indicated these genes and probably other genes around 143-kb to 234-kb position of the symbiosis island were missing after transfer. The region that makes the different between the type 1 and type 2 transconjugant is located around 32-kb to 143-kb position of the symbiosis island. This region includes *msi031, nodM (msi038), msi042, msi063, nodD1 (msi115), nolL (msi116)* and *nodD2* (*msi118*), which were detected in type 2 transconjugant (S7Sym3 and S7sym4) but not in the type 1 transconjugant (S7Sym1 and S7sym2).

Table 4.4 Presence of ICEMlSym^{R7A} genes obtained from *M. loti* R7AZ donor and *B. yuanmingense* strain S7 transconjugants based on PCR and sequencing.

ICEMlSym^{R7A} genes	Bacterial strains					
	R7A	**S7Sym1**	**S7Sym2**	**S7Sym3**	**S7Sym4**	**S7**
attP	+	-	-	-	-	-
attB	+	-	-	-	-	+
attL	+	+	+	+	+	-
attR	+	+	+	+	+	-
msi031	+	-	-	+	+	-
nodM	+	-	-	+	+	-
msi042	+	-	-	+	+	-
msi063	+	-	-	+	+	-
nodD1	+	-	-	+	+	-
nolL	+	-	-	+	+	-
nodD2	+	-	-	+	+	-
nodB	+	-	-	-	-	-
nolO	+	-	-	-	-	-
nodJ	+	-	-	-	-	-
nodI	+	-	-	-	-	-
nodC	+	-	-	-	-	-
nodA	+	-	-	-	-	-
nodS	+	-	-	-	-	-
msi158	+	-	-	-	-	-
msi170	+	-	-	-	-	-
traR	+	-	-	-	-	-
msi195	+	-	-	-	-	-
kbl	+	+	+	+	+	-
msi280	+	+	+	+	+	-
nptII	+	+	+	+	+	-
nifB	+	+	+	+	+	-
nolK	+	+	+	+	+	-
nodZ	+	+	+	+	+	-

+; positive for PCR and sequencing, _; negative for PCR and sequencing

4.4.4 Effect of symbiosis island transfer on symbiotic characteristics

ICE*Ml*Sym^{R7A} transconjugants including S7Sym1, S7Sym2, S7Sym3, and S7Sym4 were evaluated for their symbiotic performances on *Lotus* and *Glycine max*. The nodulation ability was tested on *L. japonicus*, *L. corniculatus* and *G. max*. All of ICE*Ml*Sym^{R7A} transconjugants formed root nodule on neither *L. japonicus* nor *L. corniculatus*, whereas they formed root nodule on *G. max*. On *G. max*, S7Sym3 and S7Sym4 (type 2 transconjugants) produced normally nodule number when compared with the wild type stain S7and *B. japonicum* USDA110 (Figure 4.6). In contrast, S7Sym1 and S7Sym2 (type 1 transconjugants) produced significantly lower nodule number when compared with the wild type *B. yuanmingense* S7 and *B. japonicum* USDA110 (Figure 4.6).

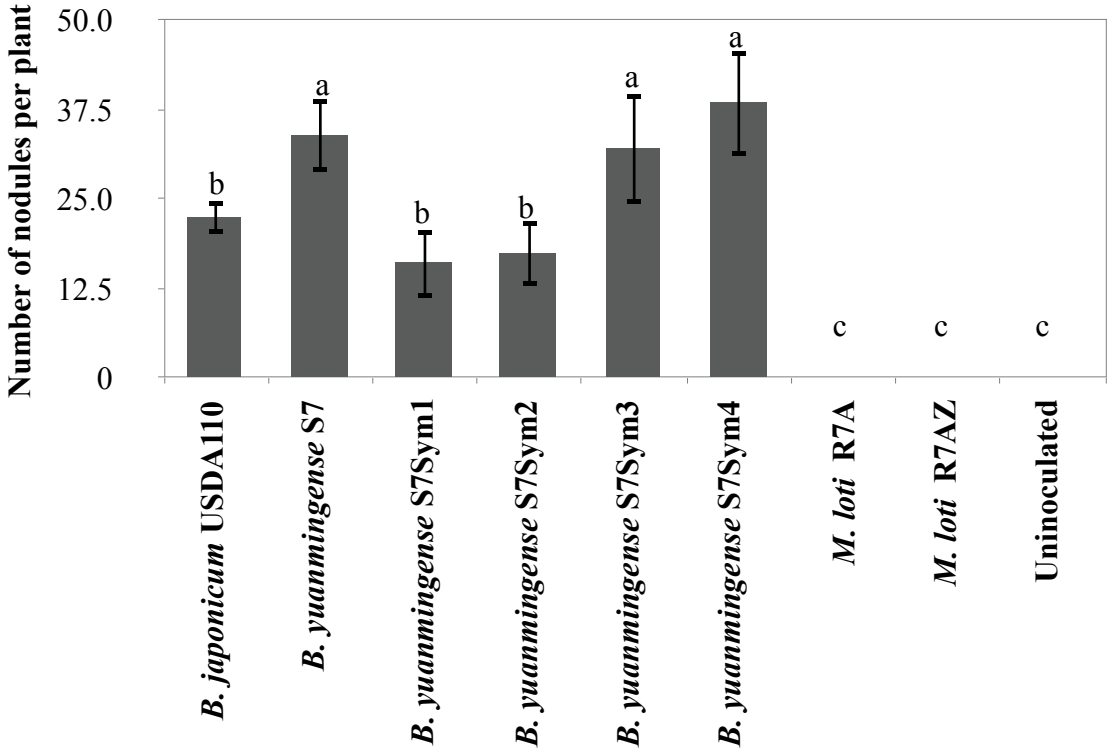

Figure 4.6 Root nodule number of 45-days old soybean inoculated with *B. japonicum* USDA110; *B. yuanmingense* S7; ICE*Ml*Sym^{R7A} transconjugants strain S7Sym1, S7Sym2, S7Sym3, and S7Sym4; *M. loti* R7A and *M. loti* R7AZ. Each bar represents a mean (±SD) of four replicates. Different letters above the bars indicate that means were significantly different, as determined by an an Duncan's Multiple Range Test (P = 0.05).

The bacterial nitrogen fixing performance on soybean was evaluated by measurement of plant dry weight, analysis of nitrogenase activity via acetylene reduction analysis, and observation of nodule characteristics. Although S7Sym3 and S7Sym4 produced high number of nodules, these nodules were pale green indicating no apparent sign of leghaemoglobin (Figure 4.7). As a result, plants inoculated with S7Sym3 and S7Sym4 failed to reduce acetylene as compared with the wild-type S7 (Figure 4.8). Plants inoculated with S7Sym3 and S7Sym4 showed nitrogen deficient symptoms including poor plant growth, pale green, and yellow leaves (Figure 4.7). In addition, means of plant dry weight were lower than that of plants inoculated with the wild-type S7 and were not significantly different to that of uninoculated plants (Figure 4.9). Taken together, these results indicated that the strain S7Sym3 and S7Sym4 absolutely lost the nitrogen fixing ability.

Although, the strain S7Sym1 and S7Sym2 produced lower number of root nodule than the wild-type S7 and the transconjugants S7Sym3 and S7Sym4, nodules produced by S7Sym1 and S7Sym2 had the nitrogen fixing ability. After cutting nodules transversely, nodules collected from plants inoculated with S7Sym1 and S7Sym2 showed softer pink than as compared with wild-type produced nodules, but remaining significantly higher than those collected from plants inoculated with S7Sym3 and S7Sym4 (Figure 4.7). The color indicated the presence of leghaemoglobin throughout the nodule. The plants inoculated with S7Sym1 and S7Sym2 showed significantly lower levels of acetylene reduction activity than plants inoculated with the wild-type S7. The S7Sym1 and S7Sym2 showed an average of 68% and 59% of the activity of the wild-type S7, respectively (Figure 4.8). In addition, we found

decreases (about 18% and 16%) in shoot dry weight of plant inoculated with S7Sym1 and S7Sym2 (Figure 4.9). These results indicated that S7Sym1 and S7Sym2 transconjugants contained incomplete nitrogen fixing ability and the transfer of symbiosis island affected their nodulation ability.

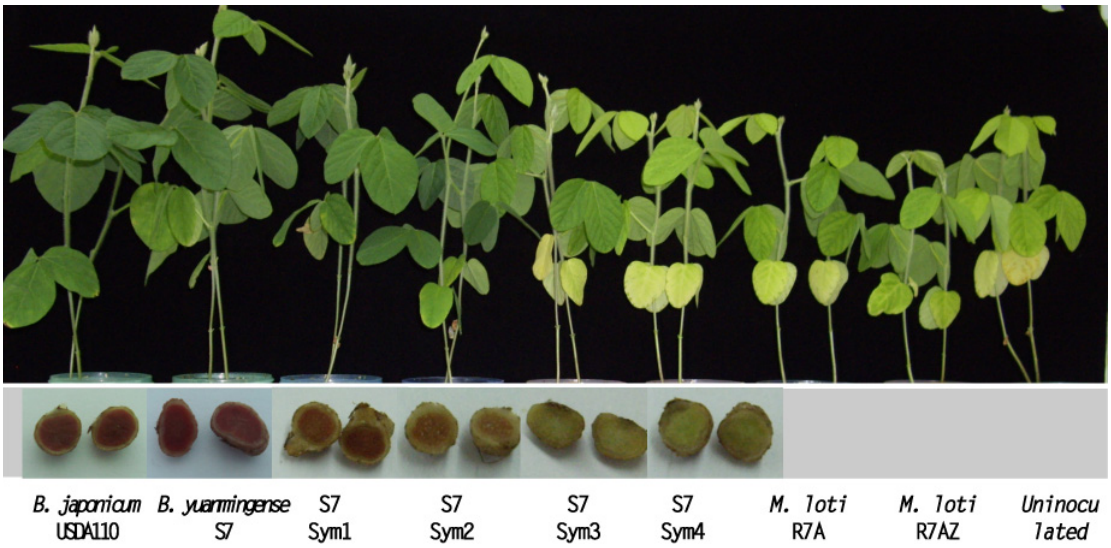

Figure 4.7 Forty five days old soybean post inoculated with *B. japonicum* USDA110; *B. yuanmingense* S7; ICE*Ml*Sym^{R7A} transconjugants strain S7Sym1, S7Sym2, S7Sym3, and S7Sym4; *M. loti* R7A and *M. loti* R7AZ. Half section of nodules harvested from the plant nodulated by each inoculated strain were shown below.

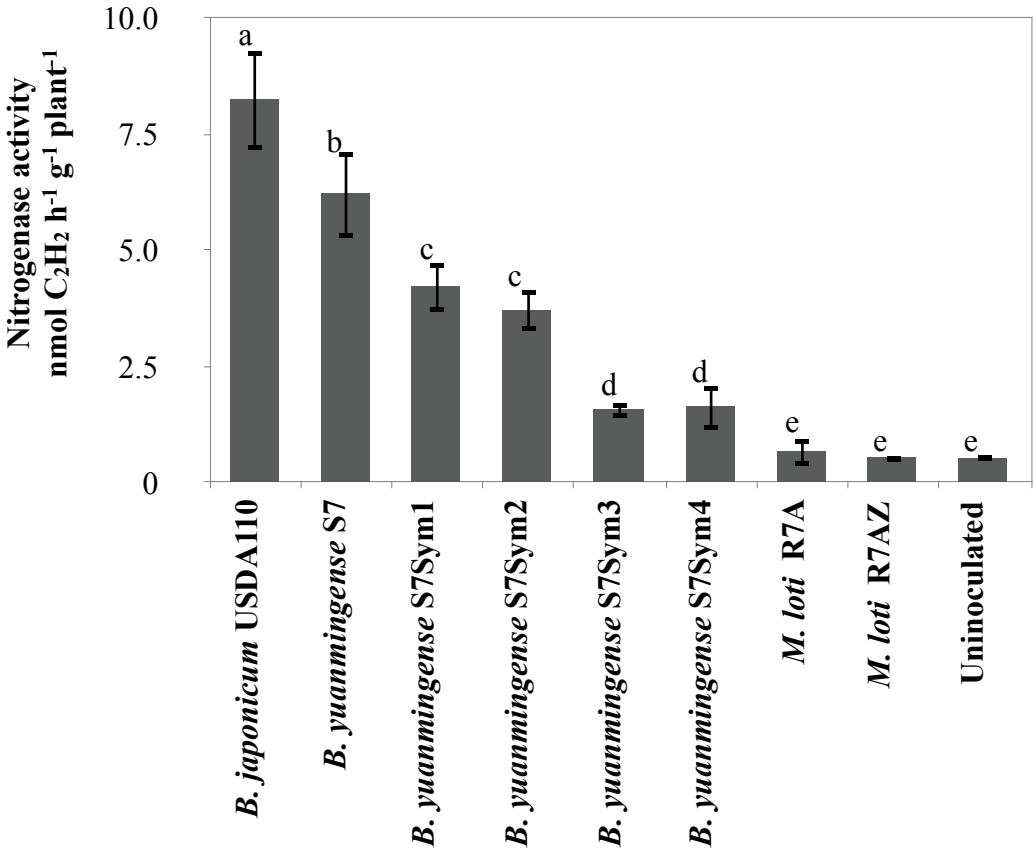

Figure 4.8 Nitrogenase activity (nmol C_2H_2 h^{-1} g^{-1} $plant^{-1}$) of the 45-days soybean inoculated with *B. japonicum* USDA110; *B. yuanmingense* S7; ICE*Ml*Sym^{R7A} transconjugants strain S7Sym1, S7Sym2, S7Sym3, and S7Sym4; *M. loti* R7A and *M. loti* R7AZ. Each bar represents a mean (±SD) of four replicates. Different letters above the bars indicate that means were significantly different, as determined by an Duncan's Multiple Range Test (P = 0.05).

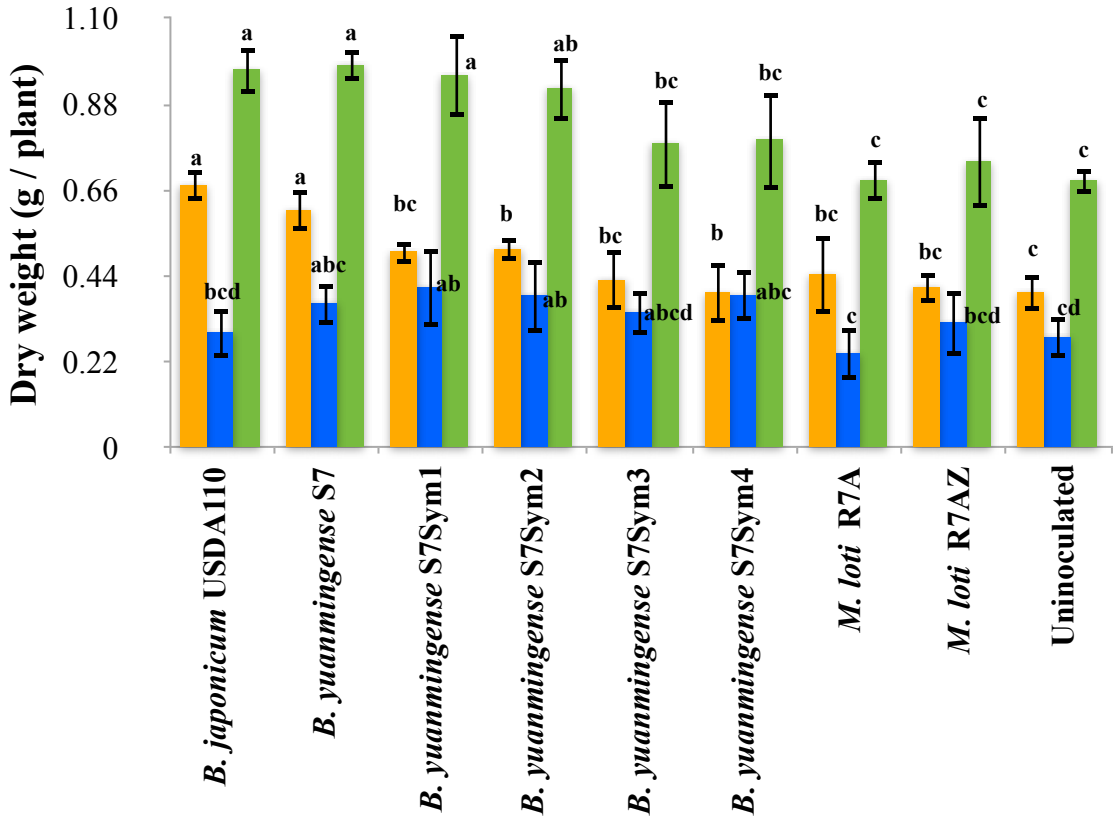

Figure 4.9 Dry weight of 45-days soybean inoculated with *B. japonicum* USDA110; *B. yuanmingense* S7; ICE*Ml*Sym^{R7A} transconjugants strain S7Sym1, S7Sym2, S7Sym3, and S7Sym4; *M. loti* R7A and *M. loti* R7AZ. Each bar represents a mean (±SD) of four replicates. Different letters above the bars indicate that means were significantly different, as determined by an Duncan's Multiple Range Test (P = 0.05). , root; , shoot; , whole plant.

4.4.5 *intS* promoter and localization of region essential for the *intS* promoter

To map transcription start site on the *intS*, the cDNA was used in 5‘RACE experiment using a primer complementary to *intS*. Sequencing of the PCR product reveal that the transcription start site was located at 16 nt upstream the first ATG of the *intS* (Figure 4.10).

To determine which region is essential for expression of integrase promoter the transcriptional fusions of variable upstream *intS* regions were were introduced to these bacteria. The levels of β-galactosidase expression of the different missing region of the *attP*-i*ntS* in *M. loti* R7ANS are presented in Figure 4.11. Missing to all analyzed regions caused the lower expression indicating that these regions are essential for the integrase transcription. The binding region for integrase might be huge. In addition, it is possible the the *M. loti intS* is an arm-type. Therefore, the *intS* needs the arm binding domain sites that occur outside the region of strand exchange (*attP*) (Han et al., 1993).

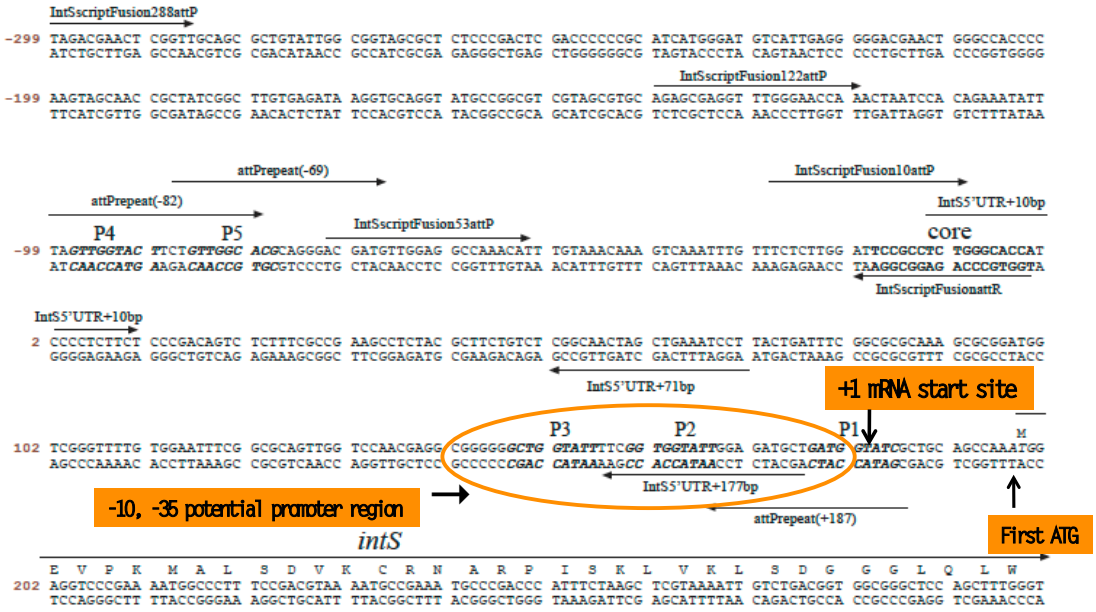

Figure 4.10 *M. loti attP–intS* region. (A) Nucleotide sequence of the P4 *attP–intS* region is from Rhizobase database. The 5' end of intS mRNA, -10 and -35 consensus sequences and potential ATG start codons are indicated. Sequences of primers used to constructed transcriptional fusions are indicated above the arrows.

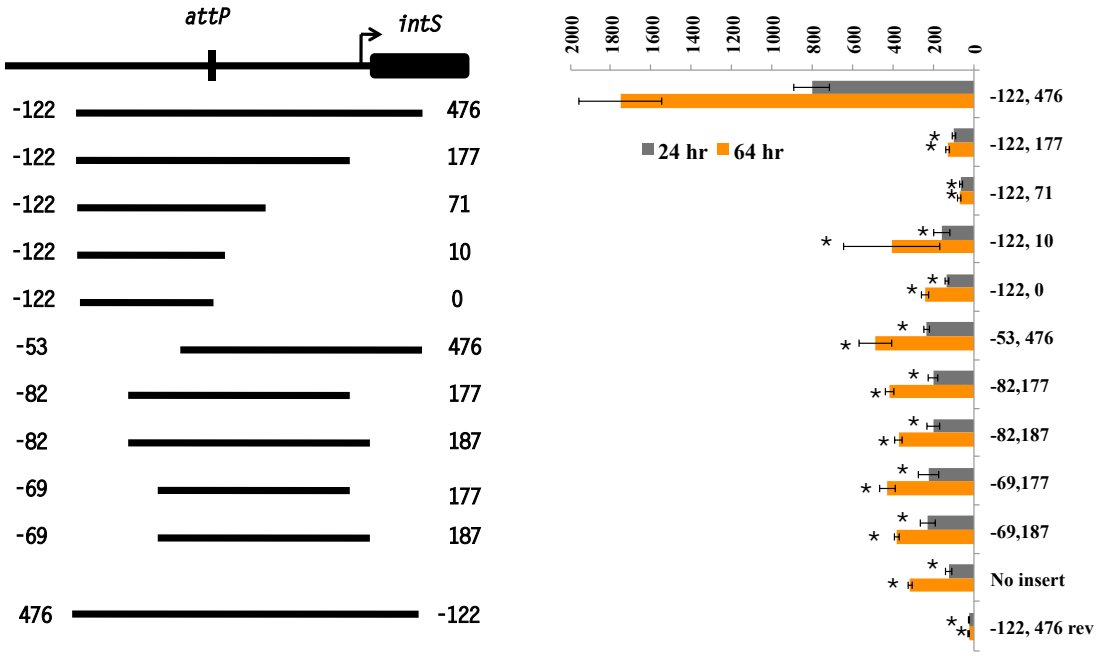

Figure 4.11 Expression of *attP-intS* promoter regions in *M. loti* R7ANS at 24 h and at 64 h. Activity values are the average of 5 assays ± SD. *M. loti* R7A carrying pFX, which contains *intS* promoter (-122,476) region in reverse, were used as a negative control. Each bar represents a mean (±SD) of five replicates. Asterisks above the bars indicate that means were highly significantly different from the expression value of the -122,476 region ($P < 0.01$), as determined by an Duncan's Multiple Range Test.

4.5 Discussion

We have shown that site-specific recombination system of *M. loti* integrase (*intS*) can function in several genomic species of *Bradyrhizobiaceae* other than *M. loti*, the native host of the *intS*. It has been reported previously (Sullivan et al., 2002) that 502-kb element of *M. loti* symbiosis island integrates into the end of a *phe*tRNA gene in the chromosome of *M. loti* strains. The entire *phe*tRNA gene is reconstructed at the left end of the element upon integration, whereas the 3' 17 nucleotides of the tRNA gene are present as a direct repeat at the right end. Integrative recombination takes place within a 17 nt-long sequence, called the core, identical in both donor and host DNAs. In similar fashion, *intS* integrated into the *phe*tRNA gene in *Bradyrhizobiaceae* species with the 17-bp 3' portion of the *phe*tRNA comprising the island attachment site.

In this study, a plasmid pJZ608 derived from *M. loti* R7A integrative *attP-intS* element was constructed. We found that this plasmid has a wide host range, since pJZ608 integration has been demonstrated in *Mesorhizobium* strains (Ramsay et al., 2006) and also in distantly related species such as *B. japonicum* USDA110, *B. yuanmingense* strain S7, *Bradyrhizobium sp*. strain S9, *Rhs. palustris* DSMZ123^T, and nonsymbiotic *Bradyrhizobiaceae* strains (NS1, NS23 and NS28) (Table 4.3). Since the target sequence for integration is the phetRNA gene, bacterial species belonging to the family *Mesorhizobiaceae* and *Bradyrhizobiaceae* have the target sequence. In addition, the high score of homology to the target sequence was also obtained from a putative tRNA gene of several other bacteria. It is possible that these bacteria harboring the cognate tRNA site could be targeted by the *intS*. Integration host factor

(IHF) is another important key for the integrative recombination. The integration of pJZ608 into the chromosome of the provided host-bacteria, in this study, suggests the existence of a putative related host factor(s) required for the integration. IHF might be conserved among these bacteria. Many other genomes of related bacteria which harbor appropriate integration target site along with the cognate integration host factor (s) could be targeted by the *intS* integrative system. Therefore, the data are useful for construction of a minimum chromosome-integrative vehicle for *Mesorhizobium*, *Bradyrhizobium*, *Rhs. palustris*, and possibly for other related bacteria.

Comparison of R7A *attP* sites and *attB* sites of these *Bradyrhizobiaceae* host strains showed that 1 nt of the core was different in the cases of the strains USDA110, S7 and S9; and 2 nt of the core were different in the cases of *Rhs. palustris* and nonsymbiotic strains NS1, NS23, and NS28. The data indicated that *M. loti* integrase was flexible and able to catalyze recombination between *attP* and *attB* with mismatches within the core sequence. In addition, the *intS* used a conserved tRNA gene as the site of integration and the recombination between *attP* and *attB* core regions did not need perfect homology, suggesting that the *intS* has a broad host range. It has been reported that integrase adopts specific conformations when bound to *attP* and *attB* sites that enable the formation of a synapse (Ghosh et al., 2005; Smith et al., 2004). Recombination defective *attB* mutants that could still bind to integrase with affinities not dissimilar to the wild-type *attB* site were found to be blocked either at synapsis or at DNA cleavage. Mutations at positions distal to the crossover site inhibit recombination by destabilizing the synapse with *attP* without significantly affecting DNA-binding affinity (Gupta et al., 2007). Therefore, the imperfect

homology of *Bradyrhizobiaceae-attB* to the *attP* could disrupt the coordination of strand exchanges so that the yield of recombinant products is drastically decreased.

Integration of symbiosis island was examined in all strains that the integrative vector pJZ608 could mediate site-specific integration. We have demonstrated that symbiosis island integrated into chromosome of *B. yuanmingense* strain S7. However, integration of the symbiosis island into the other strains were not detected. This might be because low affinity or specificity of *intS* to target site of these strains that caused the low expression of *intS* (Figure 4.5) in *Bradyrhizobiaceae* strains, as well as caused the lower integration frequency in *B. yuanmingense* strain S7 compared to in *M. loti*. After integrative recombination, missing of ICE*Ml*Sym^{R7A} genes were detected (Table 4.4) in all four ICE*Ml*Sym^{R7A} transconjugants. Possibly, sets of missing genes were deleted after transfer and integrative recombination. These genes include ICE*Ml*Sym^{R7A} specific genes and also nodulation genes. These losses could be related to genomic rearrangements that reduced the size of chromosomes. Bacterial evolution toward endosymbiosis with eukaryotic cells is associated with extensive bacterial genome reduction and loss of metabolic and regulatory capabilities (Nilsson et al., 2005). The simplest possibility would be that reduced genomes converge on a set of universal genes that underlie the core processes of cellular growth and replication, with also containing some loci corresponding to that host relationship (Moran, 2002). Since bacterial genomes are not growing ever larger in size, the acquisition of DNA must be counterbalanced by the DNA loss, after ICE*Ml*Sym^{R7A} integration. Acquired genes that provide functions allowing for niche expansion may be maintained, while genes providing smaller overall selective benefits may be lost (Lawrence, 1999).

Similar to pathogenicity islands, the symbiosis island are integrated into chromosome of recipient bacteria and convert them to be able to form close associations with their eukaryotic hosts (Hacker and Kaper, 2000; Sullivan and Ronson, 1998). The symbiosis island of *M. loti* have ability to transfer to nonsymbiotic mesorhizobia and convert them to symbionts (Ramsay et al., 2006; Sullivan and Ronson, 1998). The symbiosis island provides genes required for nodule formation and symbiotic nitrogen fixation, as well as other genes that contribute specifically to the success of the plant-microbe interaction (Sullivan et al., 2002). In this study, we found that the symbiosis island transferred to soybean-symbiotic *B. yuanmingense* strain S7 via site-specific integration ICE*Ml*Sym^{R7A} in similar fashion to nonsymbiotic *Mesorhizobium* strains. We have shown the counter-effect of symbiosis island transfer on symbiotic abilities of *B. yuanmingense*. We found that the ICE*Ml*Sym^{R7A} transconjugants could not form root nodule on *Lotus* plant and interestingly, they lost their symbiotic abilities on their own soybean host plant. Transfer of the symbiosis island could not convert *B. yuanmingense* strain S7 to be able to form symbiotic association with *Lotus* plant, in laboratory experiment, because several ICE*Ml*Sym^{R7A} genes were not maintained in their chromosome after integrative recombination (Table 4.4). The absence of these genes after transfer suggests they are not necessary for their life stage in the laboratory condition. Possibly, the ICE*Ml*Sym^{R7A} genes would be advantage and maintained in environment that the host legume is present.

Another suggestion is the symbiosis island integration is harmful or disadvantage to symbiosis lifestyle of *B. yuanmingense* strain S7. Two different

genotypes of ICE*Ml*Sym^{R7A} transconjugants were obtained from two separate mating experiments. In type1 transconjugant (S7Sym1 and S7Sym2), most of ICE*Ml*Sym^{R7A} genes were thought to be deleted after integration. Therefore, their symbiosis ability on soybean host was maintained with imperfect nitrogen fixing capability and impaired nodulation ability. In type 2 transconjugant (S7Sym3 and S7Sym4), just some parts of ICE*Ml*Sym^{R7A} genes, such as cluster of *nod* operons, were deleted. However, genes located around 32-kb to 143-kb position of the symbiosis island were remaining in S7Sym3 and S7Sym4. These regions included *nodD1* operon; *trb* operon, in which the *msi031* is located; and *vir* operon, which the *msi042* is located upstream. The complete loss of nitrogen fixing ability in S7Sym3 and S7Sym4 possibly caused by maintaining of several parts of ICE*Ml*Sym^{R7A} genes.

In the present of host plant, the competition between indigenous soil bacteria and introduced strains occurs for survival and persistence in the soil. The competition leads to arising of the transfer of symbiotic genes that causes of changing of genetic structures of natural soil populations (Barcellos et al., 2007; Rao and Cooper, 1994; Sullivan et al., 1995). The indigenous nonsymbiotic bacteria evolve into symbionts by acquisition of symbiotic functions (Sullivan et al., 1996). Recently, the transfer of symbiotic genes was also found between symbiotic bacteria (Barcellos et al., 2007). This study showed that an indigenous *B. elkanii* strain S 127 acquired a *nodC* gene from the inoculant *B. japonicum* and *Ensifer fredii* strain CPAC 402 received the whole symbiotic island from the *B. japonicum* inoculant strain. In addition, an extra copy of the original *nifH* gene was maintained in the recipient strain CPAC 402, showing the strategy of the strain to obtain ecological advantages. On the contrary,

our study is the first report of counter-effects of symbiotic-gene transfer between symbiotic bacteria associating with different host plants. The transfer of the symbiosis island did not enhance the symbiotic ability to another host plant and it caused the impaired symbiotic abilities on the original host plant. Therefore, it should be considered if the inoculant strain will be used in field sites that have different genetic structure of soil populations.

4.6 Conclusion

The data suggest that *M. loti* integrase is flexible and has broad host range. The expressions of *intS* were relatively low in members of *Bradyrhizobiaceae*, therefore it may not sufficient to allow the integration of the symbiosis island in many strains. The symbiosis island has been reported to be able to transfer to nonsymbiotic bacteria and convert them to symbionts. In this study, the integration of the symbiosis island into the symbiotic bacteria caused impaired symbiotic abilities of their own host plant. Therefore, the symbiosis island genes were removed after integration. Only acquired genes that provide advantages functions may be maintained. This finding reveals the influence of horizontal transfer of symbiotic genes on the efficiency of inoculated strains introduced in field site, especially if host plants are present. The introduced strain may lose the symbiotic ability in field soil containing the bacteria that have unstable symbiotic properties.

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APPENDICES

APPENDIX A

GROWTH MEDIA AND N-FREE NUTRIENT SOLUTION

A.1 Mineral salts-succinate media

Constituents per liter:

KH_2PO_4	0.33	g	
$MgSO_4.7H_2O$	0.33	g	
NaCl	0.33	g	
NH_4Cl	0.5	g	
$CaCl_2.2H_2O$	0.05	g	
Sodium succinate	1.0	g	
Yeast extract	0.02	g	
Trace salts solution[1]	1.0	ml	(added after autoclaving)
0.02% $FeSO_4.7H_2O$ solution	0.5	ml	(added after autoclaving)

Preparation:

Make stock solutions of trace salts and $FeSO_4.7H_2O$ in distilled water.

Dissolve the ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Check pH and adjust to 6.8 - 7.0.

Autoclave at 121° C for 15 min.

Add trace salts solution and 0.02% $FeSO_4.7H_2O$ solution and mix well.

[1]*Trace salts solution (pH 3-4)*

$ZnSO_4.7H_2O$	10	mg
$MnCl_2.4H_2O$	3	mg
H_3BO_3	30	mg
$CoCl_2.6H_2O$	20	mg
$CuCl_2.2H_2O$	1	mg
$NiCl_2.6H_2O$	2	mg
Na_2MoO_4	3	mg

Dissolve the ingredients in 1 l of distilled water.

Check pH and adjust to 3-4 and filter-sterile and store at 4ºC.

A.2 Yeast-mannitol medium (YM)

Constituents per liter:

Mannitol	10	g
K_2HPO_4	0.5	g
$MgSO_4.7H_2O$	0.2	g
NaCl	0.1	g
Yeast extract	0.5	g

Preparation:

Dissolve the ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Check pH and adjust to 6.8 - 7.0.

Autoclave at 121° C for 15 min.

A.3 Rhizobium defined medium (RDM)

Constituents per liter:

Salts solution[1]	10	ml
BTB[2]	10	ml
NH_4Cl stock[3]	6	ml
Trace elements[4]	1	ml
Vitamins[5]	1	ml
L-Histidine	100	mg
20% glucose	20	ml (added after autoclaving)
Phosphate solution	10	ml (added after autoclaving)

Preparation:

Dissolve the ingredients in 970 ml of distilled water.

Add 15 g of agar if a solid medium is required.

Check pH and adjust to 6.8 - 7.0 and autoclave at 121° C for 15 min.

Add glucose and phosphate solution and mix well.

[1]*Salts solution*

$MgSO_4.7H_2O$	25	g
$CaCl_2.2H_2O$	2	g
FeEDTA	1.5	g
NaCl	20	g

Dissolve the ingredients in 1 l of distilled water.

Autoclave at 121° C for 15 min.

[2]*BTB (2 mg/ml)*

Bromothymol Blue	2.0	g

Dissolve the ingredients in 1 l of distilled water.

Autoclave at 121° C for 15 min.

[3]*NH_4Cl stock*

NH_4Cl	18	g

Dissolve the ingredients in 1 l of distilled water.

Autoclave at 121° C for 15 min.

[4]*Trace elements*

$ZnSO_4.7H_2O$	3	mg
$Na_2 MoO_4. 2H_2O$	40	mg
H_3BO_3 (orthoboric acid)	50	mg
$MnSO_4. H_2O$	40	mg
$CuSO_4. 5H_2O$	4	ml
$CoCl_2. 6H_2O$ (0.2 g/L)	1	ml

Dissolve the ingredients in 200 l of distilled water.

Autoclave at 121° C for 15 min.

[5]*Vitamins*

Thiamine HCl	5.0	mg
Ca Panthothenate	100	mg
Biotin (1 mg/ml in fridge)	1.0	ml

Dissolve the ingredients in 50 ml of distilled water.

Filter sterile and store at 4ºC.

[5] *Phosphate solution*

KH_2PO_4	100	g

K_2HPO_4	100	g

Dissolve the ingredients in 1 l of distilled water.

Check pH and adjust to 6.5 - 7.0.

Autoclave at 121° C for 15 min.

A.4 Tryptone-yeast extract (TY) medium

Constituents per liter:

Tryptone	5.0	g
Yeast extract	3.0	g
$CaCl_2.2H_2O$	0.65	g

Preparation:

Dissolve the ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Check pH and adjust to 6.8 - 7.0.

Autoclave at 121° C for 15 min.

A.5 Luria-Bertani (LB) medium

Constituents per liter:

Tryptone	10	g
Yeast extract	5.0	g
NaCl	5.0	g

Preparation:

Dissolve the ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Check pH and adjust to 6.8 - 7.0.

Autoclave at 121° C for 15 min.

A.6 Van Niel's medium

Constituents per liter:

K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	0.5	g
Yeast extract	1.0	g

Preparation:

Dissolve the ingredients in 1 l of distilled water.

Add 15 g of agar if a solid medium is required.

Check pH and adjust to 7.0 - 7.2.

Autoclave at 121° C for 15 min.

A.7 N-free nutrient solution

Stock Solution	Chemical	g/l
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7. 3H_2O$	6.7
	$MgSO_4. 7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4. H_2O$	0.338
4	H_3BO_3	0.247

$ZnSO_4 . 7H_2O$	0.288
$CuSO_4 . 5H_2O$	0.100
$CoSO_4 . 7H_2O$	0.056
$Na_2MoO_2 . 2H_2O$	0.048

Preparation:

Prepare stock solutions by using warm water to get the ferric citrate into the solution.

Make 10 liters of full-strength plant culture solution as follows.

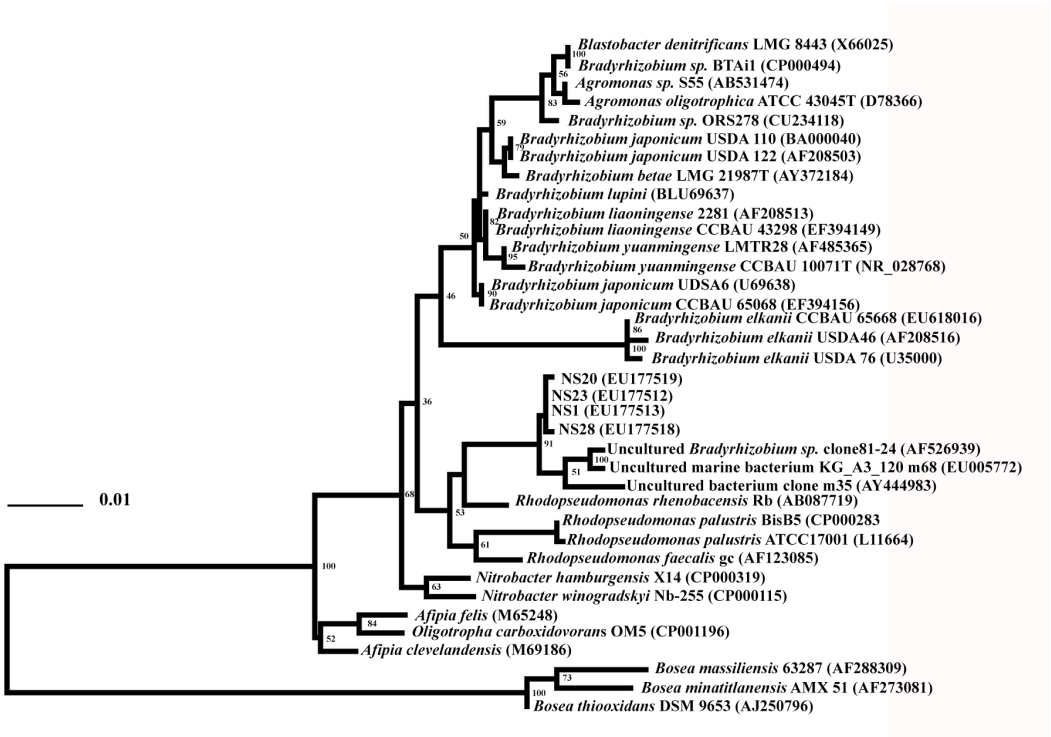
To 5 liters of water, add 5 ml of each stock solution and mix.

Dilute to 10 liters by adding another 5 liters of water.

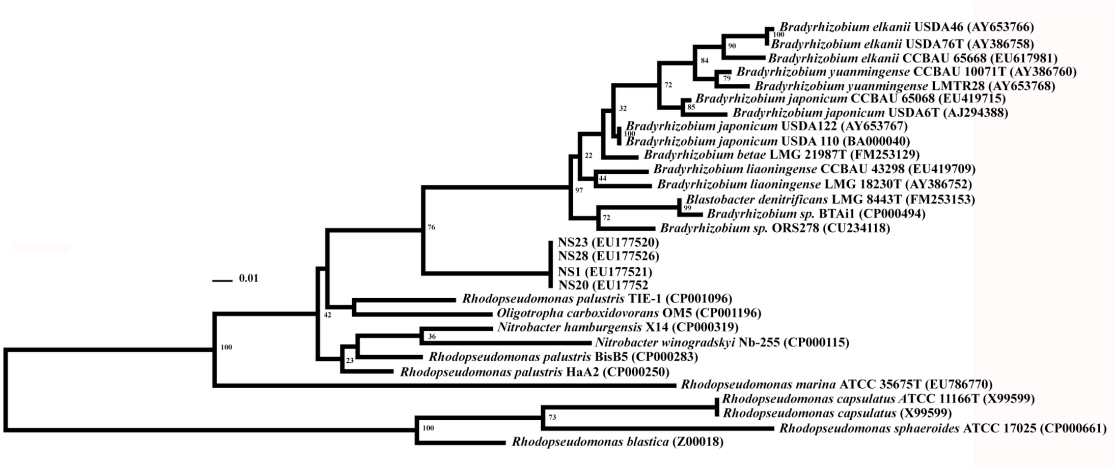
Adjust pH to 6.6-6.8 with 1N NaOH.

APPENDIX B

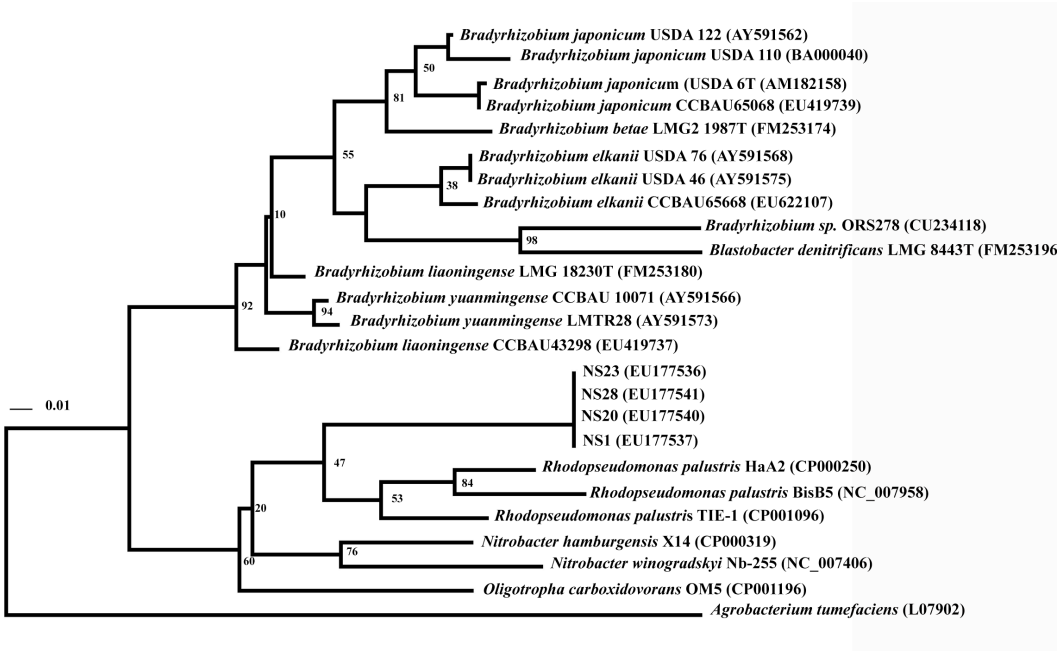
MAXIMUM LIKELIHOOD (ML) TREES AND 5‘RACE



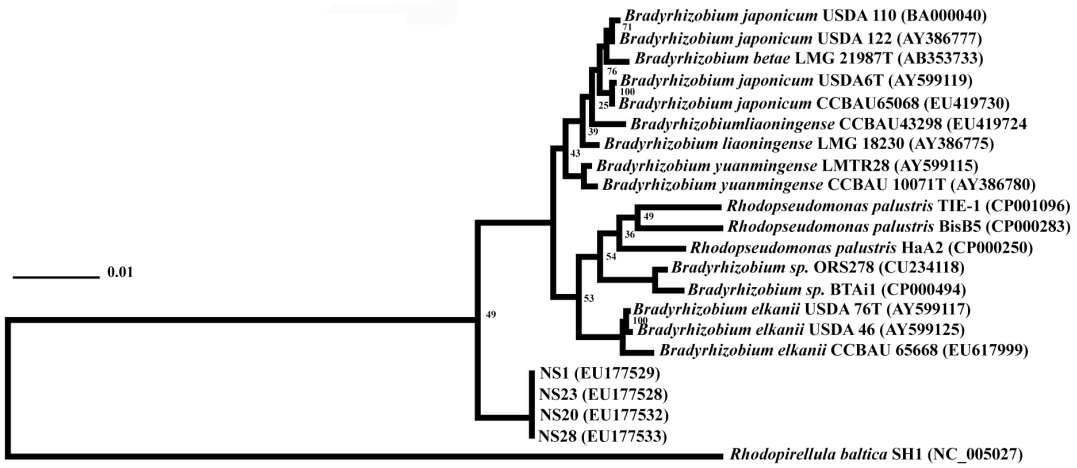
APPENDIX B1 Maximum-likelihood (ML) trees based on sequences of 16S rRNA gene. Bootstrap value is expressed as percentages of 500 replications. The bar represents 1 estimated substitution per 100 nucleotide position. Multiple alignments were performed with MUSCLE from phylogeny.fr. Unaligned regions and gaps were excluded from the analyses. The ML analyses were performed using the HKY85 substitution model with PhyML program.



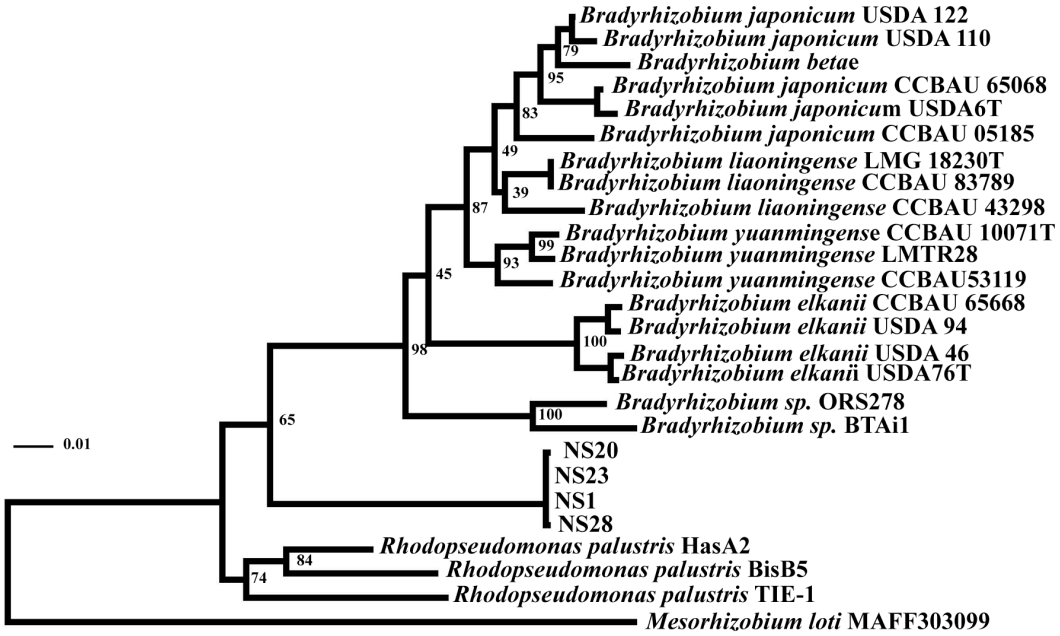
APPENDIX B2 Maximum-likelihood (ML) trees based on sequences of *atpD* gene. Bootstrap value is expressed as percentages of 500 replications. The bar represents 1 estimated substitution per 100 nucleotide position. Multiple alignments were performed with MUSCLE from phylogeny.fr. Unaligned regions and gaps were excluded from the analyses. The ML analyses were performed using the HKY85 substitution model with PhyML program.



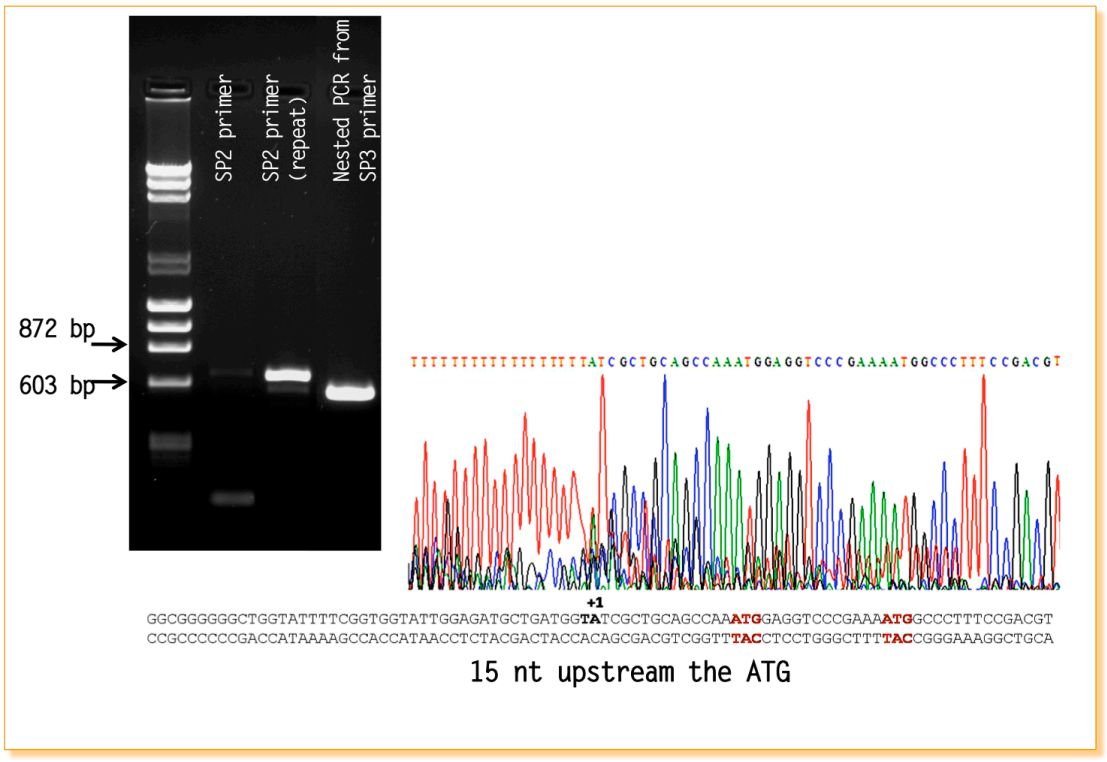
APPENDIX B3 Maximum-likelihood (ML) trees based on sequences of *recA* gene. Bootstrap value is expressed as percentages of 500 replications. The bar represents 1 estimated substitution per 100 nucleotide position. Multiple alignments were performed with MUSCLE from phylogeny.fr. Unaligned regions and gaps were excluded from the analyses. The ML analyses were performed using the HKY85 substitution model with PhyML program.



APPENDIX B4 Maximum-likelihood (ML) trees based on sequences of *glnII* gene. Bootstrap value is expressed as percentages of 500 replications. The bar represents 1 estimated substitution per 100 nucleotide position. Multiple alignments were performed with MUSCLE from phylogeny.fr. Unaligned regions and gaps were excluded from the analyses. The ML analyses were performed using the HKY85 substitution model with PhyML program.



APPENDIX B5 Maximum-likelihood (ML) trees based on sequences of combination of 16S rRNA gene, *atpD, reAc, and glnII*. Bootstrap value is expressed as percentages of 500 replications. The bar represents 1 estimated substitution per 100 nucleotide position. Multiple alignments were performed with MUSCLE from phylogeny.fr. Unaligned regions and gaps were excluded from the analyses. The ML analyses were performed using the HKY85 substitution model with PhyML program.



APPENDIX B5 R'RACE of *M. loti intS*. (A) Lane1, DNA size standard (F-303SD, Finnzymes, Biolabs nIc, Finland); Lane 2, PCR product of *intS* amplified with Oligo-d(T) anchor primer and SP2_ intS5RACE; Lane 3, nested PCR product of *intS* amplified with anchor primer and SP2_ intS5RACE; Lane 4, PCR product of *intS* amplified with anchor primer and SP3_ intS5RACE. (B) Peak of direct sequencing of the PCR product amplified from anchor primer and SP3_ intS5RACE.

BIOGRAPHY

Miss Rujirek Noisangiam was born on July 31st, 1980 in Chiangmai, Thailand. She graduated with the Bachelor Degree of Food Technology, Suranaree University of Technology, Thailand in 2002. During her Doctoral Degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (2003-2010), she presented research work in the 6th National Symposium on Graduate Research, October 13-14, 2006, Chulalongkorn University, Bangkok, Thailand (Poster presentation; in "Distribution of genes involved nodulation in non-symbiotic and symbiotic-soybean *Bradyrhizobium* isolated from Thai soil"), in the 8th European Nitrogen Fixation Conference, August 30 - September 3, 2008, Ghent University, Ghent, Belgium (Poster presentation; in "Transfer of *Mesorhizobium loti* symbiotic gene into family *Bradyrhizobiaceae* chromosome"), and in the RGJ-Ph.D. Congress XI "Research Towards Sustainability", April 1-3, 2010, Jomtien Palm Beach Hotel & Resort, Pattaya, Chonburi, Thailand (Oral Presentation; in " Symbiotic genes transfer from *M. loti* to *Bradyrhizobiaceae* strains and their effects on their symbiotic ability"). Her works have been published in Systematic Applied Microbiology in the topic of "Heavy metal tolerant *Metalliresistens boonkerdii* gen. nov., sp. nov., a new genus in family *Bradyrhizobiaceae* isolated from soil in Thailand" 2010.