CHAPTER 3
MATERIALS AND METHODS

3.1 Source of isolates

Five fast-growing nitrogen-fixing bacterial isolates (S171-S175) and four slow-growing symbiotic nitrogen fixing bacterial isolates (S76, S78, S162 and S178) were obtained by Mr.Suwat Saengkerdsub (2000) from nodules of soybean (*Glycine max* cv.SJ 5) grown in acidic soil from Mahatsajan slope, Kao Kaw district, Petchaboon province in the northern part of Thailand. The average soil pH was 5.25.

3.2 Strain identification

Since the isolates may be the same strains, the following methods will be used in strain identification :

3.2.1 Gram staining

One loop of each culture was inoculated into 2 ml of yeast extract mannitol broth in a test tube. The culture was grown at room temperature, 200 rpm, for 16 h. Cell were used in Gram staining by the standard method (Singleton & Sainbury, 1998).

3.2.2 RAPD-PCR fingerprinting

3.2.2.1 Isolation of chromosomal DNA

One loop of each of the test strains kept in agar slants at 4°C was activated by spreading onto fresh agar slants and incubated at 30°C for 2 days. One loop of activated cells was inoculated into 50 ml of yeast mannitol broth for growth at 200 rpm, 30°C until mid log phase. Cells were harvested by centrifugation one ml cell suspension at 5,000 rpm, 4°C for 5 minutes. 80 µl 2.5 $mg.ml^{-1}$ lysozyme was added to cell pellet and left standing in 37°C water bath for 1 h before 4 cycles of freezing at –20°C for 5 minutes and thawing at 80°C for 5 minutes. One volume of DNAzol® was added to the solution which was gently mixed by inverting eppendorf tubes. The mixture was centrifuged at 10,000 rpm, 4°C for 5 minutes. The supernatant was transferred to a fresh eppendorf tube. 500 µl ice-cold ethanol was added to the mixture which was gently mixed by inverting the tube before centrifugation at 10,000 rpm, 4°C for 15 minutes. The precipitate was washed with 70% ice-cold ethanol and air dried in a

laminar flow hood. Thirty µl high-purity distilled water was added to dissolve the nucleic acid precipitate at room temperature for 1 day. Quality of the isolated chromosomal DNA was checked by 0.8% agarose gel electrophorsis by standard method (Sambrook & Russell, 2001).

3.2.2.2 RAPD-PCR fingerprinting

Sequences of RPO1 and CRL-7 were as described by Richardson et al (1995) and Mathis & McMillin (1996) as follows :

RPO1 : 5'AATTTTCAAGCGTCGTGCCA3', CRL-7 : 5'GCCCGCCGCC3'

All the primers used in this Section and Section 3.2 were synthesized by Invitrogen Life Technologies, USA. To obtain reproducibility all RAPD-PCR fingerprinting experiments were repeated at least twice.

Either RPO1 or CRL-7 primer was used in RAPD-PCR fingerprinting in the following mixture (Taq Master Kit, Eppendorf) :

Mixture		Program		
5x Taq Master	10.0 µl	95°C	15 seconds	5 cycles
10X Taq Buffer	5.0 µl	50°C	30 seconds	
10 mM dNTPs	1.0 µl	72°C	90 seconds	
10 mM primer	5.0 µl	95°C	15 seconds	25 cycles
60-100 ng DNA template	2.0 µl	55°C	30 seconds	
Taq polymerase (5U. l^{-1})	0.5 µl	72°C	90 seconds	
High quality double distilled water	30.5 µl	72°C	10 minutes	
	Total 50.0 µl			

PCR products were separated by 1.25 % agarose gel eletrophoresis by standard method (Sambrook & Russell, 2001). RAPD-PCR fingerprints were viewed and photographed on a UV transilluminator (Bio-rad). RAPD-PCR patterns when the same primer was used were compared to determine if the isolates were the same strains.

3.2.3 16S rDNA sequencing

3.2.3.1 Amplification of 16S rDNA fragments

16S rDNA fragments of strains S172, S173, and S174 were obtained by PCR amplification by using 27f and 1492r as the forward and the reverse primers.

Nucleotide sequences of 27f and 1492r were as reported by Blackall (1999) as follows :

27f : 5'GAGTTTGATCCTGGCTCAG3', 1492r : 5'ACGGCTACCTTGTTACGACTT3'

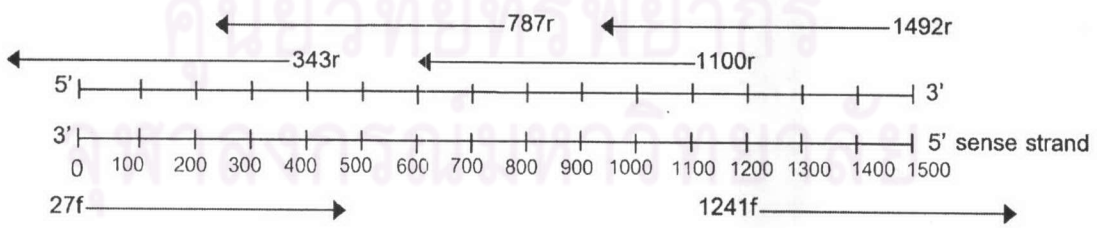
Content of the PCR mixture for the PCR run was the same as described in Section 3.1.2 except 0.2 μl each of the primers 27f and 1492r (200 ng. μl^{-1}) was used. PCR program was set as described by Blackall (1999) as follows :

95°C	30 minutes	
95°C	60 seconds	30 cycles
48°C	60 seconds	
72°C	120 seconds	
48°C	60 seconds	1 cycle
72°C	300 seconds	

Each PCR product was separated by 1.25% agarose gel electrophoresis, viewed and photographed on a UV transilluminator (Bio-rad). The expected molecular size of the 16S rDNA was approximately 1,500 bp.

3.2.3.2 Sequencing of 16S rDNA fragments

Tubes containing 16S rDNA PCR products obtained as described in Section 3.2.1 were sent to the BioService Unit, National Centre for Genetic Engineering and Biotechnology for sequencing by the ABI PRISM (Dye terminator Cycle Sequencing Kit (Applied Biosystems) The following primers were supplied with the samples (Blackall, 1999, Dorch & Stackebrandt, 1992)



Sequences of the primers from 5' end to 3' end with numbers in brackets indicating positions in the 16S rDNA of *E. coli* were as given by Blackall (1999) as follows :

27f (9-27)	GAGTTTGATCCTGGCTCAG
343r (343-357)	CTGCTGCCTCCCGTA
787r (787-803)	CTACCAGGGTATCTAAT

1100r (1100-1115)	AGGGTTGCGCTCGTTG
1241f (1224-1241)	TACACACGTGCTACAATG
1492r (1492-1512)	ACGGCTACCTTGTTACGACTT

The BioEdit program (http://www.mbio.ncsu.edu) was used to analyse the overlapping sequences of amplified fragments. Complete sequences were compared with available sequences deposited in GenBank using the NCBI BLAST program.

3.2.4 Electron microscopy and negative staining for type of flagella

One loop of cells of each of strains S172, S173, or S174 was inoculated into 2 ml of yeast mannitol broth and left standing at room temperature for 16h in the dark. The cultures were taken to the Scientific Research and Technological Equipment Centre, Chulalongkorn University for negative staining for type of flagella. Cells were stained with 0.3% phosphotungstic acid in ethanol and observed under an electron microscope.

3.2.5 Nodulation responses : determination of plant dry weight and nodule dry weight

Seeds of seven cultivars of soybeans (*Glycine max* cv. SJ4, SJ5, Chiangmai 2 , Chiangmai 60, Sukhothai 1, Sukhothai 2, and Sukhothai 3) were surface-sterilized as described by Jordan (1984). The seeds were washed with 95% ethanol for 10 seconds followed by surface-sterilization with 5% H_2O_2 for 5 minutes with intermittent shaking. Seeds were washed 6 times with sterilized distilled water. Imbibition of seeds was carried out by soaking sterilized seeds in sterized distilled water and placed at 4-8^{o}C for 4 hours. Forceps were aseptically used to place surface-sterilized seeds onto 0.75% seedling agar and incubated at 25^{o}C in the dark for 2 days. Three germinaing seeds were placed in each Leonard jar. Nitrogen-free medium,either pH 5.0 or pH 6.8, was used to water the subsequent seedlings and plants for 28 days. Plants were thinned to two plants per Leonard jar after growth for 2 weeks. Plant dry weight was obtained by cutting the shoot portions at the cotyledon scar for drying at 70^{o}C for 72 h. Nodule dry weight was obtained by weighing all dried nodules of the two plants grown in each Leonard jar. Dry weight per plant and nodule dry weight per plant were reported.

Preparation of Leonard jars was as described by Somasegaran & Hoben (1994). Statistic analysis was obtained by Duncan Multiple Test (Kenji,1999) One gram of sand

in Leonard jar where S172 was grown was added to 1 ml of sterilized distilled water, swirled, and left standing at room temperatures in the dark for 1 day. Bacteria were isolated from the solution using yeast mannitol agar containing 0.25 $\mu g.ml^{-1}$ Congo red. Bacterial isolates were identified by RAPD-PCR fingerprinting and negative staining for type of flagella as described in Sections 3.1 and 3.3 respectively.

3.3 Determination of changes in protein profiles

One loop of cells of each of the following strains was inoculated into 50 ml of yeast mannitol broth in 250 ml Klett flasks : fast-growers S172, S173, S174 ; slow-growers S76, S78, S162, and S178. Growth was monitored by measurement of optical density at 660 nanometer. Time to reach mid-log phase was obtained from growth curves. Seed culture of each strain was obtained by inoculating one loop of cells into 50 ml yeast mannitol broth and the culture was grown to mid-log phase. Five ml of the seed culture were inoculated into 45 ml of yeast mannitol broth in 8, 8, 10 and 15 250 ml Klett flasks and incubated at 30°C, 35°C, 40°C and 45°C respectively at 200 rpm for 16 h for the fast-growers, and 48 h for the slow-growers. Cells were harvested at 12,000 rpm, 4°C for 15 minutes, washed twice with extraction buffer (0.5 M Tris HCl, pH 7.5) before transferring cell pellets to fresh 1.5 ml eppendorf tubes. Two-thirds volumes of sterilized glassbeads (Sigma) were added then vortexed for 5 minutes at 30 seconds intervals with tubes on ice in-between the intervals. Cell debris was removed by centrifugation at 12,000 rpm, 4°C, 45 minutes. Soluble protein content was determined by Bradford method (Bradford, 1976). Protein profiles of each strain when grown at high temparatures were obtained by loading 50 μg of each sample in a sample well for SDS-PAGE by standard method (Laemmli, 1970). Gels were stained with Bio-rad silver staining kit as described by the manufacturers. Silver stain SDS-PAGE standards (Low range) (Bio-rad) were used as the molecular weight markers : Rabbit muscle phosphorylase b 97.4 kd ; Bovine serum albumin 66.2 kd ; Hen egg white ovalbumin 45.0 kd ; Bovine carbonic anhydrase 31.0 kd ; Soybean trypsin inhibitor 21.5 kd ; Hen egg white lysozyme 14.4 kd. Silver stain SDS-PAGE standards (High range) (Bio-rad) were used as the the molecular weight markers : Myosin 200 kd ; *E. coli* β-galactoside 116.2 kd ; Rabbit muscle phosphorylase b 97.4 kd ; Bovine serum albumin 66.2 kd ; Hen egg white ovalbumin 45.0 kd.