CHAPTER IV

DISCUSSION

In this study, there were problems about isolation of chromosomal DNA from *Rhizobium* and *Bradyrhizobium*, because they produced slime which may block activity of RNase and pronase, and reduced yield of DNA. Washing of bacterial cells with 1 M sodium chloride was found necessary to remove slime before isolation of chromosomal DNA. Since there were previous reports that *nod* genes and *nif* genes in many diazotrophs, such as *R. leguminosarum*, *R. meliloti*, *R. phaseoli*, *R. trifolii*, are located on megaplasmid DNA (Hirsch, 1992) and *A. brasilense* (Elmerich *et al*., 1987) but megaplasmid from these rhizobia and *A. brasilense* Sp7 could not be isolated and purified in this study because megaplasmid has vary large size, must be prepared by a very gentle procedure to avoid shearing of the isolated DNA and its loss. Molecular weight of megaplasmid of *R. meliloti* strain 41 is 140 Mdal (pRme41a) and more than 300 Mdal (pRm41b) (Banfalvi *et al*, 1981), megaplasmid of *A. brasilense* Sp7 is 90 Mdal which contain host specific *nod* genes regions (Elmerich *et al*., 1987). Therefore, the information of *nod* and *nif* genes homology on megaplasmids in this study were not reported. It is noted that contamination of these megaplasmids in the chromosomal DNA preparation could occur easily because of their large size.

Nonradioactive DNA-labeling using DIG-11-dUTP and random primer was applied for labeling DNA probes in this study in order to avoid hazardous radioactive method, but maintained high sensitivity (Matthews and Kricka, 1988). The reaction of DIG-DNA labeling and detection system is by ELISA principle (enzyme linked immunosorbent assay) (Kassler *et al.*, 1992; Lion and Hass, 1990; and Martin *et al.*, 1987) and catalyzed by Klenow fragment of DpI. The steroid hapten digoxigenin is linked with uracil at position C_5 of the deoxyribonucleotide by an 11-atom spacer. The denatured single strand templates are annealed to the random mixture of haxanucleotides. The synthesis is started by adding Klenow fragment of DpI and all deoxynucleotides (one is the hapten modified substrated (DIG-dUTP). During the polymerization reaction, Klenow fragment of DpI incorporates not only the nonmodified deoxynucleotide but also DIG-dUTP. The primers are able to bind all possible target sequences (both single strands may act as templates). However, a small amount of DNA can be used and gives high specific activity (Martin *et al.*, 1987). Detection of nucleic acid bound to a cardenoid-steroid digoxigenin in this research was done by using chemiluminescent, resulted from the emission of light catalyzed by alkaline phosphatase linked to digoxigenin antibody, using Lumigen PPD as a substrate. Chemiluminescent detection can be considered as a better method than colorimetric detection and also need smaller amounts of target DNA (Carlson *et al.*, 1990). Reuse of the nylon membrane for hybridization with other probes is also possible by

removal of color precipitate and DNA probe by dimethylformamide, although it is a poisonous chemical.

In the Southern hybridization non-specific hybridization sites on membrane surface were blocked by casein as blocking reagent, in the prehybridization step, where loss of DNA from nylon membrane can occur (Sittipraneed, 1985), thus the excess amount of DNA (3.0 μg) must be used to cover this loss. The hybridization temperature used (65-68 °C) following instruction of Boehringer Mannheim; Biochemica (1993), may not be suitable for the chromosomal DNA from N_2-fixing bacteria since some showed only a weak signal or no signal appeared. These results may have been influenced by inappropriate condition. The hybridization in small volume solution (2.5 ml/100 cm^2 of membrane) should increase the sensitivity of detection of hybridization bands by using larger amount of DNA (3.0 μg) on the nylon membrane. Finally, the post hybridization (washing) step, where non-bound probe is removed from the membrane, high temperature (65 °C) and low salt concentration (0.5 x SSC) has been chosen as stringent condition, since in general washing should be carried out at 10-15 °C below the T_m of the hybrid where aqueous solution have been chosen (Maniatis *et al*, 1982).

Dot hybridization for detection of specific activity of DNA probe shows the highest specific activity of *nif*HDK > *nod*D1 > *nod*AB > *nod*C when compared with control pBR328 labeled DNA which was provided in the kit of nonradioactive DIG-DNA labeling and detection kit. Because DIG will incorporate 20-25 nucleotides of DNA template.

The specific activity of the long DNA template should be higher than the short DNA template, but the specific activity of *nod*D1 was apparently high more than *nod*C, because the amount of *nod*D1-DNA spotted was twice. Dot hybridizationof *nod*D1, *nod*AB, *nod*C and *nif*HDK at 3.0 μg level of the total chromosomal DNA of 10 strains of N_2-fixing bacteria and Southern hybridization indicated similar results when probed with *nod*D1. Since *nod*D1 is a transcriptional activator of other nodulation genes in rhizobia which can be activated by plant signal, this result confirmed the presence of *nod*D1 in all symbionts and associative strains except, the free-living *K. pneumoniae* M5a1. Dot hybridization and Southern hybridization with *nod*AB and *nod*C at 3 μg of chromosomal DNA gave different results, where dot hybridization showed weak homology with *nod*AB and *nod*C in other strains of N_2-fixing bacteria, but Southern hybridization detected homology only with *R. meliloti* strain TAL380 and TAL1372. Because dot hybridization should be varied with broader concentration of DNA and selected for the concentration which give strong signal with the probe, in case of *nod*AB and *nod*C which confer lower specific activity, comparing to *nod*D1, higher amount of DNA should be used for Southern hybridization. However, the results of this study confirmed the adjacent position of *nod*AB and *nod*C in chromosomal DNA of *R. meliloti* strain TAL380 and TAL1372 as evident by similar RFLP patterns with *Bgl*II, *Eco*RI and *Sal*I, when hybridized with either *nod*D1, *nod*AB or *nod*C. In these results the smallest DNA fragments of *R. meliloti* TAL380 and TAL1372 which

hybridized with *nod*AB and *nod*C was 3.7 kb (Table 3.2. and 3.3.), which approximately equal to the size of *nod*AB (2.2 kb) plus *nod*C (1.3 kb), indicating that organization of common *nod* genes on chromosomal DNA of *R. meliloti* TAL380 and TAL1372 should be similar in size and position to *nod*D1 ABC of *R. meliloti* strain 1021, from which *nod*D1 gene was cloned from and used as probe in this study (Egelhoff and Long, 1985). According to Banfalvi *et al.* (1981) and Kondorosi *et al.* (1991 a), in the fast-growing *Rhizobium* species, the *nod* genes are located on a lagre plasmid, known as pSym. Specific flavones, flavonones and chalcones are the inducers of *nod* genes in the fast-growing species. In the slow growing *Bradyrhizobium* species, the *nod* genes are chromosomal borne. Besides flavonoids, a wide range of compounds interact with the *Bradyrhizobium nod*D gene (Gyorgypal *et al.*, 1991). In *R. meliloti nod*C encodes for an N-acetyl-glucosaminyl transferase, which polymerizes the oligo-N-acetylglucosamine (oligo-GlcNAc) backbone of the "nod factor". *Nod*B codes for a deacetylase, which removes the non-reducing N-acetyl-glucosamine residue of oligo-GlcNAc (John *et al.*, 1993). In the pathway of nod factor synthesis, deacetylation of the nonreducing end of the oligosaccharide backbone was necessary requirement for attachment of the fatty acyl chain. *Nod*A codes for an acyl transferase which transfer an acyl group to the deacetylated residue (Ehrhardt *et al.*, 1994.). The *nod*C product appeares as two polypeptide bands at 44 and 45 kD, where *Nod*A and *Nod*B proteins are 21 kD and 28 kD respectively. The *nod*D produced a single polypeptide of 33 kD which

is a transcriptional regulator. Mutants of Nod^+ genes, if all 3 copies (D1, D2 and D3) were mutated, then Nod^- phenotype will be observed on alfalfa (Hirsh, 1992). In this study the homology of *nod*ABC with 3 µg of chromosomal DNA of *A. brasilense* Sp7 was not found although homology with *nod*D1 was observed. This result contradicted with Elmerich *et al.* (1985), when digested total chromosomal DNA from several *Azospirillum* strains were found hybridized with 3.5 kb *Eco*RI fragment carrying *nod*D1 ABC from *R. meliloti* 41 as probe. Difference in size of DNA fragments were also reported in restriction cut, *Eco*RI yielded 7.2 kb, and *Sal*I yielded 3.3 kb, where in this study shorter fragments 6.8, 4.9 and 4.0 kb were observed only when cut with *Bam*HI and hybridized only with *nod*D1 probe indicating that separation of *nod*D1 0.6 kb, *nod*AB 2.2 kb and *nod*C 1.3 kb, which are much smaller comparing to *nod*D1 ABC in 3.5 kb lead to lower sensitivity and might give different RFLP patterns.

Southern hybridization by *nif* structural genes confirmed that the *nif* structural genes of the associative *Klebsiella* strains have strong homology with the free-living *K. pneumoniae* M5a1, although some restriction enzymes can distinguish associative strains from the free-living one. These results agree with previous report by Suthisukon (1992) using the same *nif*HDK probe, but labeled with α-^{32}P dCTP by nick translation reaction. But the method used in this study showed higher background than the ^{32}P-radioactive labeling method, although more or less comparable sensitivity. In this results when compare between *nod* and *nif* genes on chromosomal DNA

of N_2-fixing bacteria, the organization of *nod* genes was distant from *nif* genes, because there was no DNA fragment which hybridize both *nod* and *nif* probes. As reviewed by Banfalvi *et al.* (1981) when *nif*HDK of *K. pneumoniae* M5a1 was used as probe and hybridized with megaplasmid of *R. meliloti* 41 they discovered that *nif* genes were located on 100 kb segment of symbiotic megaplasmid. According to Prakash *et al.* (1981), *nif*HDK located on large plasmids in *R. leguminosarum*, *R. phaseoli* and *R. trifolii*. In *R. meliloti* there were strains that carried *nif* genes on large plasmid, pRme (MW 90-250 x 10^6 kb), some strains carried separate *nif* genes on megaplasmid, pSym (MW $>$ 300 x 10^6 kb). The *nif* genes in *A. brasilense* are scattered and span a region of about 30 kb of chromosomal DNA (Elmerich, 1987) discovered when chromosomal DNA of *A. brasilense* Sp7 was cut with *Eco*RI which give 7.2 kb fragment when hybridized with 6.7 kb *Eco*RI fragment of *nif*HDK of *K. peumoniae* M5a1. Several reports have shown that genes required for nodulation (*nod*) and for nitrogen fixation (*nif*) are plasmid-borne in *R. trifolii*, *R. leguminosarum* and *R. phaseoli* (Beringer *et al.*, 1980). Hybridization of the structural genes coding for enzyme nitrogenase of *K. pneumoniae* M5a1 to DNAs from different *Rhizobium* species indicated interspecies homology of the *nif* structural genes (Ruvkun and Ausubel, 1980). For *R. leguminosarum*, *nif* structural gene were reported to be specific to an indigenous plasmid (Nuti *et al.*, 1979). As reviewed by Pankhurst *et al.* (1983), *R. meliloti* harbours a very large plasmid which carries both *nod* and *nif*

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genes. In most fast-growing rhizobia some nodulation and nitrogen fixation genes are carried by the same Sym plasmid. In *R. meliloti nod* fragment hybridized with a DNA region on the Sym plasmid about 30 kb away from the *nif* structural genes. In this study only chromosomal DNA were used, therefore *nif* hybridization bands were not observed in most strains of rhizobia.

In this study many electrophorograms, showed curved DNA bands, which could result from many possible factors; 1) over staining with ethidium bromide, 2) excess voltage during electrophoresis that cause overheating in some area of the gel, 3) residual ethanol in the DNA sample or 5) overloading of DNA per well.

Plasmid pUC18 was used in *nod*D1 cloning from *K. oxytoca* NG13 because of its properties as follows; 1) small size (2.686 kb), 2) relaxed plasmid, high copy number and it can be amplified with chloramphenical, 3) has N-terminal of β-galactosidase which complementary with C-terminal of β-galactosidase of *E. coli* JM101 (host cell), and 4) insertion sites located in multiple cloning site polylinker that contains sites for 13 different hexanucleotide specific restriction enzymes and select by amplicillin resistance, and β-galactosidase negative. Then ligation was performed after dephosphorylation of the linearized plasmid to prevent self-ligation and increase the efficiency of ligation. The application of polymerase chain reaction for amplification of these *nod*D1-liked DNA fragments from this cloning are proposed for further study on *Klebsiella*-rice interaction.

Conclusion

Restriction Fragment Length Polymorphism (RFLP) analysis by using *nod*D1, *nod*AB and *nod*C from *R. meliloti* 1021 and *nif* HDK from free-living *K. pneumoniae* M5a1 as probes indicated that the *Bam*HI digested DNA from associative *K. oxytoca* strain R15, R17 and NG13 contained two DNA fragments of 4.0 and 4.9 kb that hybridized with *nod*D1 probe. Cloning of these 4.0-4.9 kb fragments of chromosomal DNA from *K. oxytoca* NG13 into *Bam*HI site of pUC18 resulting two recombinant plasmids R1 and R2 that hybridized with *nod* D1 probe. All these results are evident for homology of *nod*D1 gene among associative and symbiotic N_2-fixing bacteria which can not be detected in the free-living *K. pneumoniae* M5a1. Southern hybridization with separate *nod*AB and *nod*C probes which contain low specific activity by their small size could not detect homology by RFLP of the 8 restriction enzymes used in this study, although dot hybridization indicated for weak homology. On the other hand by using both *nod* and *nif* probes, the results indicated distant organization of *nod*D1-liked gene away from *nif* genes in chromosomal DNA of associative *Klebsiella*, and the potential of using *nod* and *nif* probes to identify different species of rhizobia.