CHAPTER IV

DISCUSSION

Since there are evidences reported that in some diazotrophs *nif* genes are located on plasmid DNA, such as *Rhizobium* spp. (Uozumi *et al.*, 1982), only *nif*HDK in *A. vinelandii* AVY15 (Yano *et al.*, 1982) and all *nif* genes in *Enterobacter agglomerans* (Singh *et al.*, 1988), therefore the first investigation is to find whether *Klebsiella* R15 and R17, the associative strains isolated from the rhizosphere of rice contain any plasmid. Three procedures of plasmid detection and preparation were used in this study : modified Eckhardt's method, Kado and Lui's method and rapid alkaline extraction. The advantages of the modified Eckhardt's method are mild condition used in releasing plasmid from the spheroplasts by SDS lysing during the first period of electrophoresis so that only supercoiled (covalently closed circular, ccc) form of plasmid are obtained. The covalently closed circular (ccc) plasmid migrated through pore of agarose gel without facing any drastic condition and no plasmid loss during the procedure, because cells are lysed in gel. Moreover, the number of plasmid band detected indicates accurately the number of plasmid in cell since the only ccc form of plasmid is present. This method is also suitable for large plasmid detection since the plasmid sizes ranging form 4 to 86 kb (pBR322, 4.36 kb; pSA30, 10.3 kb and pRD1, 86 kb) can be detected (Figure 3.1). So it is used appropriately for obtaining preliminary data about the

plasmid content of bacteria. However, the sensitivity of this method is limited since only small amount of cells can be applied to agarose gel. Therefore, plasmid that is present in very low copy number might be difficult to detect.

In Kado and Lui's method, the alkali-SDS was used for rapid lysis of cell and the lysate was cleared by heat treatment. Normally for E. coli heat treatment is not needed, only SDS at high pH condition can lyse the cells effectively (Figure 3.2 a). Like the previous method, plasmid cannot be detected in Klebsiella R15 and R17 even in drastic alkali pH and high temperature condition. The advantages of this method over the previous one are that the extracted plasmid can be used directly, scaled up for large quantity preparation and moreover the plasmid band close to chromosomal DNA is easily detected. The disadvantages of this method are the use of toxic chemicals (phenol and chloroform) and the ccc form of plasmid extracted might be changed to relaxed form during heat treatment especially for large plasmid (pRD1, lane 9, 10 in Figure 3.2 a). Therefore, the number of plasmid band obtained after heat treatment may not be the true number of plasmid in the cell. So the strains with more than one size of plasmid need to be confirmed by other method.

The third performed method of plasmid detection was rapid alkaline extraction. As compared to the first method, the conditions offered by this method are quite drastic. The plasmid are exposed to such the alkali and high salt condition resulting in the alteration of ccc to relaxed form of most plasmid extracted. Thus, this method is not suitable for detection of plasmid in cell containing more than one or large plasmid as by modified Eckhardt's method. This method gives less contamination of chromosomal DNA and ribosomal RNA, so it is a suitable method for large scale preparation of plasmid.

From three procedures of plasmid detection, modified Eckhardt's method (Figure 3.1), Kado and Lui's method (Figure 3.2 b), and rapid alkaline extraction (Figure 3.3), it is clearly shown that both K. R15 and R17 do not contain any plasmid. This result is opposite to that reported by Anchan Choonhahirun (1986) using Kado and Liu's method that both strains R15 and R17 show large smeared plasmids (>23 kb) only by heating at 65°c for 30 min, and this band disappears when heating at 65°c for 60 min. Another procedure of large plasmid isolation (Hansen and Olsen, 1978) has been performed and confirmed that no plasmid has been detected in extracts, R15 and R17 (data not shown). So the former report might result from overloading of sheared chromosomal DNA or both K. R15 and R17 might loss their plasmids during long term maintenance of cultures. However, the nitrogen-fixing ability of these diazotrophs still exist, since they can grow on nitrogen-free medium. These data indicate that the nif genes are located on the chromosomes as in K. oxytoca NG13 (You and Wang, 1990).

For DNA hybridization probes (pSA30 and pAM51) further purification of plasmid after rapid alkaline extraction have been performed. In this study, the plasmids were highly purified ($A_{260nm}/A_{280nm} \geq 2$) by isopycnic centrifugation in cesium chloride (CsCl) gradient to separate the closed circular DNA (plasmid) from linear DNA (chromosome), RNA and proteins (Figure 3.4).

For restriction endonuclease digestion, accurate interpretation of the restriction pattern after digestion of chromosomal DNA by restriction endonucleases would rely on two factors. Firstly, chromosomal DNA should be of high molecular weight with high purity and secondly, complete digestion should be obtained.

In general, extracted chromosomal DNA which were prepared from all cell type would not be intact form, since at all lysis steps and

among isolation procedures, chromosomal DNA would be sheared by shearing force. The isolated chromosomal DNA with high purity (A_{260nm}/A_{280nm} of 1.8-1.9) prepared from this study are of high molecular weight DNA, since only one nearly sharp band of slow mobility on agarose gel electrophoresis has been obtained (Figure 3.5). So, they were suitable for digestion.

In this experiment, the excess restriction enzymes (10 unit per µg DNA) were used to ensure complete cutting of chromosomal DNA since the enzymes may loss their activity (refered from supplier) during transfering and storage. It was ensured that the amount of enzymes used (10 units) were enough for complete cutting, since the restriction patterns were the same as those using 20-unit enzyme digestion in the same manner (Figure 3.6). Since all restriction endonucleases are stored in 50% glycerol and high concentration of glycerol (>5%) can inhibit the activity of restriction endonucleases, in this study, the concentration of glycerol in all digestion reaction using restriction endonucleases ranging from 1 to 20 unit(s) is always kept less than 5%.

The restriction patterns on agarose gel electrophorogram of Klebsiella strains reveal a number of discrete bands of different molecular sizes (Figure 3.7). Moreover, the position (size) and intensity of ethidium bromide staining bands (restriction patterns) are characteristics of the enzyme used and the source of DNA. From previous study, Klebsiella R15 and R17 have been classified as Klebsiella-like strain since their % G+C content (54.9-56.4) and the reassociation time of hybrid DNA are very close to K. oxytoca NG13 (Anchan Choonhahirun, 1985) except difference in fatty acid composition (Jariya Boonjawat et al., 1986). Their restriction patterns from eight restriction enzymes compared with K. pneumoniae M5a1 and K. oxytoca NG13 indicate the different patterns between free-living K. pneumoniae M5a1 and the

associative Klebsiella strains : NG13, R15 and R17, but similar restriction patterns among these 3 associative strains. Thus, it is possible that K. R15 and R17 might be the same species as K. oxytoca NG13. However, their restriction patterns could not differentiate the strains among these associative Klebsiella whereas their fatty acid composition could. Since examination of genetic variation of DNA by means of restriction endonucleases could not detect all genetic variation of DNA as complete as DNA sequencing could (Nei and Tajima, 1980), these results suggest that all 8 enzymes (BamHI, BglII, EcoRI, HindIII, PstI, SalI, SmaI and XhoI) used in the RFLP studies, could be unsuitable to display polymorphism among these strains although phenotypic variation e.g. particular protein, lipid or carbohydrate influenced by autogenetic or environmental factors may be observed (Curren et al., 1985); or the variations occurred are apart from the sequences recognized and cut by restriction enzymes but cause remarkable change in physiological property.

Labelled probes were prepared by using nick translation. The reaction is carried out by the activity of DNase I and DNA polymerase I. 3′ Hydroxyl terminis are introduced into the DNA duplex by DNase I. Then, new nucleotides including labelled nucleotide determined by the opposite strand are added to the 3′ hydroxyl side of the nicks by polymerase activity at the same time as existing nucleotides are removed from the other side of the nicks by the 5′ to 3′ exonuclease activity (an integral part) of DNA polymerase I. Consequencely, the nick is translated along the DNA molecule in 5′ to 3′ direction and the existing nucleotides in the DNA molecule are renewed without net synthesis occurring. Since rate of nick translation reaction is under kinetic rate of enzymes reaction, kinetic study has to be performed to determine when the maximum incorporation of

^{32}P-nucleotides into DNA molecules with the highest specific activity should be obtained.

In general, the hybridization process can be divided into three steps : prehybridization, hybridization and washing. The prehybridization step is designed to block all sites on the nitrocellulose membrane that would bind non-specifically with the probe, thereby reducing the background. The nonspecific sticking of single-stranded DNA can be prevented by preincubating the nitrocellulose membrane in an albumin solution which is composed of BSA, Ficoll and polyvinylpyrrolidone (Denhardt's solution). However, this prehybridization step can cause the loss of DNA from nitrocellulose membrane (Siriporn Sittipraneed, 1985), thus the amount of DNA (1.5 µg) transferred must be quite excess to cover the loss.

Since, nucleic acids hybridize most efficiently at approximately 25°c below the temperature at which they are 50% dissociated (melting temperature, T_m) (Meinkoth and Wahl, 1984) and the T_m of *K.* R15 and R17 are 91.9 and 92.5°c respectively (Anchan Choonhahirun, 1986), thus the hybridization should be conducted at 66.9-67.5°c in aqueous solution. Since each increase of 1% formamide concentration in the reaction solution lowers the T_m of a DNA duplex by 0.65°c (Casey and Davidson, 1977), hybridization in 50% formamide is conducted at 34.4-35°c. Therefore, hybridization reaction in this study which has been performed in 50% formamide at 37°c are quite stringent. Hybridization at low temperature (37°c) is easier to set up, with less evaporation problem, and milder condition for the nitrocellulose and DNA probes than hybridization at 67°c in an aqueous solution, however the rate of reaction in 50% formamide should be two times slower than in an aqueous solution (Casey and Davidson, 1977). This problem is solved by performing hybridization in smaller volume of solution (50-100µl/cm^2),

and increase the sensitivity of detection of hybridization bands by using larger amount of DNA (1.5µg) on the nitrocellulose membrane. In the washing step, high temperature (55°c) and low salt concentration (15 mM NaCl) 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).

All steps of hybridization process have been carried out under stringent conditions, so that the hybridization bands which have been detected in this study should be complementary hybrid molecules between nif structural genes probes and glnA gene probe of Klebsiella pneumoniae M5a1 and those of associative Klebsiella strains : NG13, R15 and R17.

Southern hybridization by nif structural genes show that the nif structural genes of the associative Klebsiella strains : K. oxytoca NG13, K. R15 and K. R17 are strongly homologous to those of K. pneumoniae M5a1 with the same RFLP patterns when cutting with BamHI, EcoRI, HindIII, SalI and XhoI, although there are some differences when cutting with BglII, PstI and SmaI. These results agree with the report of Wang et al. (1985) that the restriction map of the cloned nif genes of K. oxytoca NG13 is the same as that of the K. pneumoniae M5a1 nif genes with respect to the BamHI, EcoRI, HindIII and XhoI sites, but differ considerably in the PstI and BglII sites when using nifHDK from pSA30 as probe. This result has been used to provide a preliminary information about the nif genes organization and it is deduced that organization of the nif structural genes of the K. oxytoca NG13, R15 and R17 is essentially the same as that K. pneumoniae M5a1, although there are some differences in the restriction patterns. The results also indicate that nif structural genes of all associative Klebsiella locate on their chromosomes.

When using nif structural gene fragments (Fa : nifHD, Fb : nifDK

and Fc : nifKDTY and part of nifE) as probes to investigate which fragment gives different RFLP patterns of BglII, PstI and SmaI compared with known restriction map of these three enzymes in the nif genes region of K. pneumoniae, the results indicate the alteration in base sequence of nifL, nifE and nifJ regions of these associative strains respectively. Since, the nifL product controls the activity of the nifA product in response to nitrogen and oxygen status, the nifE product functions in synthesis of FeMoco, and the nifJ product functions in electron transport, these differences detected should be preliminary data to indicate whether there are changes of amino acids.

In BglII cutting the difference between the associative Klebsiella strains and K. pneumoniae is detected by all Fa (nifHD), Fb (nifDK) and Fc (nifKTY and part of nifE) probes. From the work of Kim et al. (1986) on nucleotide sequence of the nifLA operon of K. oxytoca NG13 and characterization of the gene product compared with the work of Arnold et al. (1988) on nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of K. pneumoniae, it confirms the alteration in one-of six-base sequence of nifL recognized by BglII (A/GATCT). This 6-base sequence locates at nucleotide 242-247 downstream from start codon of nifL of K. pneumoniae is AGATCT whereas K. oxytoca NG13 is AGATTT. However, the substitution is in the third position of codon giving no change of amino acid, therefore the amino acid is still Ile (Isoleucine). Since the alteration site in BglII cutting of K. oxytoca NG13 located about 9.4 kb downstream from this position (estimation from different size of RFLP, 23.4 kb minus 14.0 kb) is out of nif region and the homology of amino acids (495 amino acid) deduced from nifL of K. oxytoca with that of K. pneumoniae was 99% with substitutions of 6 bases resulted in changes of 5 amino acids (K. oxytoca vs K. pneumoniae) in following

positions : 48 Ile(ATC)- Thr(ACC); 104Arg(CGC)-Ala(GCC); 250Val(GTG)-Met(ATG); 425Phe(TTT)-Leu(TTG); 465 Ser(AGC)-Arg(CGC). Kim et al. reported that the nifL product of the two strains are strongly homologous.

In PstI cutting, the difference of fragment sizes are detected by Fc (nifKDYT and part of nifE) probe. Comparison of the different fragment sizes of K. oxytoca (0.77, 1.5 kb in K. oxytoca; 0.83 kb in K. pneumoniae) with the complete nucleotide sequence of the K. pneumomiae (Arnold et al., 1988) and the work of Wang et al. (1989) on cloning of the whole nif genes of K. oxytoca, reveals that the alteration in base sequence cutting by PstI in nifE region might be approximate at nucleotide (K. oxytoca vs K. pneumoniae) 6-11 instead of 72-77 (66 bp) and 1505-1510 instead of 1026-1031 (479 bp) from the start codon. This result is highly consistent with the previous work of Wang et al. (1989).

In SmaI cutting, the difference of fragment size is detected by all Fa, Fb and Fc probes. In the same manner, the alteration in base sequence cutting by SmaI of nifJ region might be approximate at nucleotide 4438-4443 instead of 338-343 (4100 bp) from the start codon.

As the hybridization of RFLP patterns with all nif structural genes of K. R15 and K. R17 are the same as K. oxytoca NG13, it might be concluded that the nif structural genes of K. R15 and K. R17 are strongly homologous and organized in the same order as in K. oxytoca NG13 and also K. pneumoniae. There is also strong evolutionary conservation between the nif genes of the K. oxytoca NG13; R15; R17 and K. pneumoniae M5a1.

Southern hybridization by glnA probe reveals the high homology of glnA gene between the associative Klebsiella and K. pneumoniae with the same RFLP patterns after cutting with BamHI, BglII, EcoRI, HindIII,

PstI, SalI and SmaI except XhoI. Since only one difference in fragment size is obtained from XhoI cutting (23.4 kb in K. oxytoca, 17.0 kb in K. pneumoniae), and there is no restriction site of XhoI in glnA gene, so this difference in cutting is not in glnA gene. From the restriction map of glnA ntrBC of K. pneumoniae (Figure 1.2 a), this result implies that the difference detected by XhoI cutting is not in this glnA ntrBC region, which is the common important region of nitrogen regulation system, but could be upstream or downstream from this region within 6 kb. This result also indicates the strong evolutionary conservation between the glnA ntrBC region of the associative K. oxytoca NG13; R15; R17 and K. pneumoniae M5a1, and other enterobacteria such as E. coli, Salmonella typhimurium and Klebsiella aerogenous (Alvarez-Morales et al., 1984).

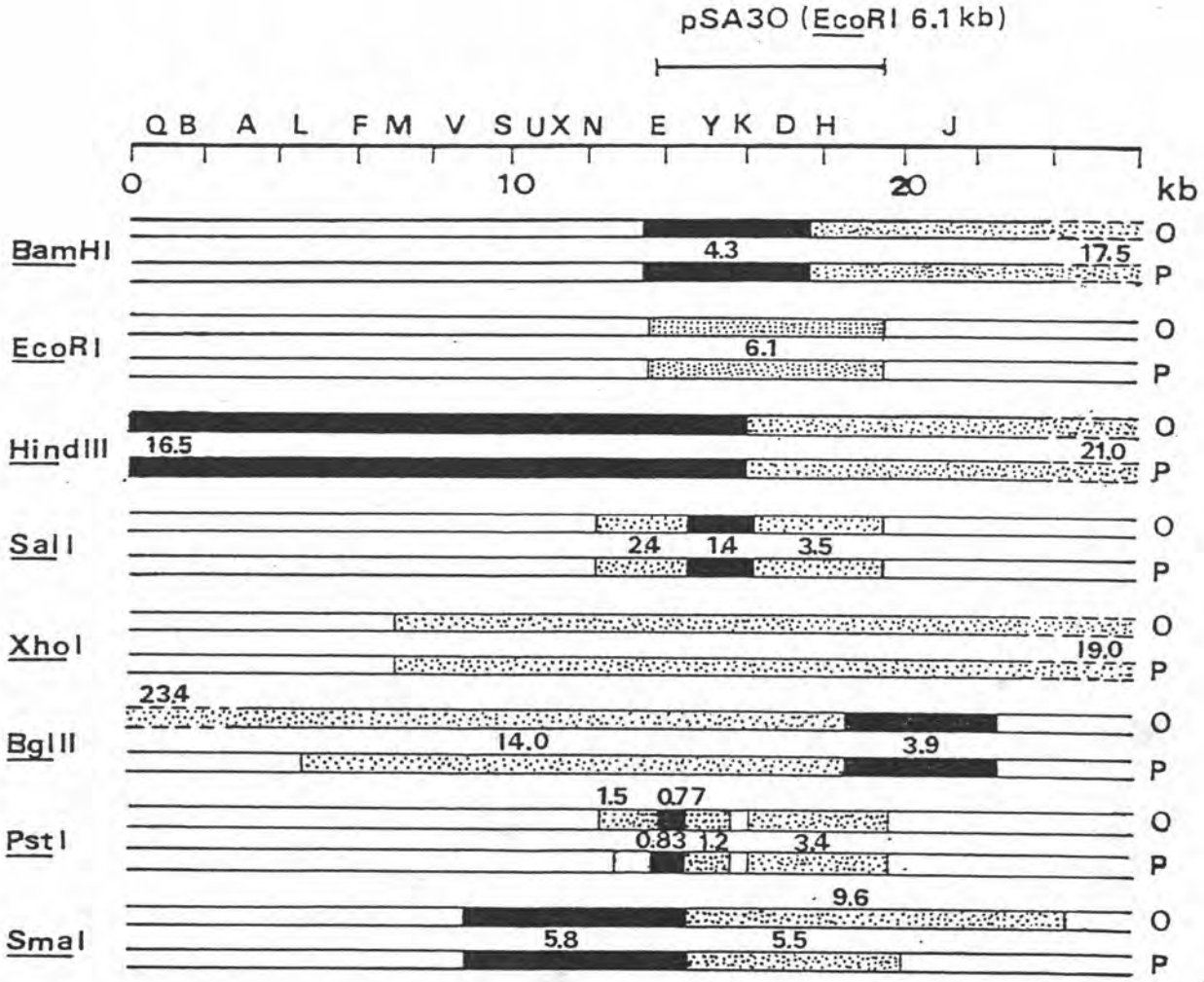
Conclusion

Both associative Klebsiella R15 and R17 isolated from the rhizosphere of rice CV RD7 and RD6 are similar to Klebsiella oxytoca NG13 in the following aspects :

1. All of them : K. oxytoca NG13, K. R15 and K. R17 contain no plasmid, and their nif structural genes are located on the chromosome.

2. The restriction patterns of BamHI, BglII, EcoRI, HindIII, PstI, SalI, SmaI and XhoI cutting in these 3 associative Klebsiella strains are strongly homologous. But they are remarkably different from K. pneumoniae M5a1, the free-living Klebsiella.

3. RFLPs obtained from all 8 enzyme-cutting when using ^{32}P-labelled nif structural genes as probes (pSA30, Fa, Fb and Fc) are the same among 3 associative strains NG13, R15 and R17, but differ from that of K.pneumoniae M5a1 when using BglII, PstI and SmaI. The differences reside in nifL, nifE and nifJ respectively. The restriction map of the nif gene clusters of these K. oxytoca strains : NG13, R15 and R17 as compared with K. pneumoniae M5a1 cited from Wang et al. (1989), Arnold et al. (1988) and obtained from this study are shown below:



This diagram shows the restriction map of the nif genes of K. oxytoca NG13 and K. pneumoniae M5a1. The upper bar (O) of each row shows nif DNA from K. oxytoca NG13, and the lower bar (P) that from K. pneumoniae M5a1. The alterations in restriction sites within the nif region marked by the hatched solid bars are demonstrated by this thesis.

4. Comparison of RFLP by glnA probe exhibits identical hybridization patterns of all 4 Klebsiella strains when cutting with 7 enzymes with restriction sites in the glnA ntrBC region. The 3 associative strains differ from K. pneumoniae in RFLP pattern only by XhoI cutting which is outside the glnA ntrBC region.

5. All of the 3 associative strains show strongly evolutionary conservation in *nif* genes and *glnA* *ntrBC* with *K. pneumoniae*. So, it is most likely that the regulation in nitrogen fixing and central nitrogen metabolism of these 3 associative strains should be the same as *K. pneumoniae* M5a1, the best known free-living diazotroph.