CHAPTER IV

DISCUSSION

All strains of bacteria isolated from Thai soil : Azotobacter and Azospirillum, which were studied in this thesis could be able to fix atmospheric nitrogen and used as sole nitrogen source because they could grow on nitrogen-free medium (Table 4 and 5). These results strongly indicated that all strains of Azotobacter and Azospirillum should be able to synthesize nitrogenase complex. Therefore, they should contain nif structural genes which code for nitrogenase.

Three procedures of plasmid detection, modified Eckhardt's method, large plasmid isolation and rapid alkaline extraction were used in this study. The advantages of the modified Eckhardt's method were using less reagents and was less time consuming. The plasmids were gently released from the spheroplasts by SDS lysis during the first period of electrophoresis and then the covalently closed circular plasmids migrated through pore of agarose gel without facing any drastic conditions. That means, the plasmids obtained from the modified Eckhardt's method should be the intacted form (supercoiled form). The plasmid lose during the procedure should be limitted since small and large plasmids (pBR322 , 4.36 kb; pSA30, 10.9 kb and pRD1, 86 kb) could be detected from this procedure (figure 4). However, the sensitivity of this method is limitted since only small amount of cells can be applied to agarose gel. Therefore plasmid

that present in very low copy number might be difficult to detect. Since generating of plasmids using modified Eckhardt's method depend on creation and subsequence lysis of spheroplasts, however, we found that freez and thaw step of cell pellet were promoted the creation and lysis of spheroplasts (data not shown). This method provides preliminary data about the plasmid content of bacteria.

For the large plasmid isolation's method, spheroplasts which were generated after lysozyme treatment will be lysed by SDS and heat treatment. After alkaline denaturation and neutralization were performed, membrane - chromosome complexes were removed by salt precipitated. Because under alkaline condition (pH 12.1-12.3) and neutral condition, plasmid remained unaffected while chromosomal DNA was diminished due to the highly denaturing conditions above pH11 (Record, 1967 and Zimmes, 1968). The secondary structure of linear DNA was completly denatured and was unable to renature under these condition. However, the conditions offered by this method were very gently, so, only one band of one plasmid (should be supercoiled form) was seen on agarose gel electrophoresis. When compared to that of modified Eckhardt's method, more reagents and time were consumed. Ribosomal RNA were contaminated in the preparation because RNase was not added in this method. From the results, the largest plasmid of A.braselense Sp7 and Azospirillum spp.A12 could not be clearly demonstrated in this method. It might be that the copy number of these plasmids were lower than the others, and because the extracted yield was very low, which was approximately 40 ng/ml culture. However, the method can be utilized for detection and preparation of plasmid because only one band could be seen from one specie of plasmid (pRD1) on agarose gel (Figure 5, lane 1). In addition, not only large

plasmid (> 86 kb) but small plasmids (11 and 9 kb) can also be isolated (Figure 5, lane 2 and 3). The disadvantages of the methods were low extracted yield, using much reagents and time consuming (2 days).

The last performed method of plasmid detection was rapid alkaline extraction. As compared to the first two methods, the conditions offered by this method were quite drastic. The plasmids were exposed to such the alkaline and high salt condition. Thus, the alternation from supercoiled to relaxed form sometimes happened to most plasmid extracted by this method. From our experiment, the large plasmid (more than 27 kb in size) could not be extracted. The advantages of the method were using less reagents and time consuming (2 hr.),and high extracted yield was obtained. Moreover, only small amount of chromosomal DNA and ribosomal RNA are contaminated in the preparation. So, this method was suitable method for large scale preparation of small plasmid.

From three procedures of plasmid detection, modified Eckhardt's method (figure 4), large plasmid isolation (figure 5) and rapid alkaline extraction (figure 6) concluded that all five strains of Azotobacter did not contained any plasmid. Therefore, we suggested that the nif structural genes might located on their chromosomes. This suggestion agreed with the previous report, derivatives strains of Azotobacter chroococcum cured of various of its six plasmids showed no loss of Nif^+ phenotype and one soil isolated containing no detectable plasmid was Nif^+ (Kennedy, Cannon et al., 1981), thatmeans the nitrogen fixation genes might located on chromosome of Azotobacter. However, this suggestion is inconsistent with the report of Yano et al.(1982)

whose studied in Azotobacter vinelandii AVY5 showed that genes homologous to nif HDK of Klebsiella pneumoniae carried on a 200 kb plasmid.

In Azospirillum, the acceptable result should be obtain from the modified Eckhardt's method (figure 4). Three out of four strains were found to contain one to four plasmids, ranging from approximate 9 to 100 kb in sizes. However, no homology between Klebsiella pneumoniae nif structural genes and the Azospirillum plasmids was obtained (Figure 26). Therefore, the nif structural genes of Azospirillum should be located on their chromosome. The corresponding results were previously reported by Franche and Elmerich (1981), Wood et al. (1982), Uozumi et al. (1982), Henlin et al. (1982), Singh et al. (1982) and Planzinsky et al. (1983). They showed that at least one to six molecular species of plasmids with a wide range of molecular weight were detected in many strains of Azospirillum. Elmerich(1983) and Planzingsky et al. (1983) also reported that plasmid location for nif genes was not observed in Azospirillum.

At the present time, the plasmids observed in Azospirillum appear to be cryptic. This suituation is very similar to that described some years ago in Rhizobium (Bechet et al., 1978 and Casse et al.,1979). Studies with Rhizobium have established that the functions related to nodulation and N_2-fixation are plasmid controlled. Furthermore, nif structural genes and nod genes have been identified on a specific plasmid of many Rhizobium spp.(Hirch et al., 1980, Banfalvi et al., 1981 and Masterson et al., 1985). Thus the plasmid function in Azospirillum is very interesting, in particular relationship with nitrogen fixation or association with plant roots.

In the restriction endonuclease digestion, accurate interpretation of the restriction pattern of the result would rely on two factors. Firstly, chromosomal DNA which were used for digest with restriction endonuclease would be high molecular weight DNA and, secondly, complete digestion would be performed.

In general, extracted chromosomal DNA which were prepared from almost cell type would not be intact form, since at cell lysis step and among isolation procedure, chromosomal DNA would be sheared by shear force. In the gentle condition of isolation, DNA fragment would nearly similar size, approximate 75 kb in size (Brock, 1974). The isolated chromosomal DNA obtained from this study are high molecular weight DNA fragments, since, only one nearly sharp band of slow mobility DNA on agarose gel electrophoresis was obtained, and plasmid DNA and RNAs did not contaminate in the preparation solution(Figure 7).

In our experiment, chromosomal DNA extracted from Azotobacter spp. which were grown in Burk's nitrogen-free medium could not be digested with any restriction endonuclease, whereas the extracted chromosomal DNA from Azospirillum spp. or E.coli obtained from the same procedure could be digested. We suggested that slime which produced from Azotobacter may interfere the digestion by shield the recognition sequence of restriction endonucleases. It has been reported by Postgate (1982) that Azotobacter will produce much polysacharide and polyhydroxy butyrate in nitrogen limited condition. This was corresponding to our experience that glutinous colonies of Azotobacter were observed on nitrogen-free agar plate medium and it was decreased by change the medium from nitrogen-free medium to rich medium (RM medium). The inhibitory effect of slime on restriction endonuclease digestion was strongly support by the evidence that

extracted chromosomal DNA from Azotobacter which were grown in RM medium could be readily digested with those restriction endonucleases.

Moreover, in our experiment the extracted chromosomal DNA of all bacterial species were purified by precipitation in presence of 2.5 M. ammonium acetate with absolute ethanol. Some inhibitors of restriction endonuclease was eliminated. Thus, the purified DNA could be completely digested with the smaller amount of restriction endonuclease (figure 8).

Observation of restriction endonuclease patterns of Azotobacter and Azospirillum chromosomal DNA reveals a number of discrete bands appearing against a smeared background. The result indicated very high reiterated sequences in the chromosome of Azotobacter and Azospirillum. Reiterated sequences are not common in bacteria, but they have been found in some strains of Rhizobium spp. (Better et al., 1983) and also Streptomyes spp. (Ono et al., 1982), Halobacterium halobium (Sapienza and Doolittle, 1982), and Psendomonas syringal p.v. "phaseolicola" (Szabo and Mills, 1984). We suggested that the presence of the reiterations in Azotobacter and Azospirillum may be related to genetic rearrangements of these organisms.

Moreover, the position and intensity of ethidium bromide staining bands were characteristic of the enzyme used and the source of DNA. These banding patterns showed reproducible differences among various species, surprisingly, down to strain differences (figure 9 to 17). Although Azotobacter vinelandii KT1 and Azotobacter vinelandii KT2 ; Azotobacter chroococcum KT and Azotobacter chroococcum NP ; Azospirillum brasilense Sp7 and Azospirillum brasilense A2 were classified by cell morphology, nutritional characteristic and some

special properties into the same species, different didested pattern could be seen between the two strains of the same species. Until recently, the classification of bacterial species relies mainly on cell morphology, nutritional characteristics and some special properties (Buchanan and Gibbons, 1974). Nutritional characteristics have been extremly useful, but it is clear from studies of perfect general that the nutritional variation can occur among members of a clearly defined species, for example Azotobacter vinelandii KT1 could be grown in LB medium whereas Azotobacter vinelandii KT2 could not be grown in this medium (Table 4), since a single mutation can cause loss of the ability to utilize any one carbon source or their nutritional characteristics. Cell morphology is useful criterion and is propably significantly less sensitive to mutation than the nutritional spectrum is. Moreover, for identification of bacterial strains may be performed by using ELISA technique which is difficult and is time consumption technique. Thus, our results lead the idea to identify the species and strains of Azotobacter and Azospirillum which were high reiterated sequences in their chromosome by examination of restriction endonuclease fingerprinting of chromosomal DNA, since the restriction endonuclease fingerprinting is the most sensitive to mutation than the nutritional spectrum and cell morphology is. The similar method has been studied for recent year to identify strains and species of yeast, Candida spp. (Magee et al., 1987).

From the result of Hae III digested pattern showed that small fragments lesser than 1 kb in size were obtained from almost strains studied. Thatmeans Azotobacter and Azospirillum DNA have a high composition of G and C base, since the recognition sequence of Hae III is GGCC. This result agreed with the previous reports, which reported

that Azotobacter spp. have a DNA G+C content of 53-70 per cent (Becking et al., 1974) and Azospirillum spp. have a DNA base composition of 67-70 mole per cent 6+C (Tarrand et al., 1978)

Labelled probes were prepared by using "nick translation". The reaction was occured by the activity of DNaseI and DNA polymerase, 3-hydroxyl terminus is introduced into the DNA duplex by DNaseI then nudeotide on the 5-phosphate side of the nick would be removed by the 5- exonuclease activity of DNA polymerase. New deoxy - nucleotide triphosphates including (α-^{32}p) -d ATP will be inserted by the polymerase activity of the DNA polymerase to replace the one removed by exonuclease. Thus rate of reaction is under kinetic rate of enzymes reaction, so, kinetic curve might be performed in almost reaction and the reaction was stopped at stationary phase (third order) of the kinetic reaction. If we did not stop the reaction, very tiny fragments of labelled DNA will be obtained and it could not be used as probe.

In general, the hybridization process can be divided into three steps; 1) Prehybridization 2) Hybridization and 3) Washing off the non-hybridized probe from the nitrocellulose filter. The solution and the condition were used in these steps are the most appropriate condition. The prehybridization step was designed to block all the sites on the nitrocellulose filter that would bind the probe non-specifically, thereby reducing the background. The nonspecific sticking of single - stranded DNA can be prevented by preincubating the nitrocellulose filter in an albumin solution which compose of BSA, ficol and polyvinylpyrrolidone (Denhardt, 1966).

Nucleic acid hybridze most efficiently at approximately 25 °C below the temperature at which they are 50% denatured (melting

temperature; Tm) (Studier, 1969). From the formular Tm= 69.3+0.41 (% G+C) (Marmur and Doty, 1962). Since the average G+C per cent of Azotobacter and Azospirillum range from 53 to 70, so that the melting temperature (Tm) of Azotobacter and Azospirillum DNA is approximate from 90 to 100 °C. Thus, the hybridization should be conducted at 65-75 °C in aqueous solutions. Since each increase of 1% formamide concentration in the reaction solution lowers the Tm of a DNA duplex by 0.7 °C (McConaughy et al., 1969 and Casey and Davidson, 1977), hybridizations involving this reagent (50% formamide) are conducted at 30-40 °C. Therefore, hybridization reaction in this study which were performed in 50% formamide at 42 °C are very stringent condition. Moreover they are easier to set up, present less of an evaporation problem, and are less harsh on the nitrocellulose filter than is hybridization at 65-75 °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), but the kinetics of nucleic acid reassociation are faster in the smaller volume of hybridization solution. Moreover, the sensitivity of detection of hybridization bands are increased by the large amount of DNA (4 μg) on the nitrocellulose filter was used. In the washing step, temperature and salt concentration are chosen as stringent condition also, since in general washing should be carried out at 10-15 °C below the Tm of the hybrid (Maniatis, 1982).

All steps of hybridization process is stringent condition, so that the hybridization bands which were detected in this study might be the hybrid molecule of nif structural genes of Klebsiella pnenmoniae and Azotobacter spp. or Azospirillum spp., certainly.

From our studies show that Southern hybridization between *nif* structural genes of *Klebsiella pneumoniae* and restriction fragments of chromosomal DNA from various species and strains of *Azotobacter* and *Azospirillum* show many differences. The number and size of the restriction homologous fragments were different from one strain to another. This result has been used to provide a preliminary information about the *nif* genes organization which they are much difference among these strains. The results also indicated that *nif* structural genes of all nine strains located on their chromosomes.

The hybridization of *nif* structural genes patterns of *A.chroococcum* and *A.vinelandii* strains which were studied in this thesis are inconsistent with the patterns of others strains of *A.chroococcum* with those reported by Jones *et al.* (1984) and Brigle *et al.* (1985), and *A.vinelandii* with reported by Ruvkun and Ausubel (1980) and Bishop *et al.*, (1985). However, the suggestive evidence report by Jones *et al.* (1984), Brigle *et al* (1985), Ruvkun and Ausubel (1980) and Bishop *et al.* (1985) also differed. Whereas *Azospirillum brasilense* Sp7 which is reference strain show similar result as report by Quiviger *et al.* (1982). Thus, we suggest that the hybridization pattern of *nif* structural genes may be used to differentiate quite easily among these strains of *Azotobacter* and *Azospirillum*, and use to confirm the result from restriction pattern on ethidium bromide-stained gel which we suggest that its can be used to dentify the strains of bacteria as mension earlier.

Moreover, one hybridization band was detected from the extracted chromosomal DNA of all strains, at least in one restriction hybridzed pattern (Table 7). For example, 5 kb of HindIII fragment

of Azotobacter vinelandii KT1; 5 kb of BamHI fragment of Azotobacter paspali B; 4.3 kb of PstI fragment of Azospirillum spp. A12 ; 3.8 kb of Hind III fragment of Azospirillum brasilense Sp7 ; 6.9 kb of BamHI fragment of Azospirillum brasilense A2 and 6.2 kb of BamHI fragment of Azospirillum lipoferum SpMRA1. These fragments should be long enough to code for nif H, nif D and nif K. Therefore, we suggested that the nif structural genes of Azotobacter spp. and Azospirillum spp. should be cluster on their chromosome. This suggestion agreed with the previous reports of Azotobacter vinelandii (Brigle et al, 1985 and Beynon et al, 1987) and Azospirillum brasilense (Quiviger et al., 1982), they reported that the nitrogenase structural genes are organized as a single operon in these species and the arrangement of genes is similar to that of Klebsiella pneumoniae. Our data may also be interpreted that nitrogenase structural genes are not reiterate, Since only one of hybridization band is observed at least in one restriction hybridization pattern. However multiple copies of nif genes have been observed in a number of diazotrophs such as Rhodopsennomonas spp. (Scolnik and Haselkorn, 1984), Anabaena spp. (Rice et al., 1982), Rhizobium phaseoli (Quinto et al., 1985), Azotobacter chroococcum (Johnes et al., 1984) and Azotobacter vinelandii (Jacobson et al., 1986).

Surprisingly, some restriction digestion of chromosomal DNA reveal undetectable hybridization band. For example, SmaI digested chromosomal DNA of Azotobacter vinelandii KT1, Azotobacter vinelandii KT2 and Azotobacter paspali B and PstI digested chromosomal DNA of Azospirillum brasilense A2 and Azospirillum lipoferum SpMRA1. Whereas the others restriction digestion of these strains showed strong hybridization band. It is estimated that the nitrogenase structural

genes have many clevage sites for these enzymes, so tiny restriction fragments of nitrogenase structural genes are obtained. Then, they did not retaine on the agarose gel in the electrophoretic condition. The similar evidence has been observed in Rhizobium japonicum which reported by Kaluza and Hennecke (1982) and Kaluza et al.(1983).

The hybridized restriction fragment reported here (Table 7) are very useful for nitrogenase structural genes cloning of these nine strains. The appropriate restriction endonuclease which provide a single hybridizing band should be chosen for cloning experiments. For example, SmaI should be used for nitrogenase structural genes cloning of Azotobacter chroococcum NP, HindIII should be used for Azotobacter vinelandii KT1, PstI should be used for Azospirillum spp A12 and BamHI should be used for Azospirillum brasilense A2 and Azospirillum lipoferum SpMRA1. It is interesting to clone these fragments which should be carrying the whole of nitrogenase structural genes. It will be more understanding in the organization and characterization of nitrogenase genes of these organisms.

Determination of nif structural genes homology was performed by hybridization of Klebsiella pneumoniae nif structural genes probe with various chromosomal DNA of Azotobacter and Azospirillum spp. using the dot blot hybridization technique. This technique was used since a large number of different nucleic acids may be spotted and fixed on the same nitrocellulose filter sheet for testing by a single probe (Kafatos et al., 1979). Dot blot hybridization is very useful and advanced, for example, it has been used to rapid screening tests and is particularly useful as semiquantitation of DNA or RNA (Thomas, 1980). It has been successfully employed as a sensitive and

reproducible assay for the presence of rotavirus in human stool suspensions (Flores et al., 1983), furthermore, a modified procedure has also been used to detect and quantitate human cytomegalovirus in urine (Chou and Merigan, 1983). In this study, modified dot blot hybridization technique was used to determine the DNA homology, between nif structural genes fragments of Klebsiella pneumoniae and chromosomal DNA of Azotobacter or Azospirillum. DNA was fixed onto nitrocellulose filter and then hybridized with ^{32}P-labelled probe in appropriate condition as described for the Southern blot hybridization. This allowed not only estimate of hybrided DNA by autoradiography, but it also enable direct counting of the radioactivity on small pieces of filter containing the hybrided molecule of nif structural gene fragments.

The relationship between amount of chromosomal DNA and radioactivity was obtained and then the relative homology of various nif structural gene fragments can be calculated from the Δcpm/Δng DNA at the first order of the hybridization kinetic curves. This modified procedure has many advantages. Firstly, a little DNA sample was used in test, no greater than 1 μg. Secondly, a large number of different DNA can be tested in one experiment, in our experiment, the homology study between a single probe and more than ten species of DNA comparable on the same hybridization bag. So, this procedure lends itselt to rapid determination. Thirdly, sensitive and reproducible procedure was obtained, as little as 50 μg of Klebsiella pneumoniae chromosome which contained approximate 10 pg of nif structural gene can be detected very easily in our experiment.

It is evident from the hybridization experiments that the homology between Klebsiella pneumoniae nif structural genes and

Azopirillum DNA was found limited only to nif H and nif D, whereas in three strains of Azotobacter spp. the nomology was detected with nif H, nif D and also nif K, but nif K region show the lowest degree of homology. On the contary, the homology appear similar among the three genes in Azotobacter vinelandii KT1 and Azotobacter paspali B. The result illustrated that nif H and nif D sequences are very highly conserved in nitrogen-fixing bacteria. The similar evidence of the homology was limited only to nif H and nif D was found in many Rhizobium species (Ruvkun and Ausubel, 1981 ; Banfalvir et al., 1981 ; Hennecke, H, 1981; and Parkash et al., 1981) and in Anabaena 7120, the same degree of homology was observed with nif H, nif D and nif K of Klebsiella pnenmoniae (Rice et al., 1982). The high degree of conservation of nitrogenase structural genes between these diverse microorganisms indicate that the nitrogenase structural genes from Klebsilla pnenmoniae, Azotobacter spp. and Azospirillum spp. arose from a common ancestral origin, but the nif K gene appears low homology suggests that this gene product is not under the high level of selection by evolution in nature.

SUMMARY

1. No plasmid was found in all five stains of Azotobacter spp. Whereas one to four plasmid, ranging from approximate 9 to 100 kb in sizes, were randomly located in three strains of Azospirillum spp.

2. All of the nitrogenase structural genes of Azotobacter and Azospirillum were located on their chromosomes.

3. Very high reiterate sequences were observed in chromosome of Azotobacter and Azospirillum, and discrete bands of restriction endonuclease patterns showed reproducible difference among the species and strains.

4. Structural genes of nitrogenase locate in difference restriction fragment in all strains of both genera.

5. The homology of nif H, D and K of Azospirillum spp. was found to limit only to nif H (3.1-8.0%) and nif D (3.0-14.4%) as compare to Klebsiella pneumoniae. Whereas, for Azotobacter spp., the the homology was found in all three fragments of nif H (3.4-11.9%), nif D (3.4-15.9%) and also nif K (5.0-9.3%).