CHAPTER 1

INTRODUCTION

Biological nitrogen fixation

The biological availability of nitrogen, phosphorus and potassium is of considerable economic importance because they are the major plant nutrients derived from the soil. Among these three nutrients, nitrogen stands out as the most susceptible to microbial transformation. This element is a key building block of the protein and nucleic acid molecules upon which all life is based. Plants and microbes make their organic nitrogenous matters from nitrates by passing through nitrite and ammonia whereas animals make their nitrogen-containing molecules directly or indirectly from plants or microbes. The available form of nitrogen for plants are ammonium (NH_4^+) and nitrate-nitrogen (NO_3^-). Because of the critical position of the nitrogen supply in crop production and soil fertility, a deficiency markedly reduces yield as well as quality of crops; and because this is one of a few soil nutrients which is lost by volatilization as well as by leaching, it requires continual conservation and maintenance. Recent years have witnessed a remakable expansion of the fertilizer industry, yet only a portion of the agricultural need for nitrogen comes from chemical fertilizers, it production by industrial process is dependent on fossil fuel. Precipitation may add several kilograms per rai each year as ammonium or nitrate. Thus, biological reduction of atmospheric nitrogen to ammonia is an interesting process.

Nowadays, we focus on enhancing the efficiency of nitrogen fixing ability of nitrogen-fixing bacteria, energy-conserving way to reduce the nations reliance on chemical nitrogen fertilizer for maintaining its agricultural productivity. Genetic studies of nitrogen-fixing bacteria are essential for identifying the genes involved in nitrogen fixation. The better understanding of this processes might help to improve the nitrogen fixing ability of the nitrogen fixer in terms of crop yield.

The nitrogen-fixing bacteria

The ability to fix atmospheric nitrogen or dinitrogen is fairly widely distributed among the procaryotic microorganisms and they vary in complexity from the primitive bacterium, Clostridium, through the physiologically and genectically intricate legume root nodule symbiosis. So, the nitrogen-fixing bacteria are classified into three groups, they are obligatory symbiosis, associative symbiosis and asymbiosis or free-living nitrogen fixing bacteria (Burns and Hardy, 1975). More than one hundred of the species of microorganisms are reported as nitrogen-fixer (Postgate, 1982), but the agriculturally important are legume-Rhizobium symbiosis and the association of microorganisms with roots of plants. The associative bacteria differ from Rhizobium in that they are not localized in specialized nodules or protuberances on the root, without formation of differentiated structure, instead they grow on the roots surface and make use of carbonaceous exudate to satisfy their energy demands. The bacteria in this group are member of three genera : Azotobacter, Azospirillum and Beijerinckia. They are especially abundant on roots of corn, sugar cane, wheat, sorghum and some species of grasses. They are

somehow linked closely to the roots inasmuch as gentle washing does not dislodge the N_2-metabolizing activity (Alexander, 1977). The general knowledge about Azotobacter and Azospirillum are as follows :-

Azotobacter

Azotobacter species are aerobic, heterotrophic bacteria. The cells are quite large, contain granules of poly-beta-hydroxybutyrate and the average G + C per cent ranges from 53 to 70. The genus Azotobacter comprises of four species : Azotobacter chroococcum, Azotobacter vinelandii, Azotobacter paspali and Azotobacter beijerinckii, all species can fix atmospheric nitrogen in the free-living state in air or under microaerobic condition (Becking et al, 1974). Bacteria in this species is usually grown in the modified Burk's medium (Strandberg and wilson, 1968). All of them tend to be sensitive to acid pH, high phosphate concentration and temperature above 35 °C. Morphologically-distinctive encysted form is usually occured in old populations of Azotobacter. Encystment can be induced by culture in the presence of agents such as n-butanol. Cysts have somewhat enhanced the resistance to stresses such as heat and desiccation (Sadoff, 1975). During growth, a green fluorescent and a dark-brown pigments are produces by Azotobacter vinelandii and Azotobacter chroococcum, respectively. In Azotobacter vinelandii, the production of a green fluorescent pigment is increased by growing in iron-limitted media (Corbin and Bulen, 1969). It has been reported that both Azotobacter vinelandii and Azotobacter chroococcum had a very large and variable genome size : their DNA content could be up to 50 times that of E.coli in growing culture and declining to ten-fold in old culture (Sadoff, Shinmel and Ellis, 1979).

Azospirillum

Bacteria, first described in 1922 by Beijerink and rediscovered in 1963 by Becking, were called under the name of Spirillum lipoferum. Their potential agronomic importance was raised when Dobereiner and Day (1976) described their association with plants from various genographical origin. Taxonomic studies led to the creation of a new genus : Azospirillum. This genus, defined by Tarrand et al. in 1978, comprises two species : A.brasilense and A. lipoferum. A third species A.amazonense was recently discovered (Magalhaes et al, 1984). Very little is known on the genetics of these bacteria and the molecular biology of their association with plants.

The bacteria are gram-negative aerobes, curved rod shape, with a polar flagellum and contain globules of poly-beta-hydroxybutyrate. They have a DNA base composition of 67-70 moles per cent G + C. The bacteria can grow on organic acids such as malate. Strains of A.lipoferum can utilize a large number of carbohydrates including glucose, which is not used by A.brasilense. A.amazonense can utilize saccharose. In addition, Azospirillum can grow very well in the complete medium, nutrient broth. Like those in Azotobacter, Azospirillum can form cyst in old cultures (Lamm and Neyra, 1981 ; Papen and Werner, 1982). In nitrogen-free minimum medium, the nitrogenase of Azospirillum can be expressed only under the microaerobic condition (Von Bulow and Dobereiner, 1975 ; Okon et al, 1976 ; Okon et al, 1977 and Barak et al, 1981).

The nitrogenase complex

The study of the biochemistry of nitrogenase has begun before 1960. Because in 1960 a dramatic advance was reported by Carnahan Mortenson, Mower and Castle, who obtained a cell-free extract of Clostridium pasteurianum which consistently fixed atmospheric nitrogen. By the end of the 1970s, nitrogenase consisting of two proteins had been extracted and purified from many microbes such as Clostridium pasteurianum (Carnahan et al, 1960), Klebsiella pneumoniae (Eady et al, 1972), Azotobacter vinelandii (Bulen et al., 1965), Azotobacter chroococcum (Kelly, 1969 a) and Azospirillum lipoferum (Okon et al., 1977). The resolved preparations were very similar in properties : one component is a protein of relatively high relative molecular mass (200,000 - 250,000) containing iron, molybdenum and labile sulphur ; the other is smaller protein (5,000 - 6,000) with iron and labile sulphur but no molybdenum. Both protein form aggregates and can be shown to consist of sub-units. By the study of Klebsiella pneumoniae (Kennedy et al, 1976); the nitrogenase are composes of two components, component I and II. Component I , a dinitrogenase or molybdoprotein (MoFe-Protein), consists of two α -and two β-subunits. The $\alpha_2\beta_2$ subunit structure is well established for component I; their polypeptides (α and β) are coded for by the distinct genes, nif D for α-subunit and nif K for β-subunit. The component II, is a dinitrogenase reductase or iron protein (Fe-protein) which consists of two α-subunit (α_2) (Tanaka et al., 1977), coded by nif H. By the study of Azotobacter vinelandii, the activity of nitrogenase required two nonheme iron proteins: component I and component II (Bulen and Le Comte, 1966). Component I which is also designated the MoFe-protein, has been crystallized (Burns et al., 1970). It is

a tetramer ($\alpha_2\beta_2$) of 245,000 daltons (Swisher et al, 1977) containing 2Mo atoms per molecule (Bulen and Le Comte, 1966) and two different molecular species of 61,000 deltons (α and β) (Swisher et al, 1977). Molydenum was found to associate with component I as a cofactor (Nagatani et al., 1974). Component II, also designated the Fe-protein, is a dimer formed of two identical subunits of 31,200 daltons, containing 289 amino acids (Hausinger and Howard, 1980). The enzyme of Azospirillum brasilense strain Sp7 was purified and an activating factor was found to be required for component II activity (Okon et al, 1977 and Ludden et al, 1978). Using antisera prepared against Klebsiella pneumoniae MoFe-protein and Fe-protein, cross-reacting material was precipitated from nitrogen-fixing culture of Azospirillum brasilense Sp7 (Elmerich, 1984).

In general, nitrogen fixation is controlled by three factors. Firstly, oxygen, the nitrogenase complex of all notrogen-fixing organisms or diazotrophs is extremely sensitive to oxygen, both components are rapidly and irreversibly inactivated upon exposure to air. However, nitrogenase in cell-free extracts of Azotobacter is relatively oxygen stable (Bulen et al, 1964 ; Kelly, 1969). Secondly, nitrogen sources, nitrogenase can not be synthesized when ammonia or easily assimilated nitrogen sources (e.g. urea, amino acid) are present in the growth medium. In Azotobacter, when ammonium was added to a nitrogen-fixing culture, nitrogenase biosynthesis stopped and the rate of decay of activity was much higher than the expected dilution rate (Shan et al., 1972 ; Drozd et al., 1972). Finally, molybdenum, it has been known for a long time that Mo is essential for nitrogen fixation. When Azotobacter vinelandii was grown in

Mo-deficient medium containing tungstate, they produced an inactive component I that could be reactivated *in vivo* by the addition of molybdate to the growth culture (Nagatani *et al*, 1974).

Bishop *et al* (1980) showed that certain mutants of *Azotobacter vinelandii* were unable to fix nitrogen in medium containing 10 μM Mo, however they were able to grow by nitrogen fixation when Mo was absent. Form this results they hypothesized the existence of a secondary (alternative) nitrogen-fixing enzyme which was expressed under condition of low Mo concentration. This hypothesis was supported by the work of Hales *et al*, (1986) who could isolate and characterize the second nitrogenase Fe-protein from *Azotobacter vinelandii*. They suggested that this second Fe-protein was associated with the alternative nitrogen-fixing system in *Azotobacter vinelandii*.

Genetics of nitrogen fixation

Geneticists refer to the genetic information enabling bacteria to fix nitrogen by the shorthand name *nif* : *nif*$^+$ and *nif*$^-$ will refer specifically to wild type and mutant *nif* genes ; Nif$^+$ and Nif$^-$ refer to ability or inability of mutants to fix nitrogen (Postgate, 1982).

Though genetics of nitrogen fixation was initiated from the *Azotobacter* (Fisher and Brill 1969 ; Sorger and Trofimenkoff, 1970), our knownledge on the nitrogen fixation (*nif*) genes comes from the study of *Klebsiella pneumoniae*. In this species the existence of a good transduction system and the construction of plasmid pRDI (Dixon *et al*, 1976 ; Postgate 1982) that carries the entire *nif* cluster of *Klebsiella pneumoniae* are most useful in the development of *nif* genetics.

In Klebsiella pneumoniae, 17 nif genes are present in its chromosome as a contiguous cluster orgainzed into 7 transcription units (Dixon et al, 1980 ; Postgate, 1982). The nif map is presented in figure 1. The products and functions of the nif genes of K.pneumoniae are shown in table 1.

The structural genes for nitrogenase nif H, nif D, nif K and nif Y of K.pneumoniae constitute one operon. Nif H codes for the identical subunit of component II of nitrogenase , nif D and nif K code for the α and β subunits of component I of nitrogenase ; the function of nif Y is still unknown. The nif HDKY operon and a part of nif E of K.pneumoniae have been cloned into plasmid pACYC184 (Cannon et al, 1979), the restriction map of this clone pSA30 is shown in figure 2.

Using the plasmid pSA30 as a hybridization probe, DNA homology was observed between nitrogenase structural genes from Klebsiella pneumoniae and total DNA from a large number of diazotrophs. Ruvhun and Ausubel (1980) reported that cloned nitrogen fixation genes from Klebsiella pneumoniae (pSA30) hybridized to DNA from 19 out of 19 widely divergent nitrogen-fixing bacterial strains but did not hybridize to DNA from 10 different non-nitrogen-fixing species.

As compared to K. pneumoniae little is known about genetic of nitrogen fixation of Azotobacter and Azospirillum. In early experiments,

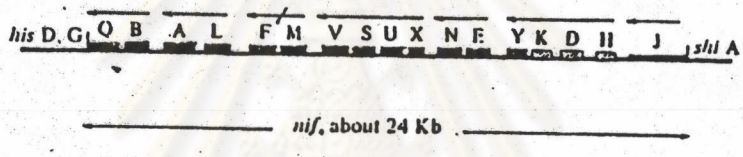

Figure 1. The nif gene cluster of Klebsiella pneumoniae M5a1.

The sketch showns the arrangement of the 17 genes accepted in 1981. The arrows represent the operons within the nif cluster and the direction of their transcription. The hatched genes are the structural genes for nitrogenase.

Table 1. The products and funtions of the *nif* genes of *Klebsiella pneumoniae*.

Gene	Rel. mol. mass of product ($X10^3$)	Function of product
Q	unknown	unknown
B	unknown	involved in synthesis or insertion of FeMoco of Kpl
A	57-60	regulatory
L	45-50	regulatory
F	17	codes for a flavodoxin
M	28	activates Kp2
V	42	modifies substrate specificity of Kpl
S	18-25	unknown
U	22-32	unknown
X	18	unknown
N	50	as B
E	40-46	as B
Y	19-24	unknown
K	60	codes for β sub-unit of Kpl
D	56-60	codes for α sub-unit of Kpl
H	31-39	codes for sub-unit of Kp2
J	120	electron input into nitrogenase

Note : A more detailed table is given by Kennedy *et al*., 1981.

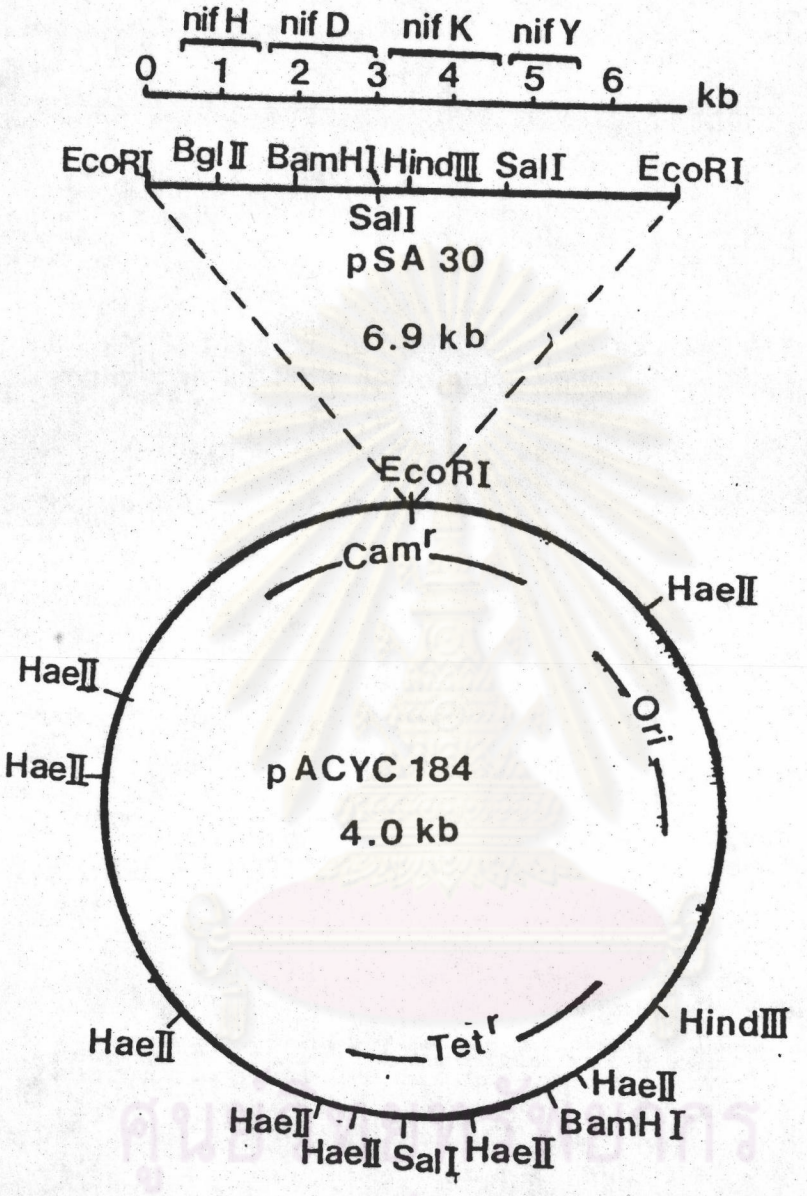

Figure 2. Physical map of pSA30.

A map of pSA30 demonstrating a number of restriction enzyme clevage sites and their position relative to the Klebsiella pneumoniae nif gene insert into EcoRI clevage site of pACYC184 and the gane coding for tetracycline resistance (Tet^r).

5EcoRI restriction fragments hybridize with pSA30 *nif* probe were detected. Homology with limited to *nif* H and *nif* D genes of *K.pneumonieae*. It is not clear why so many EcoRI restriction fragments were found to hybridize with the probe. (Ruvkun and Ausubel, 1980). The similar reuslt was presented by Jones *et al* (1984), pSA30 was used to identify restriction fragments bearing nitrogenase structural genes by hybridization to various restriction digests of genomic DNA from A. *chroococcum* and *A. vinelandii*. It was found that for each enzyme used, at least two bands of hybridization were seen for both species.

In the case of *Azospirillum*, the pSA30 *nif* probe hybridized with a single 6.7 kb EcoRI fragment of *A. brasilense* Sp7, whereas in *A. lipoferum* Br17, homology was found with two EcoRI fragments and with a single Hind III fragment (Quiviger *et al*, 1982).

Endogenous plasmid

Plasmid is extrachromosomal genetic element found in variety of bacterial species. It is double-stranded, closed circular DNA molecules that range in size from 1 kb to greater than 200 kb. Often, plasmide contain genes coding for enzymes that, under certain circumstances, are advantageous to the bacterial host. For example, it carries some genes that controlled the synthesis of specific enzyme(s) essential for bacteria, or some genes conferring resistance to antibiotics or drugs, in some cases, they also have some genes controlling toxins production. These genetic elements are sometimes nonessential for growth so that under many conditions they can be lost or gained without harm to the cell. In nitrogen-fixing bacteria, there is recent evidence that plasmid plays an important role in

determining the ability of Rhizobium to induce introgen-fixing nodules on legume roots (Banfalvi et al, 1981 and Masterson et al 1985). Moreover, some of the structural genes for nitrogenase (nif HDK) are present on some plasmids of Rhizobium spp. (Uogumi et al, 1982), such as Rhizobium japonicum (Masterson et al, 1985), Rhizobium meliloti (Banfalvi et al., 1981) and Rhizobium leguminosarum (Hirch et al, 1980).

In Azotobacter, the plasmid content has been variously reported. Sadoff et al (1979) reported that plasmids could not be detected in Azotobacter vinelandii. Robson (1981) also presented that no plasmid was found in Azotobacter vinelandii strain UW but some was found in other strains. However, Yano et al (1982) reported that Azotobacter vinelandii strain AVY15 carried genes homologous to nif HDK of Klebsiella pneumoniae on a 200 kb plasmid.

From the work of Robson et al. (1984), all of eight strains of Azotobacter chroococcum examined contained between two and six plasmids ranging from 11 to over 330 kb in size. In addition, they demonstrated their results of studying on the function of plasmid in Azotobacter chroococcum which lost some or more plasmids after curing by curing agent. Comparison between the plasmid arrays of the parent strain and the derivatives showed that the various cured derivatives could fix nitrogen since they can grow well in unsupplemented Burk's medium. The result indicated that the nif structural genes are on chromosome. Antibiotic sensitivity was also unaffected by the lost of plasmid.

Most strains of Azospirillum contain at least one to six plasmid species ranging from 6 to over 500 kb. Spontaneous loss (Franche and Elmerich, 1981) of some plasmids or curing by high temperature (Heulin et al, 1982) or by acridine organge treatment (Wood et al, 1982) was observed in Azospirillum. Phenotypic changes, in particular in glucose utilization (Heulin et al, 1982) and in heavy metals resistance (Wood et al, 1982), concomitant with plasmid loss were reported.

Detection of plasmid by Casse's alkaline lysis method and following by Southern hybridization with nif KDH and nif Q - K probes from Klebsiella pneumoniae, Uozumi et al (1982) found that Azospirillum lipoferum strain COC8 harbours a plasmid pTACOC8 (300kb), which showed homologies with nif KDH and nif Q - K. of K. pneumoniae.

OBJECTIVE OF THE THESIS

As mention earlier, the knowledge on the nitrogen fixation system of Azotobacter spp. and Azospirillum spp. were limitted, especially on genetic studies as compared to Klebsiella pneumoniae. There are no clear demonstration that a) the nif structural genes are located on plasmid on chromosome and b) which of the restriction fragments are carrying the nif structural genes, especially for strains which were isolated from Thai soil. Furthermore, it is interesting to know about the homology between the nif structural genes of Azotobacter spp. or Azospirillum spp. and Klebsiella pneumoniae. Therefore, the objectives of the thesis is to delineate the above questions. The study will be performed as follows :-

1. Detection of plasmid in Azotobacter spp. and Azospirillum spp. isolated from Thai soil. If they do not contain any plasmid, it would imply that the nif structural genes might be located on the chromosome.

2. If plasmid was found in Azotobacter spp., or Azospirillum spp., the homology between the plasmids from Azotobacter or Azopirillum and ^{32}P-pSA30 carrying nif structural genes of Klebsiella pneumoniae will be tested by Southern blot hybridization technique.

3. Localization of nif structural genes in Azotobacter and Azospirillum DNA (chromosomal or plasmid) by Southern blot hybridization technique, using the radioactive labelled pSA30, as the DNA probe. This result should show restriction patterns of nif structural genes on either chromosomes or plasmids of Azotobacter and Azospirillum.

4. Determination of homology between the nif structural gene fragments of Klebsiella pneumoniae and Azotobacter or Azospirillum by Dot blot hybridization techniques. This observation should be useful to understand the extent of divergence of each nif structural genes between Klebsiella pneumoniae and Azotobacter spp. or Azospirillum spp.