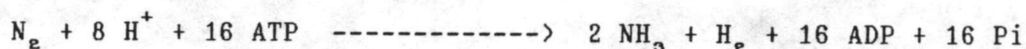
CHAPTER I

INTRODUCTION

1.1. Nitrogen-fixing bacteria

The growth and yield of agricultural crops depend, among other things, on the availability of nitrogen in the soil. It has been estimated that the need for nitrogen in agriculture will be doubled by the year of 2000 due to the increasing global population (Keeney, 1982). Although dinitrogen (N_2) are quite abundant (78%) in the air, they are not available for plants, only combined inorganic nitrogen such as NO_3^-, NH_4^+ can be taken up. These inorganic fertilizers are currently produced in factories by a chemical process called Harbor-Bosh process which requires fossil energy. Naturally molecular-dinitrogen in the air can enter the organic nitrogen pools in the biosphere through a certain groups of prokaryotes which via a reduction process mediated by nitrogenase complex that fixes N_2 by the expense of ATP, the chemical energy in living organisms, so called Biological Nitrogen Fixation (BNF). Due to the energy crisis, the price of chemical fertilizer and the risk of environmental pollution are increasing with each passing day; therefore, alternative approach are of great practical interest. One such approach is to use BNF for NH_3 production.



$$N_2 + 8\ H^+ + 16\ ATP \xrightarrow{\text{nitrogenase}} 2\ NH_3 + H_2 + 16\ ADP + 16\ Pi$$

A process examplified by a number of diversified genera of bacteria, each genus shows a certain specificity to host plants. A large number of dicotyledonous plants, from several different families, establish symbiotic associations with specific N_2-fixing bacteria. In these associations, the nodule structures develop on the root of the plant after the diazotrophs have infected the root of the plant. The root is the site where N_2 gas is reduced to ammonia, which is assimilated into amino acids; these are then used to synthesize other nitrogen-containing compounds (Hirsch, 1992). The best studied N_2-fixing bacteria-plant interaction, is that between plants of the Genus *Fabaceae* and members of the Gram negative *Rhizobiaceae*. Three genera; *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* specifically associate with legumes. Many legumes respond to *Rhizobium* inoculation by developing unique structure known as nodules on their roots. The development of a legume nodule in which rhizobia convert atmospheric N_2 into ammonia is a finely tuned process.

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1.2. <u>The nitrogenase complexes</u>

The ability to fix atmospheric N_2 of diazotroph is involved with the nitrogenase and the genetic expression of nitrogen fixation (*nif*) genes. The study of biochemistry of nitrogenase has begun before 1960. The nitrogenase, consisting of two components had been

extracted and purified from many microbes (Table 1.1). Both components form aggregates and can be shown to consist of subunits. By the study of *K. pneumoniae* (Postgate, 1982), the nitrogenase complex is composed of two components required for N_2-fixing activity, component I (Kp1) and component II (Kp2). Kp1, a dinitrogenase or molybdoprotein (MoFe protein), consists of two α- and β-subunits ($\alpha_2\beta_2$). The α- and β-subunits are coded by *nif*D and *nif*K respectively. Kp2, a dinitrogenase reductase or iron protein (Fe protein), consists of two identical subunits coded by *nif*H. These protein components are irreversibly inactivated by oxygen. An iron-molybdenum cofactor (FeMoco) containing Mo, Fe and S, the active site of nitrogenase; can be separated from the MoFe protein. Both a reductant and ATP are required for enzyme activity; approximately 15 moles of ATP are consumed per mole of dinitrogenase reduced. In view of this high energy requirement and the extreme oxygen sensitivity of the protein components, it is not surprising that nitrogenase synthesis is tightly controlled.

1.3. The nitrogen fixation (*nif*) genes and regulation of *nif* genes

To date, the best known free-living nitrogen-fixing bacterium is *K. pneumoniae*, which is closely related to *E. coli* and is therefore easily emended by all relevant methods developed for the latter. The details have been reviewed by Postgate (1982) and Merrick (1988). There are 20-21 *nif* genes involved in nitrogen fixation, located in

Table 1.1. Some organisms from which active nitrogenase has been extracted (Postgate, 1982).

Organism	Reference
Anabaena cylindrica	Stewart *et al.* (1969)
Azospirillum lipoferum	Okon *et al.* (1977)
Azotobacter chroococcum	Kelly (1968 a, 1969 a)
Azotobacter vinelandii	Bulen *et al.* (1965)
Bacillus polymyxa	Emerich and Burris (1978 b)
Chromatium vinosum	Winter and Arnon (1970)
Clostridium pasteurianum	Carnahan *et al.* (1960)
Corynebacterium autotrophicum	Berndt *et al.* (1978)
Desulfovibrio desulfuricans	Sekigushi and Nosoh (1973)
Klebsiella pneumoniae	Eady *et al.* (1972)
Mycobacterium flavum	Biggins and Postgate (1969)
Rhizobium japonicum	Koch *et al.* (1967)
Rhodospirillum rubrum	Bulen *et al.* (1965)

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contiguously cluster on the chromosome between *his*G and *shi*A, extending over 23 kb in seven operons (Figure 1.1 a.). Fifteen of these genes are responsible for the production of enzyme nitrogenase, which can reduce molecular nitrogen to NH_3; among these are three structural genes H, D and K, two regulatory genes, L and A and two other genes, F and J which provide proteins for electron transfer (Table 1.2.). The *nif*HDKYT operon and a part of *nif*E (6.11 kb *Eco*RI-fragment) of *K. pneumoniae* has been cloned into *Eco*RI site of plasmid pACYC184 (Cannon *et al.*, 1979), the restriction map of this clone pSA30 is shown in Figure 1.1 b. Using the plasmid pSA30 as a hybridization probe, DNA homology between *nif* structural genes from *K. pneumoniae* and other diazotrophs can be observed.

Although, the *nif* genes of most nitrogen-fixing bacteria are located on chromosome , there are evidences that plasmid plays a role in determining the ability of *Rhizobium* to induce nitrogen fixing nodules on legume roots (Banfalvi *et al.*, 1981 and Masterson *et al.*, 1985). In addition, the structural genes of nitrogenase (*nif*HDK) were reported on plasmids of many *Rhizobium* spp. (Uozumi *et al.*, 1982) such as *R. japonicum* (Masterson *et al.*, 1985), *R. meliloti* (Banfalvi *et al.*, 1981) and *R. leguminosarum* (Hirsch *et al.*, 1980). In some associated diazotrophs, the plasmids of several species have been isolated and detected from *Azotobacter vinelandii* strain AVY15 (Yano *et al.*, 1982), *A. chroococcum* (Robson *et al.*, 1984), *Enterobacter agglomerans* (Singh *et al.*, 1988), except *A. vinelandii* strain UW (Robson, 1981). *A. vinelandii* strain AVY15 carried genes homologous to

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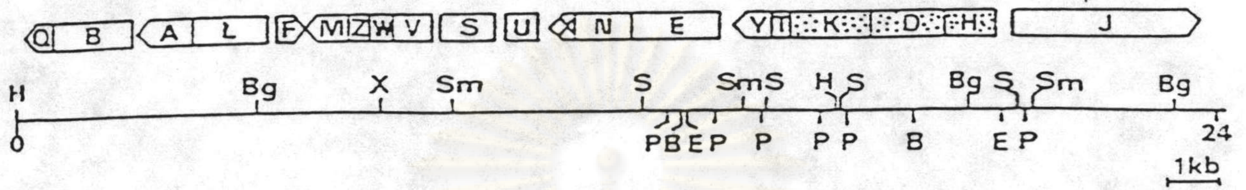
a)



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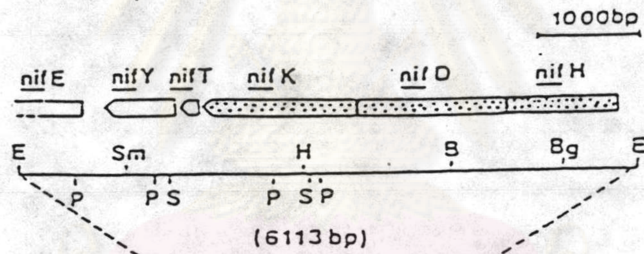

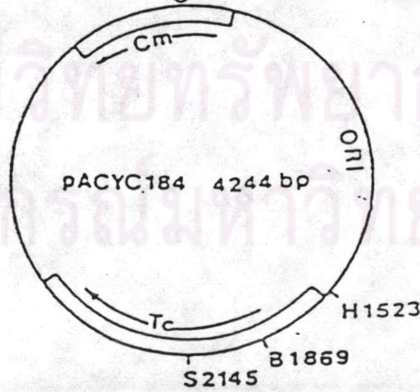

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Table 1.2. The products and functions of the *nif* genes of *Klebsiella pneumoniae* (Arnold *et al.*, 1988).

Gene	Molecular mass (kD)	Function
J	120	Electron transport:pyruvate flavodoxin oxidoreductase
H	35	Dinitrogenase reductase (Fe protein,component II)
D	56	α-Subunit of dinitrogenase (MoFe protein,component I)
K	60	β-Subunit of dinitrogenase (MoFe protein,component I)
T		unknown
Y	24	Maturation of component I
E	40	Synthesis of FeMoco
N	50	Synthesis of FeMoco
X	18	unknown
U	25	Maturation of component I
S	45	Maturation of component I or component II
V	42	Synthesis of FeMoco: homocitrate synthase
W		unknown
Z	15-17	unknown
M	28	Processing of component II
F	20	Electron transport: flavodoxin
L	50	*nif*-specific repression

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Table 1.2. (continued)

Gene	Molecular mass (kD)	Function
A	60	*nif*-specific activation
B	49	Synthesis of FeMoco
Q		Processing of Mo

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*nif*HDK of *K. pneumoniae* on a 200 kb plasmid whereas *A. chroococcum* containing 2-6 plasmids ranging from 11 to over 330 kb in size, but the *nif* structural genes are located on chromosome. In *E. agglomerans*, not only the *nif* structural genes (*nif*HDK) but also the rest of the *nif* genes are present on plasmid in strains 243, 333, 334, 335, 339. By using modified method, the plasmids of rice root-associated diazotrophs such as *Alcaligenes faecalis* A15, *E. cloacae* E26 EnSn, *K. oxytoca* NG13 (Wang *et al.*, 1989) and *Azospirillum lipoferum* strain COC8 (Uozumi *et al.*, 1982) have been isolated and detectd. Except *K. oxytoca* NG13, all bacteria described above were found to harbour plasmids, however the *nif* genes were located on the chromosomal DNA, except *A. lipoferum* COC8 that carried plasmid pTACOC8 (300 kb) which contained genes homologous to *nif*HDK and *nif*Q-K.

In free-living *K. pneumoniae*, the expression of *nif* genes is subjected to nitrogen control at two levels (Merrick, 1988). The first level of regulation is *nif*-specific and is mediated by the products of the *nif*LA operon, the *nif*A product (NifA) is a transcriptional activator which is required for the expression of all other *nif* operons. The *nif*L product (NifL) mediates O_2 repression at *nif*A activated transcription. The activity of NifA is controlled by NifL in response to nitrogen and oxygen status. NifL antagonizes NifA-mediated transcription in the presence of fixed N_2 or O_2. The second level of *nif* regulation is the control of NifLA transcription by the nitrogen regulation (*ntr*) system, a centralized system, which controls the expression of a variety of nitrogen

assimilatory genes in enteric bacteria. The *ntr* system comprises of three genes: *ntr*A, *ntr*B and *ntr*C. The *ntr*BC genes are tightly linked to *gln*A gene coding for glutamine synthetase (GS), an enzyme in ammonia assimilation, whereas the *ntr*A is unlinked. The *ntr*A and *ntr*C are necessary for activation of *nif* genes cluster through the *nif*LA operon whereas the *gln*A and the *ntr*B products are not necessary for this activation. The *rpo*N (*ntr*A) product is a sigma factor (σ^{54}) which complexes with core RNA polymerase (E) to form an RNA polymerase holoenzyme ($E\sigma^{54}$) which recognizes the *nif*LA promotor but this binding is non-productive with respect to the initiation of transcription. The activation of *nif*LA transcription under N-limitation requires *ntr*C product to bind with upstream site of $E\sigma^{54}$ in *nif*LA promotor. The *nif*A product, then in the presence of the *ntr*A product acts as a positive effector of the transcription to all other *nif* operons. The two genes: *ntr*C and *nif*A share sequence homology and the *nif*A product can substitute for the *ntr*C product to activate operons under *ntr* control (Alvarez-Morales *et al*., 1984).

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1.4. The nodulation

The nodulation of plants by *Rhizobium* or *Bradyrhizobium* species and the subsequent fixation of atmospheric nitrogen within these nodules is the result of the coordinated expression of both plants and bacterial genes (Scott, 1986). This symbiotic association is usually restricted to plant species within the family Leguminasae. Bacteria enter the root at points of bacterium-induced meristematic activity and invade cortical cells through the induction of infection threads. Infection of some rhizobia occurs through root hair curling and infection thread formation, and some through modified lateral root with an apical meristematic zone and a central vascular system, resulting in a determinate nodule structure.

1.5. The nodulation (*nod*) genes

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Genetic analysis of nodulation in the *Rhizobium*-legume symbiosis has revealed that *nod* genes, *nol* genes and *syr* genes in *R. meliloti* strain 41 are involved in nodulation (Kondorosi, 1984). In slow-growing rhizobia, now classified as *Bradyrhizobium*, *nod* genes localized on chromosomal DNA but fast growers *Rhizobium nod* genes localized on chromosomal DNA and large symbiotic plasmid, known as pSym (Gyorgypal *et al*., 1991). In *Rhizobium meliloti* there are 21 nodulation genes, located in Sym plasmid 135 kb, in six operons (Figure 1.2 a.). Nodulation genes are divided in 2 groups as shown in

Figure 1.2. The *nod* gene cluster of *Rhizobium meliloti* and plasmid pE39 and pRmSL42 derived from Kondorosi *et al.* (1991).

a) The organization of the 21 *nod* genes within the 135 kb length of DNA. The arrows represent the operons within the *nod* cluster and the direction of their transcription.

b) A physical map of pE39 demonstrating a number of restriction enzyme cleavage sites and their position in the *Rhizobium meliloti nod*D1 gene (0.6 kb) which has been inserted into *Bam*HI cleavage site of pUC8. Ap^r is the gene coding for ampicillin resistance.

c) A physical map of pRmSL42 demonstrating a number of restriction enzyme cleavage sites and their position in the *Rhizobium meliloti nod*ABC D gene (3.5 kb) which has been inserted into *Eco*RI cleavage site of pBR322. Ap^r is the gene coding for ampicillin resistance.

Restriction endonuclease which cut between and within *nod* genes are shown : B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; and P, *Pst*I.

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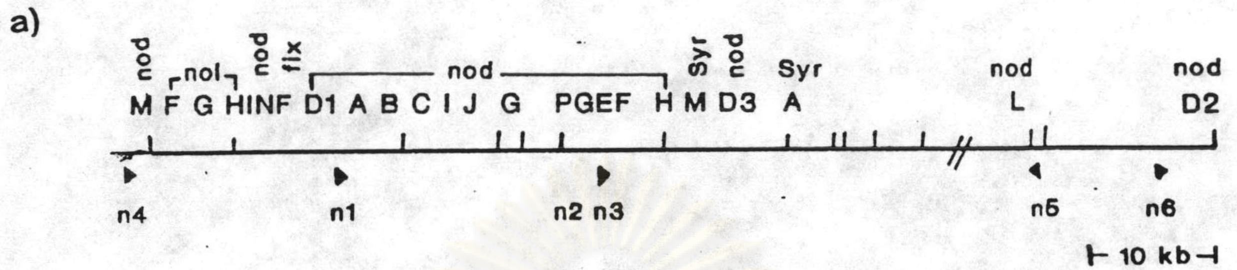

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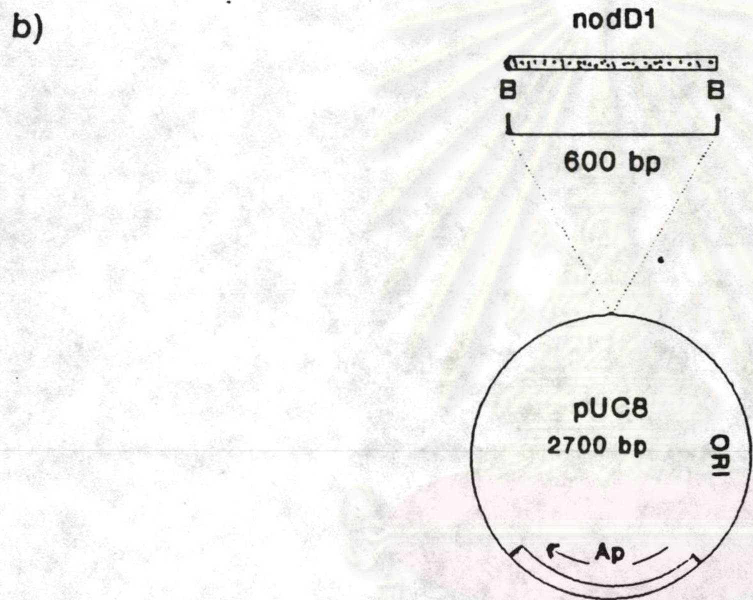

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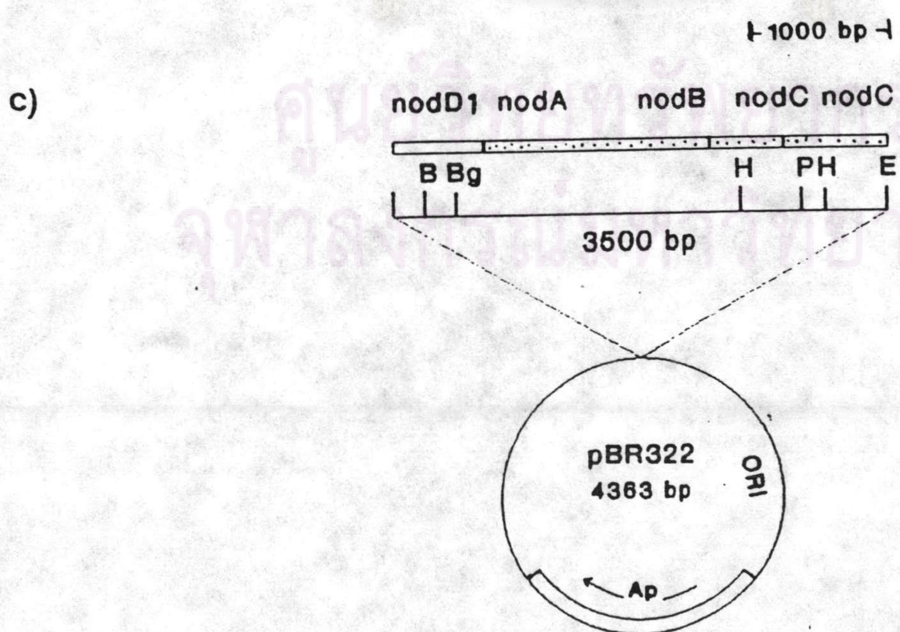

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Table 1.3.; the first group is the regulatory genes (*nod*D1, D2 and D3) which is found in all rhizobia. The part of *nod*D1 (0.6 kb *Bam*HI fragment) of *R. meliloti* 1021 has been cloned into *Bam*HI site of plasmid pUC8 (Egelhoff and Long, 1985), restriction map of this clone pE39 is shown in Figure 1.2 b. Although *nod*D is constitutively expressed, the genes of the *nod* operon are normally not expressed if host-derived molecules are absent but when plant secreted specific plant flavonoids or chalcones; derivative of flavone (Figure 1.3.) and these chemicals concerted with the product of regulatory gene *nod*D then induced structural genes. As reviewed by Hirsch (1992), The C-terminal end of the *nod*D protein determines flavonoid specificity, while the N-terminal region is involved in binding to regions of DNA known as *nod* boxes. The *nod* boxes is highly conserved 47 bp long, *cis* regulatory region found in promotor of *nod* operons. The second group is *nod* structural genes which are divided in 2 classes, firstly the common *nod* genes (*nod*ABC) are found in all rhizobia which synthesize proteins which produce precursor of nodulation factor, and secondly the host specific *nod* genes which confers specificity to host plants. These genes synthesize proteins that modify precursor nodulation factor to nodulation factor (Table 1.3.). The *nod*ABC operon (3.5 kb *Eco*RI-fragment) of *R. meliloti* 1021 has been cloned into *Eco*RI site of plasmid pBR322 (Egelhoff and Long, 1985), the restriction map of this clone pRmSL42 is shown in Figure 1.2 c. Using the plasmid pE39 and pRmSL42 as hybridization probes, DNA homology between common *nod*D ABC genes from *R. meliloti* and

Table 1.3. The functions and properties of *nod* genes of *Rhizobium meliloti* (Fisher and Long, 1992)

nod	Known function or properties
AB	Required for Nod factor production.
C	Homology to chitin and cellulose synthases; proposed to form β-1,4-glycosyl bond.
D	Transcriptional activator of inducible *nod* genes.
E	Host-specific; homology to β-ketoacyl synthase (condensing enzyme) of fatty acyl synthase; proposed to synthesize Nod factor acyl chain.
F	Host-specific; homology to acyl carrier protein; proposed to synthesize Nod factor fatty acyl chain.
G	Host-specific; homology to reductases; proposed to modify Nod factor fatty acyl side chain.
H	Host-specific; required for formation of sulphated Nod factor proposed to transfer activated sulphate (PAPs) to Nod factor.
I	Homology to ATP-binding active transport proteins; proposed to form membrane transport complex with *nod*J.
J	Homology to transmembrane proteins; proposed to form membrane transport complex with *nod*I.

Table 1.3. (continued)

nod	Known function or properties
L	Host-specific; homology to acetyl transferase; required for formation of 6-O-acetyl Nod factor; proposed to add O-acetyl group to Nod factor.
M	Host-specific glucosamine synthase; proposed to synthesize Nod factor sugar subunits.
N	Host-specific; involved in *Vicia hirsuta* nodulation.
O	Exported Ca^{++}-binding; homology to haemolysin; proposed to mediate early stage in rhizobia-Legume interaction.
P	Host-specific ATP sulphurylase; proposed to provide activated sulphate for transfer to Nod factor.
Q	Host-specific ATP sulphurylase and APS kinase; together with *nod*P makes activated sulphate (PAPs); proposed to provide activated sulphate for transfer to Nod factor.
T	Host-specific; involved in *Trifolium subterranean* nodulation; proposed to be membrane protein.
V	Homology to two-component regulatory system sensor proteins; proposed to regulate unknown target genes.
W	Homology to two-component regulatory system activator proteins; proposed to regulate unknown target genes.

Table 1.3. (continued)

nod	Known function or properties
X	Host-specific hydrophobic proteins; extends host-range to Afghanistan peas.
*nol*R	Repressor of *nod*D.

* Other genes including *nod*K, *nod*S, *nod*U, *nod*Y, *nod*Z, *nol*A, *nol*E, *nol*P, *nol*F, *nol*G, *nol*H, *nol*I have been identified, but have not been described by means of sequence homology to other published gene products, not have possible functions been proposed.

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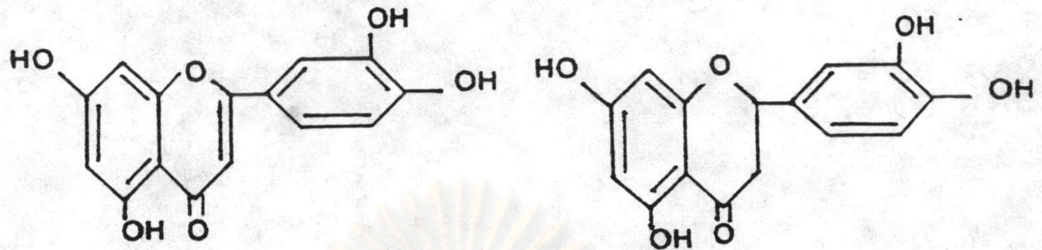
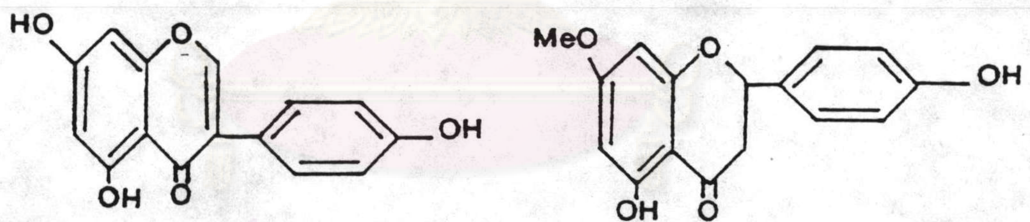


5,7,3',4'-tetrahydroxyflavone 5,7,3',4'-tetrahydroxyflavanone

"luteolin": inducer of *nod* D "eriodictyol": inducer of *nod* D



5,7,4'-trihydroxyisoflavone 5,4'-dihydroxy-7-methoxyflavanone

"genistein": inhibitor of *nod* D "Sakuranetin"

Figure 1.3. Structure of plant signal: flavonoids in legume and rice

other N_2-fixing bacteria were reported in *B. japonicum*, (Dockendorff *et al*, 1994). The common *nod* genes (*nod*ABCIJ) as well as nodulation genes involved in host specificity (*nod*FE, *nod*G, *nod*H and *nod*L) not only play a major role in root hair deformation and root hair curling but also in the initiation of cortical cell division which establish the nodule initiation. The common *nod* genes are so-called because they have been detected in all rhizobia and also because *nod* genes of *R. meliloti*, functionally complement comparable genes in other *Rhizobium* species. If any one of the *nod* ABC genes is mutated, the ability of *Rhizobium* to deform root hairs and to initiate cortical cell divisions on its host is eliminated and mutation in *nod*H enable *R. meliloti* to deform root hairs of white clover and vetch, species not normally compatible with that *Rhizobium*. The host specificity *nod* genes are not functionally conserved among the various *Rhizobium* species; they can not be genetically complemented by genes from other species. Host specific *nod* genes are also induced by plant-derived molecules (Kondorosi *et al*., 1991, Long, 1992).

Nodulation factor or nod signal is lipooligosaccharide (a substituted oligoglucosamine with a fatty acid tail on one end of the molecules) eg. nod signal of *R. meliloti* (Figure 1.4.) is 6-O-sulfated -N-(C16:2)-acyl-tri-N-acetyl-β-1,4,-D-glucosamine tetrasaccharide (Lerouge *et al*, 1990). The nod signal has specificity to its host plant and induced root hair deformation (Had^+), root hair curling and branching through which the bacteria formed infection thread into

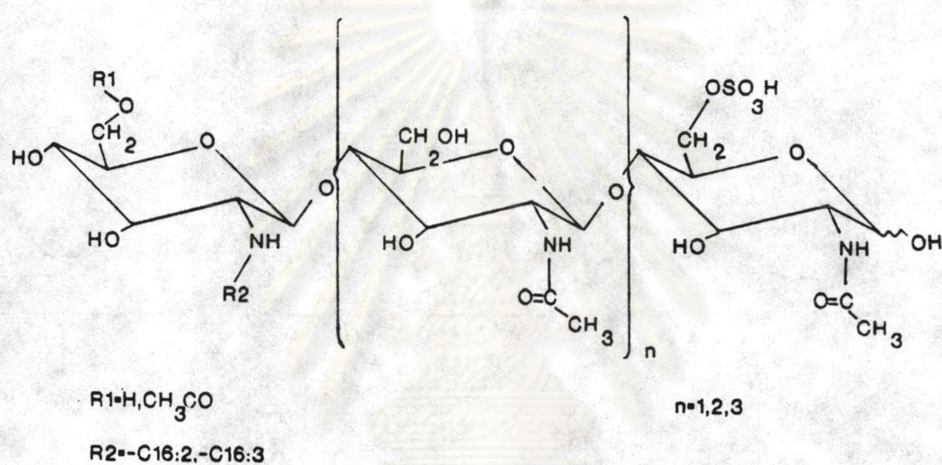


Figure 1.4. Structure of nododulation factor or nod signal in *Rhizobium meliloti*

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the cortex layer and division of plant cells in cortical layer form nodule (nodule induction (Nod^+)) and bacteria deformed to bacteroid which can fix nitrogen from atmosphere.

1.6. Biological nitrogen fixation in rice

Rice is the most important food crop of the developing countries and nitrogen is a major fertilizer in rice production, the work of many laboratories and field experiments on biological nitrogen -fixation confirm that nitrogen-fixing ability in the rice rhizosphere is rather high (Qiu *et al.*, 1980). The condition in paddy soils may be aerobic or anaerobic, and almost all major nitrogen-fixing bacteria (diazotrophs) can grow in this ecosystem. In IRRI, Philippines the great majority of bacteria associated with rice roots and rhizospheric soils belong to *Pseudomonas*, *Azospirillum*, *Enterobacter* and *Klebsiella* (Ladha *et al.*, 1982, 1983). *Azotobacter*, *Beijerinckia*, *Methylomonas*, *Flavobacterium* and *Rhodobacter* have also been reported as nitrogen-fixing inhabitants of rice roots (Wanatabe, 1985).

In Thailand, *Klebsiella* strains R15 and R17 were isolated from the rhizosphere of rice CV RD7 and RD6 (Harinasut, 1981). *K. oxytoca* NG13 is associative diazotroph isolated from rice rhizosphere in Japan. *Klebsiella* R15, R17 and NG13 are able to fix N_2 under either aerobic or microaerobic conditions and show significant increase in nitrogenase activity when associated with rice as compared to free-living condition.

One of the major problem is the criteria indicating the presence of association between diazotrophs and Gramineae. In contrast to *Rhizobium*-legume symbiosis, the diazotrophs-grasses interaction does not produce visible structure on roots which indicate successful infection. Therefore interaction between grasses and N_2-fixing bacteria has been described as associative. There are illustration of the *Azospirillum*-filled spherical structures on the root surfaces of sugarcane and deformation of root hair, and the evidence that *Azospirillum* breaks the root epidermal barrier and invades cortical and vascular tissues of the host (McClung and Patriquin, 1980). These observations are also reported in other diazotroph-plant association such as *Azospirillum*-pearl millet and guinea grass (Umali-Garcia *et al.*, 1980) and *Azospirillum*-kaller grass (Reinhold *et al.*, 1987). Association between *Klebsiella oxytoca* R15 and R17 with rice seedlings grown in sterile water resulted in curling, branching, denser and longer root hairs, together with firm adherence of bacteria on the rhizoplane as individual cells, clusters and eventually as enveloped micronodule structures of 10-15 μ in diameter. Invasion of a few bacteria clusters was also found in the epidermal and outer cortical layers of rice root and detection of lectin activity in the root exudate and bound lectin as an associative factor (Limpananont, 1987). In general, lectins are proteins or glycoproteins of non-immune origin which bind to cell surfaces via specific sugar residues and oligosaccharide determinants. Lectins in root of rice played the role as associative factor between root and N_2-

fixing bacteria *Klebsiella oxytoca*. The presence of secretory lectins in root exudate and bound lectin on root surface assist adhesion of *Klebsiella* to the rhizosphere (Limpananont, 1987 and Pitaksutheepong, 1992). Since rice lectins bind specifically with N-acetylglucosamine, the carbohydrate moiety of the nodulation factor of *R. meliloti* which mediate the first step of *Rhizobium*-legume interaction. Receptor molecules that bind the lipooligosaccharide are presumed to be present on the root hairs. The chemical nature of the receptor molecule is so far unknown, but it has been postulated to be a lectin (Lugtenberg *et al.*, 1991). Elmerich *et al.* (1985) reported that by using restriction enzymes and Southern hybridization between *nod* genes of *R. meliloti* as probe with DNA of *Azospirillum* strains there were homology between *nod* genes with DNA of *Azospirillum* (Table 1.4.). Nodulation genes in associative *Klebsiella* strains are not known but rice released Sakuranetin which is a flavanoid rice phytoalexin (5.4'-dihydroxy-7-methoxyflavanone) shown in Figure 1.3. (Kodama *et al.*, 1992). Its structure is similar to plant signal in legume, but Sakuranetin is an antifungal substance isolated from ultraviolet irradiated rice leaves, not known to be produced by root. In general flavonoids with one HO group at the 4' position inhibit IAA-induced growth by stimulating IAA oxidase whilst flavonoids with HO group at the 3' and 4' position stimulate it by inhibits IAA oxidase (Goodwin and Mercer, 1972). Sakuranetin has one OH group at the 4' position indicated inhibit IAA-induced growth by stimulating IAA oxidase.

Table 1.4. Size of DNA fragments of *Azospirillum* and *Rhizobium meliloti* 2011 which show homology when probed with common *nod* genes (*nod*ABC D), and host specific *nod* genes (*nod* EFGH) after digestion with *Eco*RI, *Sal*I and *Bgl*II (Elmerich *et al.*, 1985)

Bacterial strains	common *nod* genes probes			host specific *nod* genes probes		
	*Eco*RI	*Sal*I	*Bgl*II	*Eco*RI	*Sal*I	*Bgl*II
Azospirillum brasilense						
Sp7	7.2	3.3	nt.	12	6.8	nt.
				10	4.0	
				1.8	2.4	
RO7	7.2	3.3	14.0	17	2.9	6.4
				10	2.6	
				1.8	2.4	
Azospirillum lipoferum						
Br17	-	3.4	nt.	10	6.7	3.7
				2	3.0	
					2.1	

Table 1.4. (continued)

Bacterial strains	common *nod* genes probes			host specific *nod* genes probes		
	*Eco*RI	*Sal*I	*Bgl*II	*Eco*RI	*Sal*I	*Bgl*II
Azospirillum lipoferum						
S28	12	3.8	-	17	6.7	9.0
				10	3.0	
					2.1	
Rhizobium meliloti	8.5	6.5	4.8	1.4	4.6	7.3
2011			3.7		1.7	
			2.3			

* -: not detected, nt.: not tested, sizes are indicated in kb.

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1.7. Objective of this thesis

Since the basic knowledge on the organization of *nod* genes in associative *Klebsiella* strains are not known and these information are important for genetic improvement for better application of N_2-fixing bacteria as biofertilizer in rice, the aim of this thesis is to use Restriction Fragment Length Polymorphism (RFLP) analysis to study common *nod* genes and *nif* structural genes by using *nod*ABC D probes from *R. meliloti* 1021 and *nif*HDK probes from free-living *Klebsiella pneumoniae* M5a1 to localize *nod* genes and *nif* genes of N_2-fixing bacteria by comparing their RFLPs.

It is hoped that the accumulative data obtained from the comparative study of N_2-fixing bacteria will provide valuable insights into nodulation genes, nitrogen fixation genes organization, homology and regulation, so that when they were applied back into the paddies, nitrogen fixing efficiency in those areas should be promoted.

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