# CHAPTER I

# INTRODUCTION

## 1.1. Biological nitrogen fixation

The growth and yield of agricultural crop depends, 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 can be taken up, so called inorganic fertilizers ($NO_3^-$, $NH_4^+$) which are produced in factory by chemical process that require fossil energy. In natural condition molecular dinitrogen in the air enters the organic nitrogen pools in the biosphere through a certain groups of prokaryotes which contain nitrogenase enzyme, a complex that fixes $N_2$ by the expense of ATP, the chemical energy in living organisms, so called 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 approaches are of great practical interest. One such approach is to use biological nitrogen-fixation (BNF), a process exemplified by a number of diversified genera of bacteria, each genus shows a certain specificity to host plants.

In cereal crops there have been intensive research on the association between nitrogen-fixing bacteria and Gramineas roots only about 20 years ago, since 1972 on biological nitrogen fixation in the

root zones of tropical grass and later on of several cereal crops showing the possibility of high uptake of fixed nitrogen (Döbereiner et al., 1972 a, b). Since then biochemical methods and molecular genetic techniques have been applied in particular to elucidate the genetic basis of the nitrogen-fixation process i.e. to identify and analyse the genes responsible for this remarkable property and their regulation. The better understanding of this process might help to improve the nitrogen fixing ability of the nitrogen fixer by means of genetic manipulation. Although it is impossible now to replace absolutely conventional nitrogen fertilization with biological nitrogen fixation in growing main cereal crops, when the mechanism of associative nitrogen fixation is well understood and could be induced to contribute a significant amount of nitrogen to cereal crops, grain yield may soon become less dependent on the industrially produced nitrogen fertilizer. For developing countries, even a little saving on fertilizer will be of economically interest.

## 1.2. 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 (Watanabe, 1985; Qiu et al., 1980). The conditions in paddy soils may be aerobic or anaerobic, and almost all major nitrogen-fixing bacteria (diazotrophs) can grow in this ecosystem. The great majority of bacteria associated with rice roots and rhizopheric soils belong to Pseudomonas (Barraquio and Watanabe, 1981), 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 (Watanabe, 1985).

In Thailand, Klebsiella strains R15 and R17 were isolated from the rhizophere of rice CV RD7 and RD6 (Poontariga Harinasut, 1981). Their DNA have % G+C content of 54.9-56.4 and the reassociation time of hybrid DNA is very close to that of Klebsiella oxytoca NG13, the associative diazotroph isolated from rice rhizosphere in Japan. Both Klebsiella strain R15 and R17 differ from NG13 by their fatty acid composition (Jariya Boonjawat et al., 1986). Klebsiella NG13, R15 and R17 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.

## 1.3. The nitrogenase complexes

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 Klebsiella 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 $\alpha$- and $\beta$-subunits ($\alpha_2\beta_2$). The $\alpha$- and $\beta$-subunits are coded by nifD and nifK respectively. Kp2, a dinitrogenase reductase or iron protein (Fe protein), consists of two identical subunits coded by nifH. These protein components are irreversibly inactivated by oxygen. A cofactor (FeMOco) containing Mo, Fe and S,

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

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

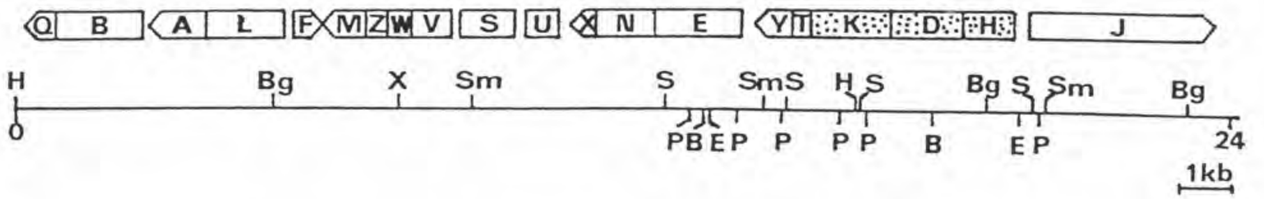
presumed to carry the active site of nitrogenase; can be separated from the MoFe protein (Shah and Brill, 1977). Both a reductant and ATP are required for enzyme activity; approximately 15 mole of ATP are consumed per mole of dinitrogen reduced. In view of this high energy requirement and the extreme oxygen sensitivity of the protein components, it is not surprising that nitrogenase synthesis is tightly controlled.

## 1.4. The nitrogen fixation (*nif*) genes

To date, the best known free-living nitrogen-fixing bacterium is *Klebsiella pneumoniae*, which is 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 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

a)

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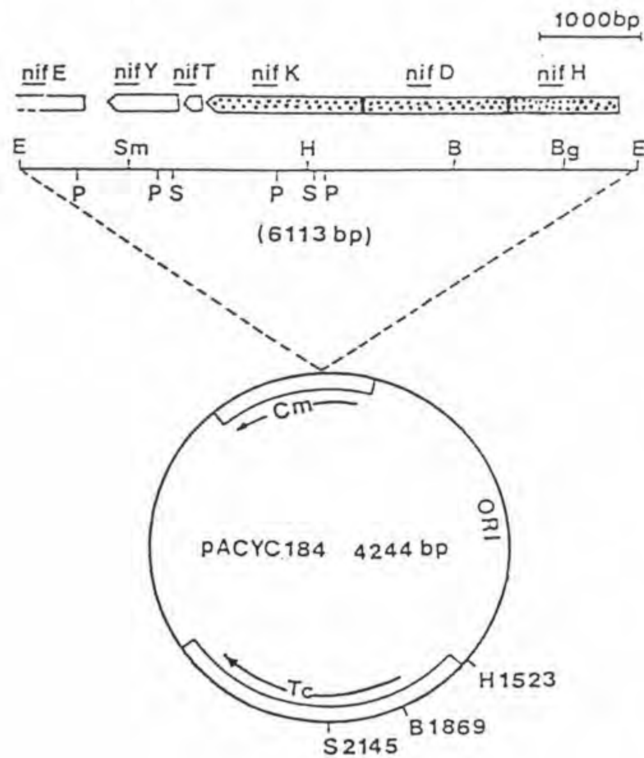


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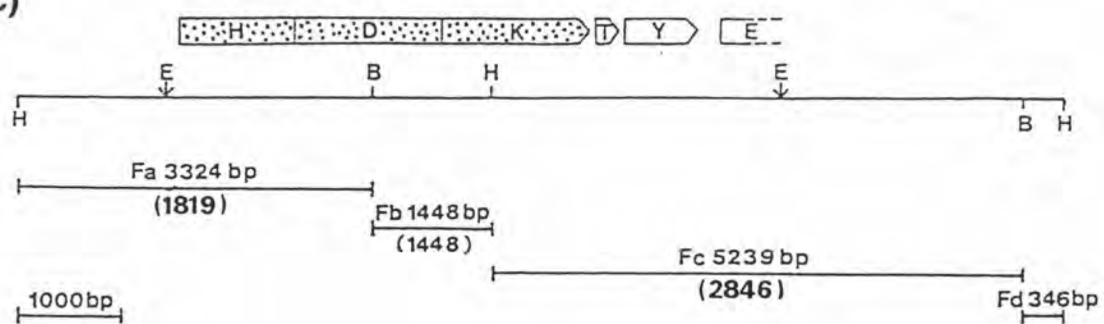


Table 1.2. The products and functions of the <u>nif</u> genes of <u>Klebsiella pneumoniae</u> (Arnold <u>et al</u>., 1988)

| 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 | <u>nif</u>-specific repression |
| A | 60 | <u>nif</u>-specific activation |
| B | 49 | Synthesis of FeMoco |
| Q | | Processing of Mo |

on legume roots (Banfalvi et al., 1981 and Masterson et al, 1985). In addition, the structural genes of nitrogenase (nifHDK) 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 (strains) 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 nifHDK 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 (nifHDK) but also the rest of the nif genes are present on plasmid in strains 243, 333, 334, 335 and 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 detected. 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 nifKDH and nifQ-K.

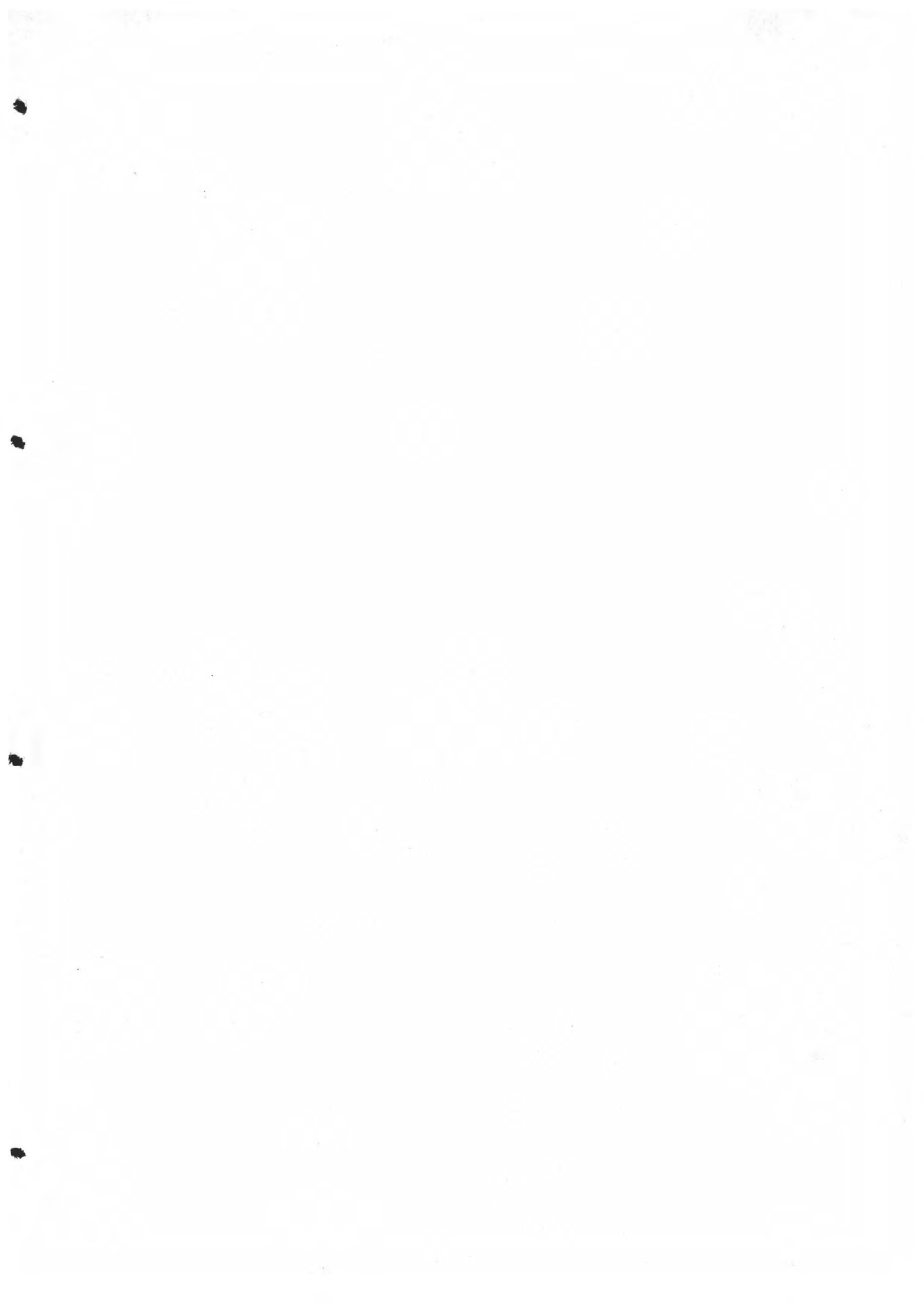
## 1.5. Regulation of nif genes

In free-living K. pneumoniae, the expression of nif genes is subjected to nitrogen control at two levels (Merrick, 1988). The first

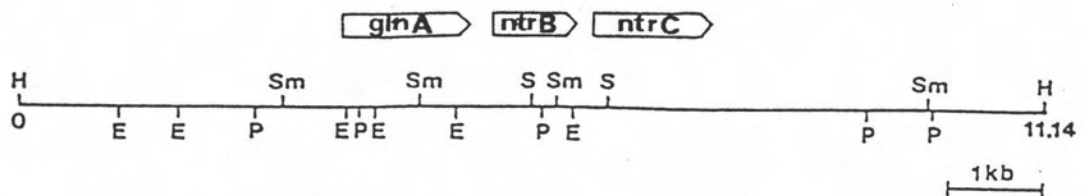
level of regulation is nif-specific and is mediated by the products of the nifLA operon, the nifA product (NifA) is a transcriptional activator which is required for the expression of all other nif operons. The nifL product (NifL) mediates $O_2$ repression at nifA activated transcription. The activity 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 three genes : ntrA, ntrB and ntrC. The ntrBC genes are tightly linked to glnA gene coding for glutamine synthetase (GS), an enzyme in ammonia assimilation, whereas the ntrA is unlinked. The ntrA and ntrC are necessary for activation of nif genes cluster through the nifLA operon whereas the glnA and the ntrB products are not necessary for this activation. The rpoN (ntrA) product is a sigma factor ($\sigma^{54}$) which complexes with core RNA polymerase (E) to form an RNA polymerase holoenzyme ($E\sigma^{54}$) which recognizes the nifLA promoter but this binding is non-productive with respect to the initiation of transcription. The activation of nifLA transcription under N-limitation requires ntrC product to bind with upstream site of $E\sigma^{54}$ in nifLA promoter. The nifA product, then in the presence of the ntrA product acts as a positive effector of the transcription to all other nif operons. The two gene ntrC and nifA share sequence homology and the nifA product can substitute for the ntrC product to activate operons under ntr control (Alvarez-Morales et al., 1984).

The gene order of the K. pneumoniae glnA region is glnA ntrB ntrC with the three genes being transcribed in the same direction, from glnA to ntrC (Epsin et al., 1982). To study their role in regulation

of *nif* gene cluster, the *gln*A, *ntr*B and *ntr*C region on a 11.2 kb *Hin*dIII fragment has been cloned into the plasmid pACYC184, resulted in pGE100 (Figure 1.2 a). Alvarez-Morales *et al*. (1984) has found that the *gln*A *ntr*BC regulon in *K*. *pneumoniae* has a similar structure, $P_1$ *gln*A $P_2$ *ntr*BC, to that in other enterobacteria. Under N-limiting condition *ntr*BC is expressed mainly from the $P_1$ promoter, whereas in N-excess the P2 promoter is primarily responsible for *ntr*BC transcription. Figure 1.3 shows the model for the regulation of *ntr*-regulated operon. Transcription from $P_1$ can be regulated in either a positive or a negative manner. In its absence, the *ntr*B product causes repression of transcription from $P_1$ which required the products of *ntr*C and also *ntr*B to modulate the repression function of *ntr*C product, but the *ntr*B product alone does not show any regulatory activity at $P_1$. By comparison, transcription from $P_2$ is only negatively controlled, repression being mediated by the *ntr*C product. As with $P_1$, the presence of *ntr*B product appears to modulate the repressor activity of *ntr*C product. In summary, the balance of transcription from $P_1$ and $P_2$ and the response of the regulon to the N-status of the cell is regulated by the activator/repressor state of the *ntr*C product which in turn is a consequence of modulation by the *ntr*B product.



a)

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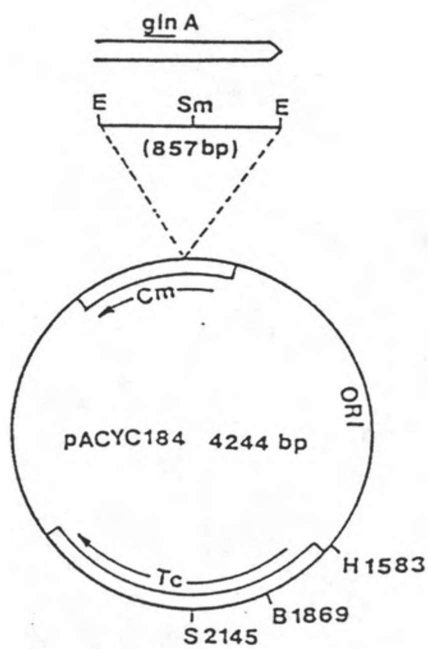

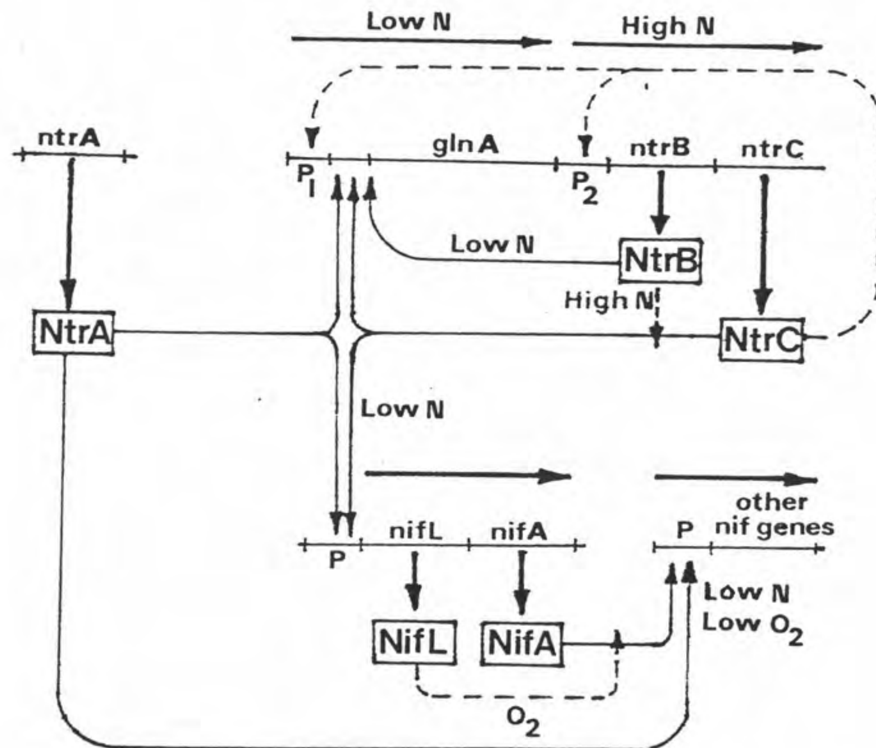


Figure 1.3. Model for the regulation of nif genes via nifLA by glnA ntrBC regulon

This model was purposed by Alvarez-Morales et al. (1984). (—) positive regulatory circuits, (---) negative regulatory circuit. Horizontal arrows indicate transcriptional organization.

### 1.6. Objective of this thesis

Since the basic knowledge on the organization of *nif* genes and *ntr* regulon in associative *Klebsiella* strains R15 and R17 are not known and these informations are important for genetic improvement for better application of these $N_2$-fixing bacteria as biofertilizers in rice, the aim of this thesis is to use Restriction Fragment Length Polymorphism (RFLP) analysis to study *nif* genes and *gln*A *ntr*BC region by using *nif*HDK and *gln*A probes from free-living *Klebsiella pneumoniae* M5a1 to localize and investigate the genetic relatedness of *nif* structural genes and *gln*A region of *K.* R15 and R17 with those of *K. oxytoca* NG13, the associative diazotroph from japonica rice, and the standard strain, the free-living *K. pneumoniae* M5a1 by comparing their RFLPs.

It is hoped that the accumulative data obtained from the comparative study of indogenous *Klebsiella* R15 and R17 with exogenous strain NG13 and the best known *K. pneumoniae* M5a1 will provide valuable insights into nitrogenase, gene organization, mechanism, and regulation, and then the chance to improve these strains by genetic manipulation, so that when they were applied back into the paddies, nitrogen fixation efficiency in those areas should be promoted.