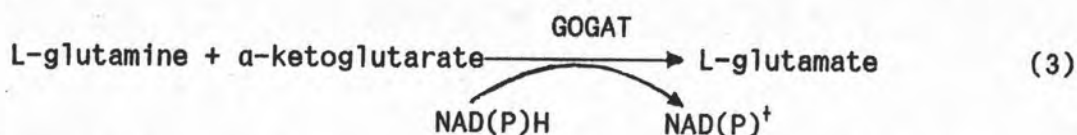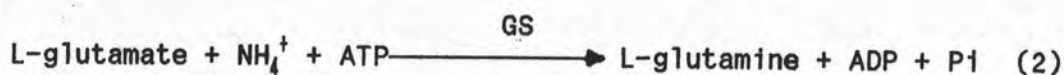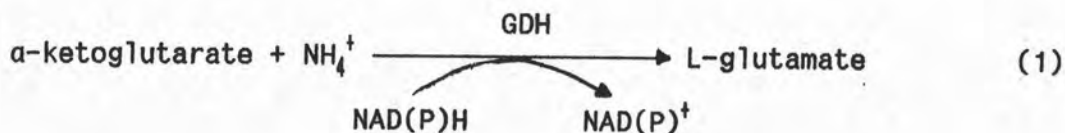CHAPTER I

INTRODUCTION

1.1 Role of glutamine synthetase in nitrogen metabolism.

In enteric bacteria, there are two known pathways for ammonium assimilation either of which may be active in a particular species depending upon the organism and the available of ammonium and energy. In high concentration of ammonium, assimilation can be catalyzed by glutamate dehydrogenase (GDH, EC. 1.4.1.4) (reaction 1) whereas in limiting ammonium it occurs by the glutamine synthetase (GS, EC. 6.3.1.2) glutamate synthase (GOGAT, EC.2.6.1.53) system (reactions 2 and 3) (Tyler, 1978)

$$\alpha\text{-ketoglutarate} + NH_4^+ \xrightarrow{\quad GDH \quad} \text{L-glutamate} \qquad (1)$$
$$NAD(P)H \qquad NAD(P)^+$$

$$\text{L-glutamate} + NH_4^+ + ATP \xrightarrow{\quad GS \quad} \text{L-glutamine} + ADP + Pi \quad (2)$$

$$\text{L-glutamine} + \alpha\text{-ketoglutarate} \xrightarrow{\quad GOGAT \quad} \text{L-glutamate} \qquad (3)$$
$$NAD(P)H \qquad NAD(P)^+$$

GS is a central control point in nitrogen metabolism flux of nitrogenous compounds (Voet, 1990). In *E.Coli*, purified GS has a molecular weight about 600 kDa and consists of 12 identical subunits of 50 kDa daltons arranged in two hexagonal units layer (Stadtman and Ginsberg, 1974). The enzyme can be regulated post-translationally by adenylylation to a tyrosine residue in each subunits. From the

physiological point of view, adenylylated GS has diminished activity for glutamine synthesis (reaction 2) (Stadtman and Ginsburg, 1974). Both adenylylated and deadenylylated forms have the same transferase activity measured by the capacity of transferring the γ-glutamyl residue of glutamine to hydroxylamine in the presence of ADP arsenate and Mn^{2+} (reaction 4) (Farden and Robertson, 1980)

$$\text{glutamine} + NH_2OH \xrightarrow[\text{arsenate, ADP}]{Mn^{2+}} \gamma\text{-glutamyl hydroxamate} + NH_3 \quad (4)$$
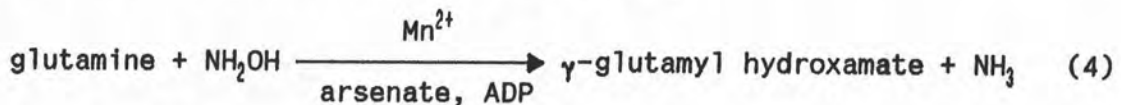
Under nonphysiological standardized assay conditions Mg^{2+} could support transferase activity of deadenylylated GS, but it selectively inhibited the Mn^{2+} dependent transferase activity of adenylylated enzyme, so that in the presence Mn^{2+} and Mg^{2+} the transferase activity of deadenylylated enzyme only can be measured. In the presence of 0.3 Mm Mn^{2+} transferase activity of both adenylylated and deadenylylated GS can be measured (Farden and Robertson, 1980).

The γ-glutamyl transfer reaction(4) has been used as a highly sensitive procedure for the determination of GS in crude extract, and also for the estimation of the average state of adenylylation of GS.

Further investigations have shown that the activity of GS is under the fine control of a bicyclic cascade system (Figure 1.1) (Stadtman, 1991). Glutamine inhibits and α-ketoglutarate stimulates the ability of adenylyltransferase to catalyze the P_{II}-dependent adenylylation of GS at the adenylylation site(AT_a) of AT_{ase}, whereas each effector exerts an opposite effect on the capacity of AT_{ase} to catalyze the deadenylylation of GS at the deadenylylation site(AT_d) of the enzyme. Similarly, glutamine was found to inhibit the ability of uridylyltransferase to catalyze the uridylylation of P_{II} at the UT_u site

of UT_{ase}, but to stimulate its ability to catalyze the deuridylylation of $P_{II} \cdot UMP$ at the UT_d site. In opposite direction α-ketoglutarate stimulates the deuridylylation reaction. The activity of GS is subjected to regulation by over 40 metabolites including ATP, CMP which have been reviewed in details by Stadtman (1991).

On the regulation of GS synthesis, extensive investigation in the laboratories of Magasanik and Kustu have shown that transcription of the structural gene for GS glnA is under the control of several gene products (Table 1.1)(Magasanik, 1988) two of which ntrC and ntrB are members of the gln operon. The product of ntrB(NRII) is a protein kinase that catalyzes the phosphorylation of ntrC product (NRI) and give rise to NRI-P which can activate glnA transcription. The cyclic interconversion of the ntrC product(NRI-P $\longrightarrow$ NRI) is dependent upon the concentration of the P_{II} protein (glnB product) which stimulates the dephosphorylation of NR-P.

Since the interconversion of P_{II} between uridylylated form and unuridylylated form is dependent on UT_{ase}-UR which via allosteric interaction controlled by α-ketoglutarate and glutamine, P_{II} acts as signal transducer between GS adenylylation-deadenylylation and phosphorylated-dephosphorelated NRI and hence the rate of GS activity and GS synthesis (Figure 1.1).
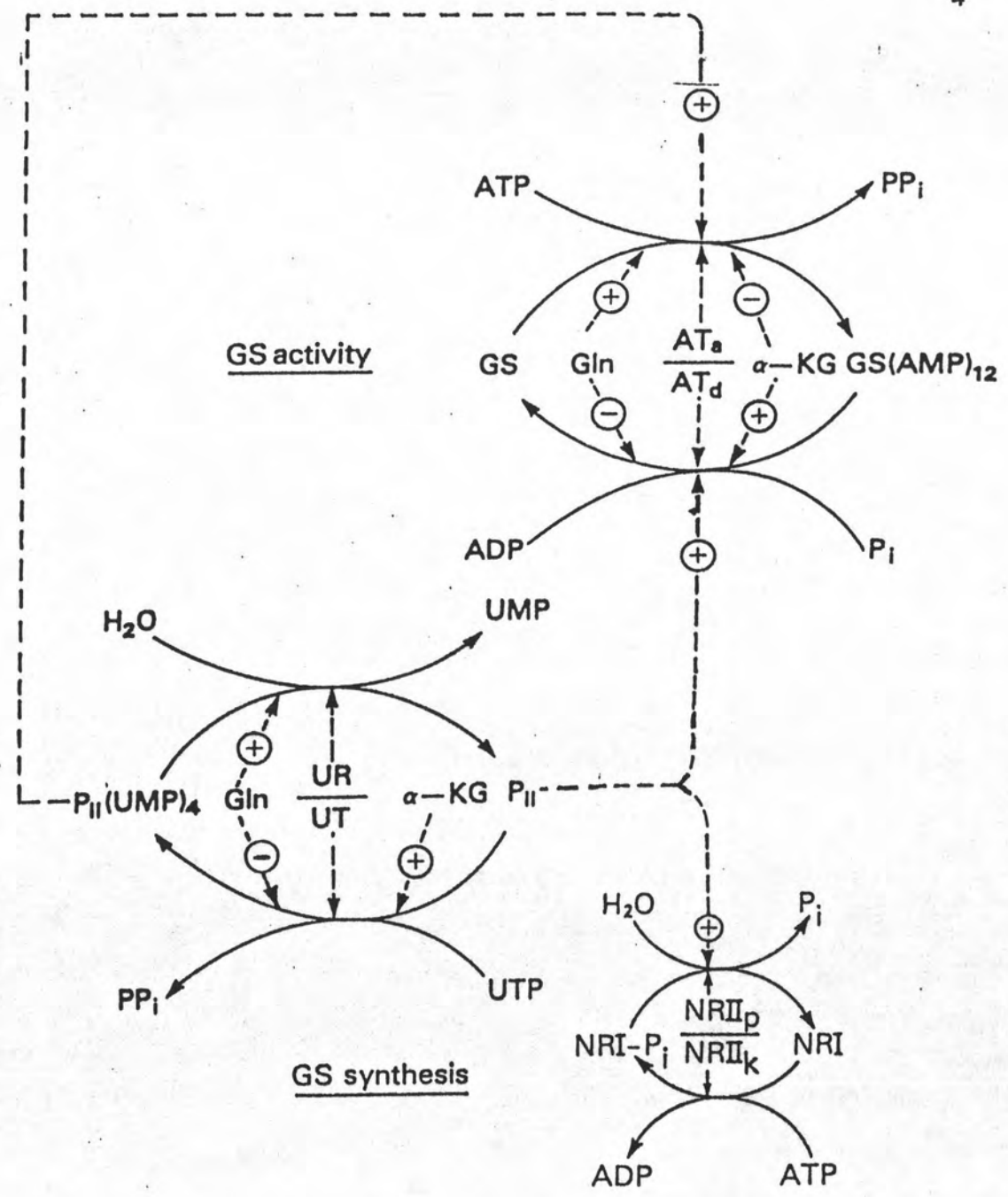
Figure 1.1 The cyclic cascade of GS regulation. Interrelationship between the uridylyation cycle, the adenylylation cycle, and the phosphorylation cycle; the reciprocal controls of these interconversion by L-glutamine (Gln) and α-ketoglutarate (α-KG) are shown; (+) indicates stimulation, (-)indicates inhibition (Stadtman, 1991).

Table 1.1   Regulatory proteins and genes involved in GS control

Gene	Protein	Role
*rpo*N(*ntr*A)	σ^{54}	RNA polymerase
*gln*A	GS	Sensor
*gln*G(*ntr*C)	NRI	Effector
*gln*L(*ntr*B)	NRII	Modulator
*gln*B	P_{II}	Signal transducer
*gln*D	$UT_{ase}-UR$	Signal transducer
*nif*A	NifA	Effector
*nif*L	NifL	Modulator

1.2 GS in N₂ fixation bacteria.

In free-living nitrogen fixing bacteria when source of combined nitrogen is limited, N_2 is reduced to ammonia by enzyme nitrogenase. In *K. pneumoniae* the enzyme system involved in nitrogen fixation is a complex and nitrogenase activity is controlled by several factors including other enzymes involved in nitrogen metabolism. Regulation of *nif* structural genes (*nif*HDK) are facilitated *ntr*BC gene along with *gln*A constitute *ntr* operon though *nif*LA, regulatory genes of the *nif* regulon. Genes and products of regulatory genes are shown in Table 1.1. The regulation scheme of *ntr*-regulated operon is shown in Figure 1.2. There are three genes, *rpo*N and *ntr*BC control expression of the *nif* regulatory
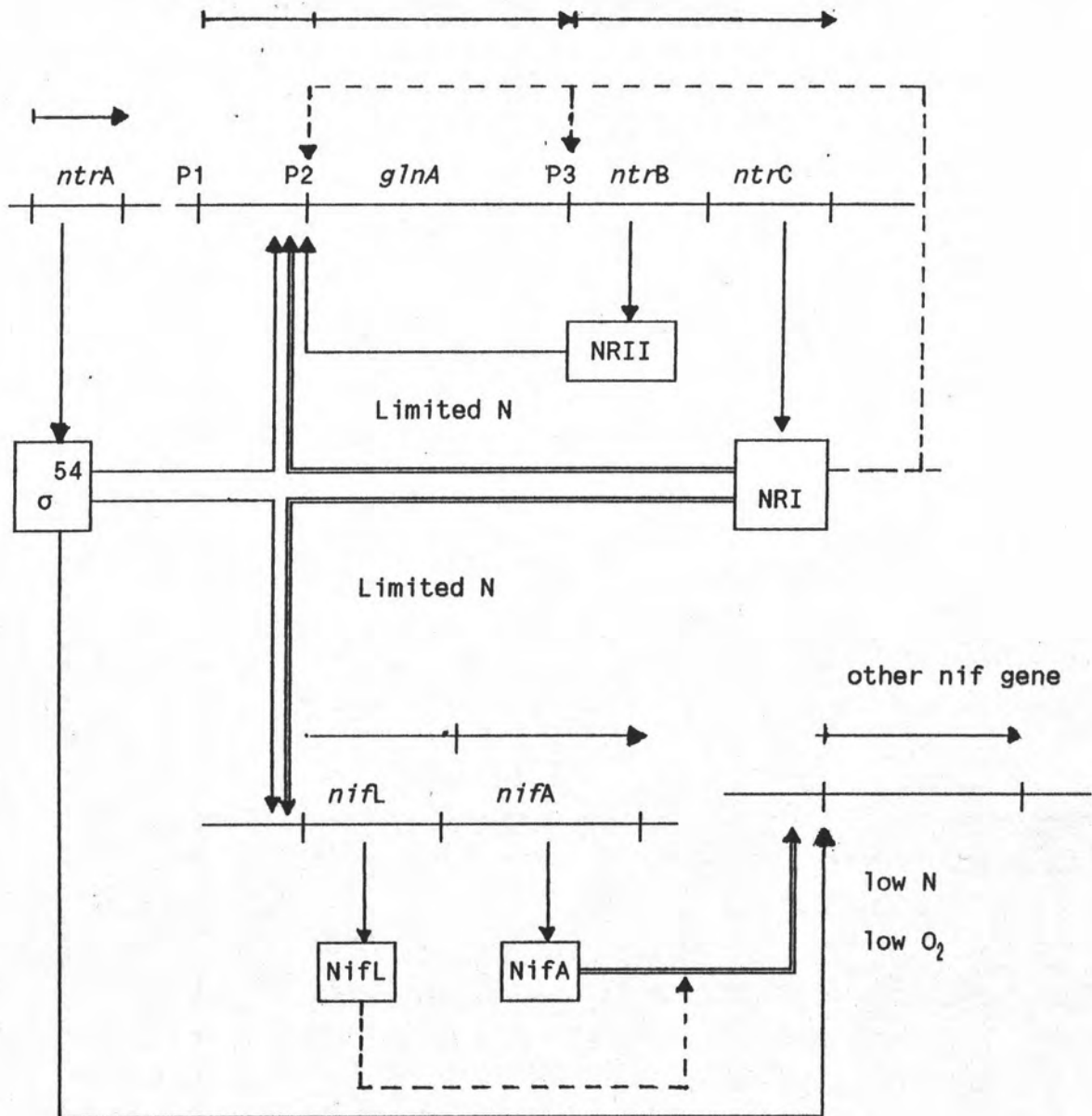
Figure 1.2 A Model for the *nif* and *gln* regulation *K. pneumoniae*.

(——) positive regulatory circuits, (--) negative regulatory circuits.

Horizontal arrows indicate transcriptional organization. Double arrows

indicate activation sequence.

(Modified from Cannon et.al., 1985)

operon. The *rpo* N(*ntr*A) product is a sigma factor(σ^{54}) which complexes with core RNA polymerase(E) and result in RNA polymerase holoenzyme(E) which bind specifically to *ntr* at P2 and *nif*LA promoter, but concentration of *ntr*C product(NRI) required for activation of *nif*LA transcription is 5-10 fold greater than that required at the P2 (Merrik, 1988). The activation of *nif*LA transcription under condition of N-limited requires NRI. NRI and σ^{54} bind to site upstream to σ^{54} in *nif*LA promoter results in the increased level of the *nif*A product which is the transcription activator of the other genes. The NifA activity is controlled by NifL in response to N_2 & O_2 status. NifL protein antagonists NifA-mediated transcription in the presence of combined nitrogen or oxygen. The gene order of the *K.pneumoniae ntr*A region is *gln*A *ntr*B *ntr*C(Espin et al., 1982) in which *gln*A product is GS, and the three genes are transcribed in the same direction under the control of P1 and P2 promoters under N-limiting condition *ntr*BC is expressed mainly from the P2 promoter, whereas N-excess the P3 promoter is primarily responsible for *ntr*BC transcription(Alvarz-Morales et al., 1984)

1.3 GS in plant-microbe symbiosis.

There are a number of plant-microbe symbiosis including legum-*Rhizobiaceae* association, nonlegumes angiosperm-actinomyectes and those between the cyanobacteria and water ferns, lichens and liverworts. The biochemistry of ammonium assimilation and nitrogen transferring between these symbiont were studied extensively by studying the role of GS in the regulation of assimilation of nitrogen compound. All member of *Rhizobium* and *Bradyrhizobium* (except. *Rhizobium.* spp strain ORS 571) (Donald and Ludwig, 1984) synthesis at least two distinct forms of GS,

that have been designated GSI and GSII (Fuchs and Keister 1980 ; Edmand et al., 1986 ; de Brunijn et al., 1989), GSI is similar to GS from enteric bacteria in many respects such as heat stable ; its regulation is controlled in responsible to extracellular ammonium assimilation by reversible adenylylation cascade system (Ludwig, 1980), transcribed by *gln*A gene (Somerville and Kahn, 1983 ; Carlson et al., 1985), and feed back inhibition by metabolites down stream from GS (Bhandari and Nicholas, 1986).

On the other hand GSII, is heat labile, not subject to adenylylation control (Somerville and Michael, 1983) and encoded by *gln*II and the amino acid sequence is highly homologous to eukaryotic GS (Carlson and Chelm, 1986). In *R. meliloti, Bradyrhizobium japonica* living symbiotically as bacteroid in leguminous nodule GS-GOGAT activities are very low. The ammonia produced is not mainly utilized by the bacteroid, but is excreted into the cytoplasm of the host cell, where it is then incorporated by plant GS (Brown and Dilworth, 1975 ; O'gara and shanmugan, 1976). In *B. japonicum,* GSI mutant shows positive effect on symbiotic nitrogen fixation by inducing higher number of nodule per plant and higher nitrogenase activity than the wild type (Carlson et al., 1987). In contrast, Moreno et al.(1991) report that in *R. leguminosarum* GSI mutation reduced the capacity of bacteroids to fix nitrogen in induced nodule. In *R. melioti* 104A14 and *B. japonicum gln*II gene is regulated by nitrogen availability by using a regulatory system similar to that of enteric bacteria (Carlson et al., 1987 ; Shatters et al., 1989), *ntr*A gene is required for the expression of GSII protein and GSII activity in bacteroid is repressed either by low oxygen level or by the presence of ammonia at the transcriptional level of *gln*II(Shatters et al., 1989).

In plant, GS was considered to be the main enzyme involved in the incorporation of either mineral nitrogen or fixed atmosphere dinitrogen into glutamine(Miflin and Lea, 1980). In legume, root specific and/or root nodule specific GS is responsible for assimilation of symbiotic fixed nitrogen, there are several distinct isoforms of cytosolic GS (GS1 and Gsn)(Cullimore et al., 1983 ; Lara et al., 1984). In soybean, transcription of GS MRNA is apparently increased due to the NH_4^+ stimulated expression of GS isoforms on the root(Hirel et al., 1987). In *Lotus corxiculatus* the ammonia-enhanced GS gene expression in plant is due to an increasing transcription, which is directly regulated by externally supplied or symbiotically fixed nitrogen (Hirel et.al., 1991).

For cyanobacteria in symbiotic association with bryophyte, *Anthroceros* studied by tracing N_2-fixed, and transfer of NH_4 in the *Anthroceros-Nostoc* symbiotic association (Meek et al., 1985), has shown that in situ, symbiotic *Nostoc* assimilates about 10 % of the N_2-derived NH_4^+, and the rest 90 % of NH_4^+ is available to *Anthroceros* tissue. In this system the GS specific activity in *Nostoc* sp. strain 7801 grown in symbiotic association with *Anthroceros* is 3-4 fold lower than in free living *Nostoc*, and based on enzyme linked immunosorbant assay(ELISA) the amount of GS protein in symbiotic N_2-fixation and NH_4^+ grown *Nostoc* are similar. These results implied that the regulation of GS is by post-translational mechanism (Joseph and Meek, 1987).

1.4 Research problem

The ammonium assimilation in the rice nitrogen-fixing bacteria association and the mechanism of transport of fixation products to the

host plant have never been studied before. *Klebsiella* R15-rice association is one of the most interesting system to study on fixed-dinitrogen assimilation. Since rice is clearly the most important food crop of the world and it is the main food crop of Thailand, in the hope to replace nitrogen fertilizer with *Klebsiella* R15 inoculation, the basic knowledge on nitrogen metabolism in associative condition should be well understood.

Klebsiella R15 is Gram-negative and rod shape structure, isolated from the rhizosphere of rice cv. RD7 (Poontariga, 1981). Association between *Klebsiella* R15 and rice seedlings grown in sterile water resulting in more branching, denser and longer root hair. Colonization of *Klebsiella* R15 on the rhizoplane can be observed as micronodule of 10-15 μ diameter. The invasion of a few bacteria have also been found in the epidermal and cortical layer of rice root (Boonjawat et.al., 1990). In free-living condition *Klebsiella* R15 assimilate N_2-derived ammonia or supplied nitrogen through GS-GOGAT pathway (Boontariga, 1988), and the activity of purified GS from *Klebsiella* R15 is regulated by adenylylation-deadenylylation system and feedback inhibition by number of amino acids. In rice plant, there are three isoforms of GS, two forms (GS1 and GS2) have been identified in leaves and one form (GSr) has been identified in root (Hirel and Gadal, 1980). Both GSr and GS1 are cytosol specific, whereas GS2 is chloroplastic enzyme. Hirel and Gadal(1980) suggested that GSr might be involved in the primary ammonium assimilation in rice root and GS1 might be involved in the recycling of ammonia during photorespiration.

There is no information on ammonium assimilation in *Klebsiella* R15 associated with rhizosphere of rice. Therefore in this research the attempt is to correlate the role of bacteria GS and rice GS in

Klebsiella R15 - rice association. These results should lead to improve the efficiency of transport of fixed-N_2 from atmosphere to nitrogen metabolism of rice. It is hoped to improve the nitrogen fixing bacteria strain *Klebsiella* R15 to enhance the potential of nitrogen fixation and transfer of fixed-N_2 to the rice plant with increasing efficacy.

The expectation of this research are as follows:

1) If NH_4^+ or product of fixed nitrogen had been transferred to rice directly, the GS specific activity in *Klebsiella* R15 associated with rhizosphere of rice should be decreased in comparison with free-living *Klebsiella* R15 , and the GS specific activity in rice roots in association with *Klebsiella* R15 should be increased as compared to free-living rice roots.

2) If the other nitrogenous compound such as glutamine or other amino acids had been transferred to rice plants, the GS specific activity in *Klebsiella* R15 and rice root should be opposite to the first hypothesis.

The experimental approach of this thesis is the following:

1) To determine the GS specific activity and the amount of GS protein in free-living *Klebsiella* R15 in comparison with *Klebsiella* R15 associated with the rhizosphere of rice cv. RD7

2) To study the relationship between GS and nitrogenase activity in *Klebsiella* R15 associated with the rhizosphere of rice.

3) To compare the GS specific activity in rice seedling roots in free-living condition and when inoculated with *Klebsiella* R15.