STUDIES ON THE REGULATION OF UPTAKE HYDROGENASE IN *Azorhizobium caulinodans*

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STUDIES ON THE REGULATION OF UPTAKE HYDROGENASE IN *Azorhizobium caulinodans*

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CERTIFICATE

This is to certify that the thesis entitled, "***Studies on the Regulation of Uptake Hydrogenase in Azorhizobium caulinodans***", submitted to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirement for the degree of ***Doctor of Philosophy in Biochemistry***, embodies the results of a *bonafide* research carried out by ***Sandhya Sanghi*** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

It is further certified that such help or source of information has been duly acknowledged by her.

Place : New Delhi
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Chapter 1

INTRODUCTION

Biological nitrogen fixation is an important biochemical process, contributing more than 70% of the input into the world's soil and water nitrogen. Among the various biological systems which are able to fix atmospheric nitrogen, the legume-*Rhizobium* symbiotic system is the most important and efficient system which contributes most nitrogen to the ecosystem and to food production. About 100 to 175 million tonnes of nitrogen are fixed annually by the process of biological nitrogen fixation and out of this, about 50% is contributed by legume-*Rhizobium* symbiosis.

The process of biological nitrogen fixation is not dependent upon nonrenewable energy resources. Hence, its use in agriculture needs maximization. Moreover, concern about our dwindling supplies of fossil fuel has stimulated an interest in the possibilities of increasing the use and efficiency of biological nitrogen fixation for agricultural purposes, thus conserving some of the fossil fuel. Increase in N_2 fixation efficiency of nodules is vital for increasing crop yield of leguminous plants.

During biological nitrogen fixation, N_2 is reduced to NH_3 by the enzyme nitrogenase. All nitrogenase preparations obtained so far have been found to catalyze an ATP-dependent H_2 evolution, even in the presence of N_2. Due to H_2 evolution, in most legumes loss of energy as ATP and reducing power may be as high as 30-50% of the total energy flux through nitrogenase (Evans *et al.*, 1981). Some strains of *Rhizobium*, however, possess an O_2-dependent enzyme system, termed as uptake hydrogenase (Hup), which recycles the H_2 evolved by nitrogenase system. Recycling of H_2 by nodule bacteroids in the oxyhydrogen reaction is known to have many useful biochemical and physiological functions especially under conditions of carbon starvation. Recycling of H_2 under *ex planta* condition has an added advantage of ecological adaptation under adverse conditions. These advantages could help *Rhizobium*-legume symbiotic system fix more N_2 and produce more biomass. In some legume-*Rhizobium* symbiotic system a consistent positive effect of the Hup system on overall plant productivity has been shown. Thus the *hup* genes are emerging as prime candidates for practical application in agriculture.

Extensive studies have been made on Hup system of *Bradyrhizobium japonicum*. However, not much is known about the Hup system of *Azorhizobium caulinodans* which nodulates *Sesbania rostrata*, a tropical

legume. Unlike all other rhizobia, *Az. caulinodans* can be derepressed for Hup activity in the presence of high organic carbon under free-living conditions (de Vries *et al.*,1984).

Azorhizobium caulinodans assumes a unique position amongst the N_2-fixing organisms. In addition to its ability to fix N_2 in aerial stem nodules as well as root nodules while in symbiosis with its specific host *Sesbania rostrata*, it is also capable of fixing N_2 in free-living state and can grow with atmospheric nitrogen as the sole nitrogen source. Moreover, *S. rostrata-Az. caulinodans* symbiotic system being the most efficient N_2-fixing system, can be adopted as a 'Model system' to work on and improve N_2-fixing efficiency of other symbiotic systems. Since presence of efficient H_2-recycling system in the microsymbiont helps in improving symbiotic energy efficiency, it is important to know more about the Hup system. Therefore, the present investigation was carried out in *Azorhizobium caulinodans* strain IRBG 46 with the following objectives :

1. To study the factors regulating the expression of uptake hydrogenase.
2. To study the relationship of enzyme uptake hydrogenase with other enzymes such as nitrogenase and RuBP carboxylase.

Chapter 2

REVIEW OF LITERATURE

During biological N_2 fixation, N_2 is reduced to NH_3 by the enzyme nitrogenase. As a by-product of this reaction, nitrogenase also evolves H_2, which adds considerably to the energy costs of the fixation. Some rhizobia possess an O_2-dependent enzyme system, termed 'uptake hydrogenase' , that is capable of recycling the H_2 thus released. Such a system regenerates chemical energy in the form of ATP or reducing power and also removes H_2 and O_2 from the active site of nitrogenase where they might act as reversible or irreversible inhibitors of N_2 fixation (Dixon, 1972; Emerich *et al.*, 1979; Ruiz-Argüeso *et al.*, 1979).

Improvement of legume productivity by increasing the N_2-fixing capability of *Rhizobium* species has become a major research objective. In order to realise this goal, it is desirable to transfer the H_2-recycling capability to commercially important *Rhizobium* strains which lack this capability. A number of experiments have shown the beneficial effects of inoculating legume crops with H_2-uptake positive (Hup^+) strains of *Rhizobium* (Dixon, 1972; Albrecht *et al.*, 1979; Eisbrenner and Evans, 1983; Evans *et al.*, 1985).

Recent developments in understanding the biochemistry of H_2-uptake system including uptake hydrogenase and advantages conferred by H_2 oxidation in Hup^+ rhizobia in plants, have been reviewed in this chapter. In addition, regulation of hydrogenase as well as progress made in transfer and expression of *hup* genes encoding uptake hydrogenase, both in free-living and symbiotic conditions in different species of rhizobia have also been reviewed.

2:1 Hydrogen evolution from nodules

In 1941, Phelps and Wilson for the first time reported the presence of a H_2-uptake system in nodules of *Pisum sativum* formed by *Rhizobium leguminosarum* strain 311. This report was confirmed more than 25 years later when Dixon (1967) demonstrated H_2 oxidation activity in bacteroids which could couple H_2 oxidation to ATP production.

Hoch *et al.* (1957, 1960) discovered that N_2-fixing soybean nodules evolved H_2 and the enzyme nitrogenase catalyzes a concurrent reduction of N_2 to NH_3 and protons to H_2. Bulen *et al.* (1965) observed that cell-free extracts of *Azotobacter vinelandii* evolve H_2 which like the nitrogenase reaction requires ATP. Also H_2 was evolved in the absence of N_2 in a reaction containing purified

nitrogenase, $Na_2S_2O_4$ and an ATP generating system. This provided direct evidence that H_2 evolution during N_2 fixation was catalyzed by nitrogenase.

Evans *et al.* (1981), have shown that in the absence of an active uptake hydrogenase, the reduction of protons to H_2 in legume root nodules results in loss of 30-50% electron flux through nitrogenase. They found very few strains which evolved little or no H_2, which had been attributed to an uptake hydrogenase. The lack of this H_2 recycling ability undoubtedly accounts for the estimate that one million or more metric tons of H_2 are produced globally each year by nodulated legumes (Evans *et al.*, 1987).

Since no plant system is known to utilize H_2, uptake hydrogenase of *Rhizobium* has acquired much significance as a means of conserving plant energy. In the fast growing rhizobial species like *R. leguminosarum, R. meliloti, R. phaseoli* and *R. trifolii*, the occurrence of an Hup system is sporadic. Sometimes, some strains exhibit very slightly active Hup system (Ruiz-Argüeso *et al.*, 1978; Nelson and Child, 1981). An active uptake hydrogenase has been found more often in slow-growing cowpea and soybean rhizobia (Schubert *et al.*, 1977, 1978; Lim *et al.*, 1981; Pahwa and Dogra, 1981, 1983). Recently, some fast growing strains of cowpea rhizobia have also been found to possess an active uptake hydrogenase (Trinick, 1982). A functional Hup system was detected in fast-growing *Azorhizobium* strain ORS 571 (Stam *et al.*, 1984), which was originally isolated from stem nodules of *Sesbania rostrata*, a tropical legume (Dreyfus and Dommergues, 1981).

Hup^+, Hup^- strains and Hup^- mutants were compared on the basis of their effect on dry matter accumulation and N_2 fixation. Plants incoulated with Hup^+ strains produced higher N-content and dry matter over the plants inoculated with Hup^- strains. This is in consonance with the belief that if energy supply is a major limiting factor for N_2 fixation in legumes as proposed by Burns and Hardy (1975) and Hardy and Havelka (1975), then the coupling of oxidation of H_2 to energy yielding processes would conceivably increase the rate of N_2 fixation. Alternatively, the conservation of energy through the H_2-recycling process might decrease the demand for photosynthate and consequently contribute to increased dry matter production. Therefore, the increase in yield and N_2 fixation observed in the above study seems to be associated with improved efficiency of symbiotic N_2 fixation by Hup^+ strains of *Rhizobium*.

Feijtel *et al.* (1985) assessed RE for Hup^- mutant of *R. meliloti* and *R. leguminosarum*. RE was 0.67 for both the mutants while for the wild type (Hup^+) strains was 0.90. Hydrogen production was also 2 and 1.5 times that of the two wild type strains, while acetylene reduction was two-third and half of the wild type strains.

2:2 Beneficial aspects of the Hup system

Production of H_2 by nitrogenase represent an inefficiency of the N_2-fixing system because the energy available for N_2 reduction is wasted in H_2 evolution. The presence of an active Hup system allows greater efficiency in biological N_2 fixation (BNF) as has been shown by many free-living diazotrophs (Arp, 1992). One mechanism by which a Hup system might enhance the overall efficiency of BNF is by increasing the ATP or reductant availability to bacteroids (Dixon, 1972) assuming that BNF is energy-and/or reductant limited.

The efficiency of the symbiosis is improved with uptake hydrogenase activity in the bacteroids because the oxidation of H_2 stimulates ATP production (Emerich *et al.*, 1979). Thus, to bring about improvement in legume productivity by increasing the N_2-fixing capability of *Rhizobium* spp., strategies are being devised to isolate and transfer the genetic determinants for H_2-uptake into those species lacking them. Since H_2 evolution appears to be inherent in the nitrogenase mechanism (Chatt, 1980; Simpson and Burris, 1984), attempts to mutate nitrogenase otherwise, such that it produces only NH_4^+ and no H_2 may be futile.

Dixon (1972) pointed out the following potential advantages of an efficient H_2-recycling system to N_2-fixing organisms:

a) The consumption of O_2 by the H_2 oxidation reaction may contribute towards the protection of the O_2-sensitive nitrogenase.

b) The oxidation and thus removal of H_2 produced via nitrogenase within the cell might prevent inhibition of nitrogenase by H_2.

c) Oxidation of H_2 may support the synthesis of ATP for use in N_2 fixation and other processes.

Lepo *et al.* (1980) observed that several slow growing *Rhizobium* strains can grow as hydrogen autotrophs, which raises another interesting possibility, that hydrogenase is of importance to the bacterium in some ecological niche in the soil. For example, H_2 and CO_2 evolved from a legume root nodule occupied by a Hup^- *Rhizobium* strain could provide the necessary energy and carbon sources for hydrogen dependent autotrophic growth of Hup^+ *Rhizobium* strain on the surface of that nodule.

Dixon and Blunden (1983) observed that the apparent fraction of nitrogenase electrons allocated to N_2 reduction in pea nodules was inversely related to nitrogenase activity under a variety of experimental conditions in which variations in nitrogenase activities were associated with water stress, ontogeny, temperature and incubation time. All the results were consistent with the conclusion that high nitrogenase activities were associated with high rates of H_2 evolution which resulted in the accumulation of H_2 at concentrations sufficient to inhibit N_2 fixation.

Minamisawa *et al.* (1983) have reported that the rate of transport of fixed N from roots of soybean plants nodulated by Hup^+ strains was greater than that of plants nodulated by Hup^- strains and proposed that an operative H_2-recycling system affected the balance of carbon utilization and the nitrogen assimilation process in nodules.

Nelson and Salminen (1982) observed that some strains of *R. leguminosarum* contain Hup system that is coupled to ATP synthesis while in others, it is not coupled. Salminen and Nelson (1984) further suggested that under conditions of substrate starvation, the uptake hydrogenase could provide reductant as well as ATP in an isolate in which the H_2-uptake is coupled to ATP formation. Lodha and Naik (1984) also reported that uptake hydrogenase from groundnut bacteroids reduced low redox potential carriers such as benzyl viologen and NAD^+. Thus, they suggested that the reduction of a primary physiological electron acceptor by H_2 may be providing a reductant for nitrogenase activity.

A number of studies also indicated that Hup system significantly benefits overall plant productivity since energy supply could be one of the major limiting factors in N_2 fixation in soybean (Hardy and Havelka, 1975).

Lepo *et al.* (1981) used nitrous acid to obtain Hup$^-$ mutants which were deficient in the hydrogenase enzyme activity. Plant growth experiments under bacteriologically controlled conditions showed that the plants inoculated with these Hup$^-$ strains had lower dry weights and contained less total N than did plants inoculated with the parent Hup$^+$ strain. The results strongly supported a beneficial role of the H_2-uptake phenotype in legume symbiosis.

Uptake hydrogenase system in fast growing strains of *Rhizobium* sp. *Sesbania* was analysed by Saini *et al.* (1987). They found a statistically significant increase (>22%) in total plant nitrogen content and dry matter yield by the inoculation of Hup$^+$ strains over Hup$^-$ strains.

It has been proposed by Evans *et al.* (1987) that any *Rhizobium* strain that is capable of chemolithotrophic growth with H_2 as the sole source of energy is capable of coupling H_2 oxidation to ATP synthesis. It has been found that chemolithotrophic growth capability is most prominent in the Hup$^+$ strains of *B. japonicum* or other rhizobia that have acquired *B. japonicum* determinant(s) for H_2 oxidation (Lambert *et al.*, 1985a). Earlier, several experiments to support Dixon's view of H_2 dependent synthesis of ATP have also been demonstrated with *R. leguminosarum* and *B. japonicum* bacteroids (Emerich *et al.*, 1979).

Thimmaiah and Lodha (1986) also reported that rhizobial strains with an active Hup system were superior in their N_2-fixing ability over H_2 evolving Hup$^-$ strain in mungbean. More recently, in 1990, Vasudev *et al.* showed that interspecies transfer of *B. japonicum hup* genes into *Cicer*-rhizobia increased the relative efficiency (RE) of nitrogen fixation from 0.73 to 0.81 and 0.70 to 0.83 in two different strains. A *Rhizobium leguminosarum* field isolate Vp1 possessing uptake hydrogenase was isolated by Garg *et al.* (1990a). Vp1 was cured by plasmid curing treatments to get isogenic Hup$^-$ mutant, Vm1. *In planta* comparison of Vpl (Hup$^+$) and Vml (Hup$^-$) showed that the presence of uptake hydrogenase activity in Vpl resulted in nodules having higher nitrogenase activity and enhanced relative nitrogen-fixing efficiency and oxygen consumption due to hydrogen-recycling.

2:3 Purification of the enzyme hydrogenase

Arp and Burris (1979) were the first to purify hydrogenase from *R. japonicum*. They reported that the enzyme is a monomer of 65.3 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

is sensitive to air, having a half life of 70 min. Subsequent purifications of the *R. japonicum* hydrogenase from bacteroids (Arp, 1985), chemolithotrophically-grown cells (Harker *et al.*, 1984) and heterotrophically-grown cells (Stults *et al.*, 1986a) all revealed that the enzyme contains two subunits with molecular weight of about 65 kDa and 35 kDa. The two subunits were present in a 1:1 molar ratio and were immunologically distinct from each other, since the molecular weight of the native enzyme was determined to be 104 kDa by sucrose density centrifugation (Arp, 1985). Hydrogenase from bacteroids and chemolithotrophically-grown cells was purified under strict anaerobic conditions using ion exchange and sizing columns, whereas the enzyme from a *R. japonicum* strain that synthesizes hydrogenase heterotrophically was purified aerobically using a "Reactive-Red" affinity column.

Antibodies raised against each of the two subunits did not cross-react with the other subunit, showing that 35 kDa subunit was not a degradation product of the larger subunit (Harker *et al.*, 1984; Stults *et al.*, 1986a). Polypeptides of 60 and 30 kDa have been detected in aerobically and anaerobically cultured *E. coli* that cross react with antibodies against the 60 and 30 kDa subunits of *B. japonicum* (Harker *et al.*, 1986).

Although the H_2-uptake hydrogenase from *R. leguminosarum* has not yet been purified because of difficulties in obtaining working amounts of hydrogenase-induced cells (Levya *et al.*, 1990), a dimeric (α, ß) polypeptide structure similar to that of hydrogenases from other N_2-fixing bacteria such as *B. japonicum* (Arp, 1985; Harker *et al.*, 1984; Stults *et al.*, 1986a), *A. vinelandii* (Seefeldt and Arp, 1986) or *Rhodobacter capsulatus* (Seefeldt *et al.*, 1987) is being expected.

2:4 Genetic determinants for uptake hydrogenase

Genes encoding uptake hydrogenase are located on plasmids in certain species of H_2-oxidizing bacteria. Reh and Schlegel (1975), Schlegel (1976) and Pootjes (1977) have shown that loss of the ability to grow chemolithotrophically and loss of hydrogenase activity were associated with the exposure of *Nocardia* species and *Pseudomonas facilis* to plasmid curing agents. Reh and Schlegel (1975) also demonstrated conjugal transfer of autotrophic capability and NAD-dependent hydrogenase activity between

different species of *Nocardia*. On the other hand, simultaneous loss of hydrogenase activity and a high molecular weight plasmid from *Alcaligenes eutrophus* was observed after exposure to mitomycin C (Lim *et al.*, 1980). The transfer of this large plasmid to the plasmid cured strains resulted in the restoration of hydrogenase activity.

2:4:1 Hup determinants in slow-growing rhizobia

Although large plasmids have been detected in slow-growing species such as *R. japonicum*, the efforts to resolve such large plasmids were not successful. Thus correlations of Hup with plasmid content have not been possible (Cantrell *et al.*, 1982; O'Hara, 1984). The plasmid profiles of a series of Hup^+ and Hup^- strains were compared by Cantrell *et al.* (1982). None of the strains of *R. japonicum* with high H_2-uptake activities exhibited discernible plasmids, while most of the strains, with little or no Hup activity, showed plasmids with molecular weights ranging from approximately 49-290X10^6. Thus, the Hup determinants whenever present seem to be chromosomal encoded.

Since the efforts to resolve such large plasmids were unsuccessful, a cosmid gene bank of *B. japonicum* was constructed by Cantrell *et al.* (1983). The cosmid clones from the gene bank were identified carrying *hup* gene(s) by the ability to complement the Hup^- derivative of *B. japonicum* 122 DES (pJ17nal).

Eisbrenner and Evans (1982) demonstrated *in vivo* biochemical complementation for methylene blue dependent H_2-uptake by mixing crude cell-extracts from groups of Hup^- mutants of *B. japonicum*. These results indicated involvement of more than one gene in the Hup system. Thus, transfer of H_2-uptake activity to wild type Hup^- strains of *Rhizobium* may require the transfer of several genes.

2:4:2 Hup determinants in fast-growing rhizobia

In contrast to the slow-growing rhizobia, in fast-growing rhizobia, H_2-recycling ability has been found to be plasmid encoded (Brewin *et al.*, 1980a; Dejong *et al.*, 1982; Kagan and Brewin, 1985; Bekhi *et at.*, 1985). Brewin *et al.* (1980b) reported that determinants for hydrogenase activity (*hup*) in a particular strain of *R. leguminosarum* 128C53 are located on the non-

transmissible plasmid pRL6JI of molecular weight $19X10^7$ and linked to the determinants for nodulation ability residing on the same plasmid. But hydrogenase activity in *R. leguminosarum,* in contrast to that in *B. japonicum,* is expressed only in symbiotic association with the host (Evans *et al.*, 1987). Consequently, screening for Hup^+ strains must be carried out using nodule bacteroids.

The variability in the H_2 oxidation systems is also reflected in the level of homology of the *hup*-specific DNA in *R. leguminosarum* and *R. japonicum.* Nelson *et al.* (1985) found that *hup* specific DNA from *R. japonicum* and *R. leguminosarum* hybridized weakly or not at all with total or plasmid DNA from four out of twelve *R. leguminosarum* Hup^+ strains examined. These observations not only reflect intergenic variability of *hup*-specific DNA, but also differences among *R. leguminosarum* strains.

2:4:3 Hup determinants in azorhizobia

Not much is known about the detailed genetic organization of *hup* determinants in azorhizobia. de Vries *et al.* (1988) isolated four hydrogenase-negative (Hup^-) mutants of *Azorhizobium caulinodans* ORS 571 by means of Tn5 mutagenesis. Recently, the Tn5 insertions in these strains have been found to map in the same region of the ORS 571 genome (unpublished work of Hilgert *et al.* as cited by de Vries *et al.*, 1988)) which shares a high degree of DNA homology with the *hup* locus of *Rhizobium leguminosoarum*, described by Tichy *et al.* (1987).

Mehta *et al.* (1993) constructed a gene bank of *Azorhizobium* IRBG 46 and isolated *hup* genes using 12.9 kb *Eco* RI fragment of cosmid pHU52 as a heterologous *hup* probe. Five positive clones were transferred to Hup^- *Cicer-Rhizobium* strain Rcd 301. Transconjugants thus, obtained were found to have *ex planta* uptake hydrogenase activity.

2:5 Genetic analysis using Hup^- mutants

Hydrogen-uptake negative (Hup^-) mutants of *Rhizobium* have been useful for the study of the biochemistry, regulation and genetics of the oxidation, as well as for assessment of the importance of H_2 oxidation to symbiotic N_2 fixation. Hup^- mutants were first selected by enriching a bacteroid suspension for cells to unable to reduce PMS with H_2, and then screening for

colonies that failed to reduce triphenyltetrazolium chloride in the presence of H_2 (Maier *et al.*, 1978). Mutants thus obtained expressed Hup$^-$ character both in free-living as well as under symbiotic conditions. The same method has been used to isolate Hup$^-$ mutants of mung bean and urd bean *Rhizobium* (Pahwa and Dogra, 1981, 1983).

Maier (1981) used the mutagen ethylmethane sulfate (EMS) to obtain *R. japonicum* Hup$^-$ mutants. The mutants were screened by their inability to grow autotrophically with H_2 and CO_2 as the sole energy and carbon sources, respectively. Two of such mutants were found to have hydrogenase activity in the presence of artificial electron acceptor such as methylene blue but were unable to oxidize H_2. These mutants presumably synthesize an active hydrogenase enzyme, but some other component may be missing in the electron transport system to O_2. Another class of EMS mutant lacked H_2-uptake activity either with O_2 or methylene blue as acceptor. These mutants can be utilised to study the mutation in structural genes. However, two dimensional gel electrophoresis of bacteroid extracts of 10 of these mutations has shown that most of them lack any detectable hydrogenase protein (O'Hara, 1984). Therefore, many of the mutants could be in genes responsible for regulation of hydrogenase synthesis. Some Hup$^-$ mutants were found to be hypersensitive to oxygen mediated repression of hydrogenase. These mutants were useful in studying the regulation of O_2 which plays a key role in the expression of symbiotic properties in *Rhizobium* spp.

Site-directed insertion of the transposon Tn5 into the genome of the strain 122 DES nal was carried out by Haugland *et al.* (1984) in order to determine the extent of *hup*-specific DNA. For this, one of the *hup* cosmid pHU1 was subjected to transposon Tn5 mutagenesis. This mutant was integrated into the genome. The results indicated that *hup*-specific sequences spanned about 16 kb of DNA within pHU1.

Hom *et al.* (1984) isolated Hup$^-$ transposon mutant using the suicide vector system pSUP1011. This carries the transposon Tn5, encoding kanamycin and streptomycin resistance. Such mutants would be very useful in studying the regulation and genetics of H_2 oxidation in *R. japonicum*. Moshiri *et al.* (1983) obtained a mutant that was Nif$^-$ Hup$^-$. This mutant lacks both

of the nitrogenase component proteins as well as hydrogenase. It is possible that this strain contains a regulatory mutation preventing the synthesis of all these three proteins.

Yates and Robson (1985) used nitrosoguanidine for mutagenizing *Azotobacter chroococcum* and Hup$^-$ mutants were screened by ^3H uptake. Four mutants having H_2 evolving activity and not showing any H_2-uptake were complemented for hydrogenase activity by conjugation with *E. coli* carrying plasmid pHU1 containing *R. japonicum* hydrogenase genes. One mutant strain, MCD-124, expressed hydrogenase activity similar to the solubilised wild type enzyme. A pHU1 transconjugant of this mutant, however did not express any additional hydrogenase activity.

2:6 Regulation of hydrogenase synthesis and activity

2:6:1 Effect of carbon substrates, O_2 and H_2

Expression of high rates of H_2 oxidation activity in bacteroids from Hup$^+$ strains of *R. japonicum* can be easily demonstrated. Maier *et al.* (1978) for the first time, were able to demonstrate H_2 oxidation activity in *R. japonicum* when cells were cultured under 1% partial pressure of O_2, limiting substrates, and in the presence of H_2. These conditions are similar to those that undoubtedly occur inside the legume nodules (Appleby *et al.*, 1975) where bacteroids actively synthesize hydrogenase if they possess the genetic capability. The expression of H_2-uptake in liquid cultures of *R. japonicum* enabled studies of the factors that regulate the expression of H_2 oxidation (Maier *et al.*, 1979; Simpson *et al.*, 1979). The expression of the H_2-uptake system is regulated by a number of factors, including H_2, O_2, and carbon-substrates. The addition of H_2 was required for the induction of H_2-uptake system, while high levels of O_2 and carbon-substrates repressed the H_2-uptake system.

Experiments done by Maier *et al.* (1978) showed that *R. japonicum* cells removed from slants under aerobic conditions contained hydrogenase activity that was approximately equal to that of comparable cells removed from slants under anaerobic conditions. The inhibitory effect of O_2 on hydrogenase expression, therefore, probably was due to repression of hydrogenase synthesis rather than inactivation of preformed hydrogenase.

Maier *et al.* (1978) also demonstrated H_2-uptake by *R. japonicum* grown asymbiotically under conditions in which carbon substrates were limited and nitrogenase activity was not detected. Under these conditions, however, it was necessary to pretreat the cultures with H_2 before the expression of hydrogenase activity was detected. These results indicated the requirement of H_2 for induction of the synthesis of hydrogenase. However, on the other hand, H_2 may be needed as a substrate for the generation of the energy required for the synthesis of the hydrogenase system (Schlegel and Eberhardt, 1972).

Since an environment low in O_2 is required for hydrogenase expression in free-living cells, it is possible that the microaerophillic environment of the root nodule may be important for the enzyme synthesis in bacteroids. However, it is interesting that bacteroids are supplied with adequate carbon substrates by the plant, whereas free-living cells must be incubated in a medium lacking carbon substrates for expression of hydrogenase (Maier *et al.*, 1978, 1979, Simpson *et al.*, 1979).

The mechanism of regulation by O_2 and carbon substrates is not known, but it seems to be related to the redox state of the pyridine nucleotide pool and/or the energy charge of the cells (Maier, 1986). Maier and Merberg (1982) isolated mutants hypersensitive to repression of H_2 oxidation by O_2. These mutants were found by screening free-living Hup^- mutants that retained H_2-uptake activity as bacteroids at low (0.1% partial pressure) oxygen tensions. These mutants were also hypersensitive to carbon substrate repression of H_2 oxidation indicating that carbon and O_2 repression acts through a common regulatory element.

In an effort to answer some basic questions concerning the regulatory role of O_2 in *Rhizobium*, Merberg and Maier (1983) isolated *R. japonicum* mutant strains that express H_2-oxidizing activity in the presence of high concentration of oxygen. These mutants also showed increased hydrogenase activity when harvested from soybean nodules. H_2-uptake was completely repressed by 10% O_2 in the wild type strain but not in the mutant strains. Moreover, mutant strains also produced more hydrogenase activity than the parent strain when derepressed in 1% O_2. The mutant strains having higher hydrogenase activity in culture, also had high activity in nodules, suggesting

thereby that common elements might be involved in regulating the Hup system in free-living cells and bacteroids. These mutants were also partially relieved of the normal repression of hydrogenase by carbon substrates such as succinate, arabinose, glycerol and gluconate (Merberg *et al.*, 1983). Since these strains make hydrogenase constitutively even in the absence of H_2, they have been designated as hydrogenase constitutive (Hup^c).

Merberg *et al.* (1983) also revealed by two dimensional polyacrylamide gel electrophoresis (PAGE) that during heterotrophic growth, the Hup^c *R. japonicum* mutant strain SR 470 synthesized at least six peptides not found in the wild type strain. Presumably, one of these peptides is the hydrogen-activating enzyme; other peptides may be involved in regulation of hydrogenase or may be co-regulated with hydrogenase. This observation is consistent with a hypothesis that the gene affected in the Hup^c mutant controls the synthesis of several enzymes.

Lim and Shanmugan (1979) reported a 10-fold increase in intracellular cAMP levels associated with the induction of H_2-uptake activity in *R.japonicum*. However, Merberg *et al.* (1983) could not find much difference in the concentration of cAMP in Hup^c and wild type strains cultivated heterotrophically. Also there was no significant difference in the amount of ppGpp observed in heterotrophically grown SR and Hup^c mutants, thereby not providing a very clear picture of the Hup system being subject to a catabolite repression-like regulation, a phenomenon that has been linked to regulation by carbon substrates and respiratory electron acceptors (Botsford, 1981; Lee and Dobrogosz, 1983).

A fast growing *Rhizobium* strain, ORS 571 has been isolated from stem nodules of the tropical legume *Sesbania rostrata* (Dreyfus and Dommergues, 1981). This strain forms nodules on both the stem and roots of the host plant. Under N_2-fixing conditions, an uptake hydrogenase is induced in *Rhizobium* ORS 571 (Stam *et al.*, 1984). It has also been shown that N_2-fixing cultures of *Rhizobium* ORS 571 contain considerable hydrogenase activity and for the induction of hydrogenase in ammonia-assimilating cultures, addition of H_2 to the gas phase is required. This induction of hydrogenase in N_2-fixing cultures suggests that H_2 formed intracellularly by nitrogenase is recycled.

de Vries *et al.* (1984) described the influence of H_2 oxidation on the behaviour of *Rhizobium* ORS 571 in free-living, energy-limited cultures. They provided the first report that in this bacterium, the presence of a carbon source does not repress or inhibit the synthesis and activity of hydrogenase. In this respect, *Rhizobium* ORS 571 differs from *R. japonicum* in which the synthesis of hydrogenase is repressed by carbon substrates (Maier *et al.*, 1978, 1979).

de Vries *et al.* (1984) also reported that the Hup system of *Rhizobium* ORS 571 seems to function not only in carbon-limited N_2-fixing cultures but also in O_2 limited N_2-fixing cultures. Though Hardy and Havelka (1976) suggested N_2 fixation in nodules to be limited by the supply of carbohydrates, later studies by Larue *et al.* (1984) suggested the condition of bacteroids in the nodule to be oxygen limited.

Studies by Saini *et al.* (1987) on the uptake hydrogenase in fast growing strains of *Rhizobium* sp. *(Sesbania)* in relation to N_2 fixation have shown derepression of uptake hydrogenase in both low as well as high carbon containing media. In addition to H_2-uptake medium, all the eight strains also expressed uptake hydrogenase activity on media containing high carbon, *viz.* nitrogenase induction (Dadarwal *et al.*, 1981) and YEMA media. The time required for derepression of hydrogenase on all the media was found to be the same (about 40 h). The specific activity of uptake hydrogenase, however, was high on H_2-uptake medium as compared with that on YEMA and nitrogenase induction media.

The activity of uptake hydrogenase in the nodules of *Sesbania* was sufficiently high (30-70 μmol H_2/h/g fresh nodules) as compared with that in pea nodules (0.2-0.8 μmol H_2/h/g fresh nodules) and soybean nodules (3.7-6.7 μmol H_2/h/g fresh nodules) (Ruíz-Argüeso *et al.*, 1978; Hanus *et al.*, 1981). The high uptake hydrogenase activity observed in the *Sesbania* nodules produced by Hup^+ strains indicates that this activity would be sufficient to recycle all the hydrogen produced by nitrogenase. The uptake hydrogenase in these *Sesbania* strains might be less sensitive to high carbon repression.

Expression of uptake hydrogenase activity in free-living cultures of mungbean *Rhizobium* strain S-24 was found to be influenced by the quality and quantity of carbon source present in the induction medium (Thimmaiah *et al.*, 1986). Carbon sources (15 mM) like sucrose, fructose, arabinose and

sorbitol induced the expression of the enzyme while ribose, sodium citrate, glycerol and sodium gluconate which enhanced the growth of the strain completely repressed the expression. The enhancement in the expression of the enzyme was maximum with sorbitol and fructose (about 55%) followed by arabinose and sucrose (about 22%). With the increase in concentration of glucose from 5 to 25 mM, inhibition on the expression of enzyme also increased. The repression effect (64%) brought about by 25 mM glucose was reversed by both cAMP and cGMP. In the presence of glucose, cAMP stimulated the expression by 63% and cGMP by 145% compared to the standard medium. This is contrary to the studies on *R. japonicum* Hup^c mutant by Merberg *et al.* (1983). The concentrations of cAMP were almost similar in the wild type and Hup^c mutant strains during heterotrophic growth. This also contradicts the earlier observation by Lim and Shanmugan (1979) that in *R. japonicum* strain 110 when the hydrogenase activity is low, intracellular concentration of cAMP also remains low. Thus, variation in cAMP concentration alone may not completely account for catabolite repression.

Thimmaiah *et al.* (1986) also studied the effect of combined nitrogen sources. All the N-sources tested (10 mM concentration) supported the expression of hydrogenase equally well. Only NH_4Cl had a stimulatory effect to the extent of 42% as compared to standard medium. Growth was not affected by the N-source tested except sodium glutamate which inhibited the growth by 43%. The stimulatory effect of NH_4Cl on the expression of uptake hydrogenase in mungbean *Rhizobium* may be ascribed to secondary effects which act when there is no repression by carbon sources. Maier *et al.* (1979) could not observe any effect of KNO_3 or NH_4Cl (10 mM) addition on hydrogenase formation. Repression by glutamate was probably associated with its utilization as a source of carbon rather than N, since neither KNO_3 nor NH_4Cl repressed hydrogenase formation.

Effect of nitrogen sources on the derepression of the Hup activity in free-living *B. japonicum* was also investigated by Fukai *et al.* (1990). It was found that nitrate and ammonium stimulated hydrogenase synthesis in free-living *B. japonicum* cells. No reason was found for the discrepancy between these results and those of Maier *et al.* (1979) which concluded that neither nitrate nor ammonium had any effect on hydrogenase derepression.

In *A. eutrophus* the synthesis of hydrogenase is repressed by pyruvate and succinate - two preferentially utilized carbon sources (Friedrich and Schwartz, 1993). Growth on poor substrate such as glycerol derepressed the hydrogenase system to various levels, even in the absence of H_2. Thus, the production of hydrogenase in *A. eutrophus* H16 was not strictly dependent on H_2. It appeared that a physiological parameter such as reductant supply is the signal triggering hydrogenase derepression.

Recently in 1994, Sellstedt *et al.* studied the effect of carbon sources on growth, nitrogenase and uptake hydrogenase activities in *Frankia* isolates from *Casuarina* sp.. The carbon sources pyruvate, propionate and Tween 80, which gave the greatest growth were found to suppress uptake hydrogenase activity. Earlier Sellstedt and Smith (1990) have shown that low concentrations of carbon source(s) and oxygen and also the presence of hydrogen and nickel were required for expression of measurable uptake hydrogenase activity in *Frankia*.

In some H_2-oxidizing bacteria, H_2 is needed to induce hydrogenase, whereas in others, hydrogenase synthesis occurs without H_2 (Schlegel and Eberhardt, 1972). In the initial reports of expression of hydrogenase in free-living *R. japonicum* low levels of H_2-uptake activity were detected in free-living N_2-fixing cultures (Maier *et al.*, 1978) and these results led to the conclusion that H_2 either added or produced by nitrogenase was required to obtain H_2-uptake activity. In the presence of carbon substrates that permit nitrogenase expression, there might be a coordinate regulation of expression of both nitrogenase and hydrogenase. Graham *et al.* (1984) observed a positive correlation between the level of nitrogenase and corresponding hydrogenase activities among the nineteen carbon substrates screened for their ability to support nitrogenase, and then hydrogenase activities. Only media containing mannitol, gluconate, α-ketoglutarate, malate and succinate gave cells which were able to express nitrogenase and O_2-dependent Hup activity with the highest values yielded by α-ketoglutarate. Coordinate relationships have been observed earlier also in other H_2-oxidizing N_2-fixing bacteria (Robson and Postgate, 1980). Contrary to these studies, Lim and Shanmugam (1979) have reported Hup activity to be lowest in *R. japonicum* when cells were incubated in media that supported highest nitrogenase activities.

Further experiments have been performed by Graham *et al.* (1984) to show that the positive correlation between nitrogenase and hydrogenase is due to the stimulatory effect of nitrogenase produced H_2 on hydrogenase expression. They observed high levels of Hup activity in cells incubated under N_2-fixing conditions, i.e., in α-ketoglutarate containing medium in the presence of H_2. Also H_2-oxidation was observed in the nitrogenase induction medium without the addition of H_2, as the cells were producing H_2 via nitrogenase.

Though these results suggest there is coordinate regulation of the expression of both these enzymes involved in H_2 metabolism, there may be some regulatory differences between the two enzyme systems. This is demonstrated by the fact that the Hup^c mutants isolated by Merberg and Maier (1983) expressed much greater hydrogenase activity in the nitrogenase-induction medium , but nitrogenase activity was no greater than the wild type. Therefore, the regulatory factor(s) in the Hup^c mutants causing increased hydrogenase activity do not correspondingly affect nitrogenase activity.

The mechanism by which carbon substrates repress Hup activity of cell suspensions were shown to be changes in the pH of the induction medium or limitations in the supply of O_2 to the cells caused by carbon substrate metabolism (van Berkum and Maier, 1988). In this study, expression of *ex planta* Hup activity in *B. japonicum* induced in the absence or presence of carbon substrates was compared. The results showed an increased demand for O_2 in cells induced in the presence of a carbon substrate compared with those induced in the absence of a carbon substrate. This is due to the presence of a higher respiration rate in the presence of a carbon substrate. A limitation in O_2 supply results in lower Hup activity because O_2 is necessary for the induction of Hup activity in *B. japonicum* (van Berkum, 1987). Also, Hup activity was influenced by pH, indicating that acidification of induction medium with low buffering capacity resulting from carbon substrate metabolism inhibited Hup activity. These studies thus, showed that the expression of Hup activity by whole cell preparations was sensitive to the pH of the induction medium and on the type of carbon substrate used.

All *R. japonicum* Hup^c mutants isolated so far produced significantly more cytochrome *'o'* than did the wild type (O'Brian and Maier, 1985). Like hydrogenase, cytochrome *'o'* is synthesized under low O_2 conditions in many

bacteria (Poole, 1983) suggesting that the regulatory gene altered in the Hup^c mutants also affects cell systems not directly related to hydrogenase (O'Brian and Maier, 1988). Bacteroids of Hup^c mutants contain four to six fold more hydrogen activating enzyme than do bacteroids of wild type stain. On the other hand, the oxygen insensitive mutants did not produce significantly higher levels of cytochrome *c*, *b* or aa_3 than did the wild strain (Merberg *et al.*, 1983).

Minamisawa *et al.* (1990) reported that rhizobitoxine produced by *Bradyrhizobium* species strongly prevented derepression of hydrogenase expression in free-living cells. Cystathionine and methionine strongly prevented the inhibition of hydrogenase derepression by rhizobitoxine suggesting that the level of sulphur containing amino acids in the cells affects the regulation of H_2 oxidation.

2:6:2 Regulation by Nickel

The *B. japonicum* hydrogenase like many other hydrogenases, is a Ni^{2+}-containing enzyme (Stults *et al.*, 1984; Arp, 1985). Ni^{2+} appears to play a role in regulating the expression of hydrogenase synthesis in free-living *B. japonicum,* since the amount of antigenically detectable hydrogenase increases with the amount of Ni^{2+} supplied to the cells (Stults *et al.*, 1986b). Kim and Maier (1990) demonstrated that Ni^{2+} is required for the synthesis of hydrogenase mRNA in *B. japonicum* and that a region upstream of the hydrogenase structural genes is responsible for transcriptional regulation. Moreover in a study carried by Kim *et al.* (1991), this cis acting region was proven to be essential for transcriptional regulation of hydrogenase expression by O_2 and H_2. They proposed a hypothetical signal transduction pathway with a membrane bound sensor protein to detect the redox condition in the cell, which is determined by O_2 and H_2 concentrations. The hydrogenase protein itself cannot be the sensor since the regulatory effect was also observed in a Hup^- mutant with a Tn5 insertion in the hydrogenase structural genes (Kim *et al.*, 1991). Two open reading frames homologous with the *Alcaligenes eutrophus hoxX and hoxA* genes (Eberz and Friedrich, 1991) were identified. The structural characteristics of both gene products indicate that they form a sensor-effector couple involved in free-living *hup* gene expression (van Soom *et al.*, 1993).

A Ni^{2+} metabolism related locus located 8.3 kbp upstream of the hydrogenase strucutural genes, was isolated from *B. japonicum* strain JH (Fu and Maier, 1991). A mutant with a gene-directed mutation at this locus requires a much higher concentration of Ni^{2+} during hydrogenase derepression than the wild type strain. To investigate the possible role of the locus in Ni^{2+} insertion into hydrogenase, a hydrogenase constitutive mutant (Hup^c) was mutated at the Ni^{2+} locus (Fu and Maier, 1992). In the presence of Ni^{2+}, the amount of hydrogenase apoprotein synthesis in the double mutant was the same as in the Hup^c strain but whole cell hydrogenase uptake activity was only half of that seen in the Hup^c strain. This supports the hypothesis that the lower hydrogenase activity in the double mutant is due to a defect in a factor responsible for incorporation of Ni^{2+} into the hydrogenase apoenzyme.

2:6:3 Supercoiling and hydrogenase expression

Certain genes that are regulated in response to environmental stimuli like anaerobiosis, show an altered expression that is dependent on DNA tertiary structure (Novak and Maier, 1989). Synthesis of *B. japonicum* hydrogenase is repressed by high concentrations of O_2 (Maier *et al.*, 1979; van Berkum, 1987), and enzyme synthesis has been shown to be prevented by inhibitors of DNA gyrase (Novak and Maier, 1987).

Hup^c mutants display a pleiotropically altered phenotype; several proteins normally specific to a microaerobic environment during derepression for hydrogenase are expressed constitutively. Hup^c mutants appear to be insensitive to the previously demonstrated inhibition of hydrogenase synthesis by DNA gyrase inhibitors, while sensitivity to growth inhibition by the inhibitors of mutant strain itself has not changed (Novak and Maier, 1989).

The Hup^c mutant may be affected in two ways. Firstly, the tertiary structure of the DNA could be altered so that the mutant senses a lower O_2 tension than the actual tension, which could in turn affect the expression of a regulatory gene normally active only under anaerobic conditions. Alternatively the regulatory gene itself could be altered, so that its product recognizes genes that are normally of the correct tertiary structure under anaerobic conditions (Novak and Maier, 1989).

2:6:4 Host control on hydrogenase expression

Keyser *et al.* (1982) investigated the legume host - *R. japonicum* strain interactions for hydrogenase activity in soybean, cowpea and siratro. Strains of *R. japonicum* of varying effectiveness were examined. The nonhomologous hosts were nodulated by all the strains tested, but effectiveness was not related to that of the homologous host. On siratro, compared to soybean, many strains reversed their relative effectiveness ranking. Both siratro and cowpea produced more dry matter with standard cowpea rhizobia CB756 and 176A22 than with the strains of *R. japonicum*. The strain USDA 122 expressed high rates of hydrogenase activity in symbiosis with cowpea as well as the soybean host. These results indicated host influence for the expression of hydrogenase activity.

Bedmar *et al.* (1983) showed that host plant determinants within a single species can affect *Hup* phenotypes. Relative efficiency was calculated in three lines of *Pisum sativum* nodulated by single strain of *R. leguminosarum*. Relative efficiency did not vary significantly among Alaska, Feltham First and JI 1205 peas inoculated with the Hup^- strain *R. leguminosarum* 300. RE of strain 128C53 was significantly greater in symbiosis with Alaska and JI1205 than in root nodules of Feltham First. On the basis of H_2-uptake strain 128C53 was Hup^+ in first two pea lines and Hup^- in the last. Another strain 3960 was also Hup^+ in Alaska and JI1205 but Hup^- in Feltham First.

Shoot/root grafting studies by Bedmar and Phillips (1984) showed organ and host cultivar effects on net H_2 evolution from *Pisum sativum* L. root nodules. The results indicated the presence of an transmissible shoot factor(s) which can increase uptake hydrogenase activity in a *Rhizobium* symbiont and showed that root genotype also can influence that parameter.

Differential expression of uptake hydrogenase activity because of host influence has also been demonstrated by van Berkum (1990). His experiments revealed the existence of an additional uptake hydrogenase phenotype besides Hup^+ and Hup^- among the soybean bradyrhizobia. The strains belonging to this phenotype showed H_2 oxidation capability only in symbiosis with specific host plants and were not capable of having H_2 oxidation induced as free-living bacteria by using standard methods. Since these characteristics were distinctly different from those used to describe the Hup^+ phenotype, the

term H_2-uptake host-regulated (Hup-hr) phenotype has been suggested to describe strains belonging to this third class of H_2-oxidizing bradyrhizobia.

Strains belonging to this Hup-hr phenotype contained DNA-sequences hybridizing with pHU 1, but the hybridisation pattern was found to be different from that of Hup^+ strains (Salzwedel and van Berkum, 1992). Nucleotide sequence analysis revealed the presence of an extra 1 kbp between the open reading frames encoding the small and large subunits of the hydrogenase enzyme in Hup-hr strains, compared with a region of 32 bp in Hup^+ strains.

Hup-hr strains were found to be highly competitive for nodulation which inhibits the efficiency of using Hup^+ inocula to increase H_2 oxidation in soybean (van Berkum and Sloger, 1991). Therefore, the identification of exotic soybean germplasm which allows hydrogenase expression by Hup-hr strains could enhance BNF in soybean production.

2:7 Coordinate regulation of H_2-uptake and CO_2 fixation

Studies on the regulation of H_2-uptake in free-living *R.japonicum* indicate that CO_2 fixation activity is correlated with H_2 oxidation activity. Simpson *et al.* (1979) found that CO_2 fixation occurs in H_2 derepressed cells as indicated by RuBP carboxylase activity in these cells. Furthermore, both H_2-uptake and CO_2 fixation activities were found to be repressed by added carbon and both activities were induced when cells were incubated in carbon-poor medium in an atmosphere containing H_2 plus CO_2. Moreover, all Hup^- mutants had very low RuBP carboxylase activity (Lepo *et al.*, 1981; Maier, 1981). These results suggest a coordinate regulatory relationship between H_2-uptake activity and RuBP carboxylase expression. Autotrophic growth of *R. japonicum* has been observed using H_2 and CO_2 as the sole energy and carbon sources (Hanus *et al.*, 1979) and RuBP carboxylase has been purified from these autotrophically grown cells (Purohit *et al.*, 1982). However, Friedrich (1982) provided evidence that RuBP carboxylase and hydrogenase activities were not correlated in *Alcaligenes eutrophus*. Manian *et al.* (1984) described another Hup^- *R. japonicum* mutant having poor RuBP carboxylase activity. This strain exhibited pleiotrophic effects on C-1 metabolism since phosphoribulokinase and formate dehydrogenase activities were also impaired. These results suggested coordinate regulation of hydrogenase and some

Calvin cycle enzymes. Tilak *et al.* (1984) demonstrated lithoautotrophic growth of four strains of *Rhizobium (R. triofolii* RCL10, *R. japonicum* S19 and SB16, and *Rhizobium* sp. NEA4) with molecular hydrogen as sole electron donor and with ammonium or N_2 as N source. All these strains showed the presence of hydrogenase activity as well as RuBP carboxylase activity. Also in *Azospirillum* spp., out of 15 strains isolated from the roots of different plants, 4 were able to grow autotrophically with H_2 and CO_2. All of them showed H_2-uptake and RuBP carboxylase activity (Tilak *et al.*, 1986).

Merberg and Maier (1984) reported that Hup^c mutants expressed both H_2 oxidation activity and RuBP carboxylase activity when cultured heterotrophically, whereas under the same conditions, the wild type had neither activity. This indicated that the mutation in the constitutive strain affected RuBP carboxylase expression in addition to H_2-uptake. Since these were spontaneous mutants, it was unlikely that these were two separate lesions -one affecting hydrogenase and another affecting RuBP carboxylase. These results also suggested that the Hup system and RuBP carboxylase could be under coordinated regulatory control in *R. japonicum*.

Unlike high levels of H_2-uptake activity, RuBP carboxylase activity could not be detected in wild type bacteroids (Simpson *et al.*, 1979). Like the wild type, bacteroids of the Hup^c mutants also express hydrogenase activity but not RuBP carboxylase activity. Therefore, the alteration in these Hup^c mutants still did not allow expression of RuBP carboxylase in the root nodules (Merberg and Maier, 1983).

Maier (1981) isolated *R. japonicum* mutants that were unable to grow chemoautotrophically and all the Hup^- mutants had no ability to fix CO_2 (Cfx^-). On the other hand, there were mutants that are Cfx^-, but Hup^+. No Hup^- Cfx^+ could be isolated. This suggest that RuBP carboxylase-mediated CO_2 fixation ability depends on the expression of hydrogenase, but not vice versa.

2:8 Transfer of plasmid-encoded Hup determinants into Hup^- rhizobia

Some of the genes needed for H_2-uptake capability are carried on plasmids in *Alcaligenes* spp. (Friedrich *et al.*, 1981) and *R. leguminosarum* (Brewin *et al.*, 1980a). Studies done by Friedrich *et al.* (1984) on H_2 oxidation

and large plasmids in *Alcaligenes eutrophus* and *A. hydrogenophilus* indicated that both structural and regulatory H_2 oxidation genes reside on large plasmids in *Alcaligenes* spp.

Transfer of H_2-uptake ability in *R. japonicum* would be facilitated if H_2-uptake determinants are located on plasmids. Although large plasmids can be detected in *R. japonicum*, correlations of Hup with plasmid content have not been possible.

Brewin *et al.* (1980b) reported that determinants for hydrogenase activity (*hup*) in *R. leguminosarum* strain 128C53 are genetically linked to determinants for nodulation ability (*nod*) and are carried on a plasmid pRL6JI of molecular weight 19×10^7. Although pRL6JI was not self-transmissible, the *nod* and *hup* determinants could be cotransferred to other strains of *R. leguminosarum* after recombination with a derivative of a transmissible *R. leguminosarum* plasmid.

DeJong *et al.* (1982) reported the effect of transfer of a recombinant plasmid pIJ1008 into several Hup^- *R. leguminosarum* strains. As plasmid pIJ1008 carries genetic determinants for Hup^+ as well as Nod^+ and other determinants for symbiotic nitrogen fixation (Fix^+), its transfer produced strains with significantly better symbiotic properties. Ruiz-Argüeso *et al.* (1979) reported that no strain of *R. meliloti* has been identified that possessed significant Hup activity. Thus, plasmid pIJ1008 was transferred from *R. leguminosarum* to *R. meliloti* without impairing the capacity of the latter species to form root nodules on alfalfa. Though the plasmid was still present in rhizobia reisolated from the root nodules of 12 different alfalfa cultivars, only low levels of Hup activity were detected in alfalfa (Bedmar *et al.*, 1984).

Plasmid pIJ1008 was also transferable to three of seven natural isolates of *R. meliloti* tested (Bekhi *et al.*, 1985). In these three strains, pIJ1008 was maintained stably with the respective *sym* megaplasmid indigenous to each *R. meliloti* strain. By reisolation and examination of the strains from alfalfa nodule tissue, it was shown that pIJ1008 continued to be maintained but the pea-nodulation ability was suppressed. In three separate plant growth experiments carried out by Bekhi *et al.* (1985), alfalfa nodules induced by each of the *R. meliloti* strains carrying both *sym* plasmids were assayed for H_2-uptake activity. The average activity was 4.0-, 3.5- and 2-fold higher than

with the respective plJ1008-free strains. This higher activity, however, was not accompanied by an increase in plant biomass or nitrogen content of shoots.

The plasmid from Hup$^+$ Nod$^-$ *R. leguminosarum* strain Vm2 was transferred to Hup$^-$ Nod$^+$ strain Vm1 by Garg *et al.* (1990b). It resulted into a Hup$^+$ Nod$^+$ transconjugant strain Vc4. The strain Vc4 showed H_2-uptake activity both *ex planta* and in nodules. Symbiotic performance of Vc4 was better than the Hup$^-$ Vm1 and comparable to the parent Hup$^+$ field isolate Vpl. The results showed nearly 26% and 51% higher accumulation of dry matter and nitrogen respectively in plants inoculated with the Hup$^+$ transconjugant Vc4 as compared to its parent Hup$^-$ Vm1. In Vp1 and Vc4, the Hup character was present on a plasmid which did not appear to carry any other gene involved in N_2 fixation. Therefore, besides conclusively proving the beneficial effect of Hup$^+$ character in legume-rhizobia symbiosis, such Hup locations are more useful, as they do not affect any other aspect of symbiosis.

Uptake hydrogenase genes of *B. japonicum* 122 DES cloned in cosmid pLAFR1 (pHU52) complemented all but one of the Hup$^-$ mutants and is therefore, a promising cosmid for intraspecies transfer of hydrogenase activity (Lambert *et al.*, 1985b). Vasudev *et al.* (1991) transferred *hup* genes cloned in pHU52 to two Hup$^-$ strains, G36-84 and BG4 of *Cicer*-rhizobia. Transconjugants thus obtained showed *ex planta* Hup activity, H_2-dependent autotrophic growth and increased relative efficiency of N_2 fixation under symbiotic conditions. Cosmid profile analysis and DNA hybridization studies indicated maintenance of introduced cosmid in the extrachromosomal state. Thus, demonstration of Hup activity in genetically manipulated strains suggested that *hup* gene expression is not repressed in natural isolates of *Cicer-Rhizobium*.

In order to produce genetically engineered Hup$^+$ strains capable of recycling all nitrogenase mediated H_2, the cosmid-borne *hup* genes must be stabilized in their new environment. This could be accomplished using new generations of more stable plasmid vectors (Hom *et al.*, 1985), or by using an integration plasmid to deliver these *hup* genes into the chromosome. The *hup* gene cosmid pHU52 was integrated into the chromosome of a Hup$^-$ *Cicer-Rhizobium* strain G36-84 by using transposon Tn5 as homologous sequence between them (Kunnimalaiyaan *et al.*, 1992). Functional

characterization of the final recombinants has demonstrated uptake hydrogenase activity in free-living cells and *hup* stability in nodules (Kunnimalaiyaan and Lodha, 1992). To reduce the genetic load, *hup* gene fragment (30.2 kb) of cosmid pHU52 has been integrated into the genome of chickpea-*Rhizobium* Rcd301 (Vijayabhanu *et al.*, 1994). The integrated *hup* genes thus have conferred an increase in specific Hup activity. In addition, the Hup^+ transconjugants have two notable features: One is the proven stability of integrated *hup* DNA, and the other is that no permanent selective pressure for vector marker is necessary to maintain the stable integration.

Chapter 3

MATERIALS AND METHODS

3:1 Materials

3:1:1 Bacterial strains

An *Azorhizobium* isolate of *Sesbania rostrata*, IRBG 46 was obtained from Dr. J.K. Ladha, International Rice Research Institute, Manila, Philippines. Pure cultures of *Escherichia coli* strains DH5α, HB101 (pRK2073) and *Cicer-Rhizobium* strain Rcd 301 (a Sm^R derivative of F75) were obtained from the National Reseach Centre for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi. *E. coli* containing the cosmid pHU52 was kindly provided by Prof. H.J. Evans, Oregon State University, USA. Relevant characteristics of these strains/plasmids are given in Table 1.

3:1:2 Plant seed material

The seeds of *Sesbania rostrata* were obtained from the International Rice Research Institute, Manila, Philippines.

3:1:3 Chemicals and Reagents

Lysozyme, ethidium bromide, cesium chloride, sodium lauryl sulfate, RNase A, Ficoll 400, proteinase K, agarose, tetracycline, nalidixic acid and ampicillin were obtained from Sigma Chemical Co., USA. Restriction enzymes *Eco* RI and *Hin*d III, Lambda DNA marker, distilled phenol were from Bangalore Genei Pvt. Ltd., Bangalore. The nick-translation kit was obtained from Promega, USA. Ethylene and Hydrogen standards were obtained from EDT Research, London. Sucrose, L-arabinose, L-glutamic acid, inositol, pyridoxine-HCl, thiamine-HCl, Tris and EDTA were from Sisco Research Laboratories Pvt. Ltd., Bombay. Bacto-agar, yeast extract and Bacto-tryptone used were from Difco Laboratories, USA. Luria broth, Luria agar and sodium chloride were from Hi-media Laboratories Pvt. Ltd., Bombay. Absolute ethanol was obtained from Bengal Chemicals Ltd. and (α-^{32}P) dCTP was purchased from BARC, Bombay.

All other chemicals used were of analytical grade obtained from SRL, Qualigens, BDH and Merck. Double glass distilled water or Milli-Q water was used throughout the studies.

Table 1. Bacterial strains/plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/Reference
E. coli		
HB101 (pRK2073)	Mob$^+$, Tra$^+$, Sm^R, Sp^R	Figurski and Helinski (1979)
DH5α (pHU52)	pLAFR1:: *hup*, Tc^R	Lambert *et al.* (1985a)
Az. caulinodans		
IRBG 46	Hup$^+$, Nd^R, Ap^R	Dr. J.K. Ladha IRRI, Manila, Philippines
Cicer-Rhizobium		
Rcd 301	Spontaneous Sm^R derivative of F 75	Khanuja (1991)
R. leguminosarum		
B164 (pIJ1008)	Tra$^+$, Hup$^+$, Nod$^+$, Fix$^+$ Km^R	Brewin *et al.* (1982)

3:2 Methods

3:2:1 Maintenance of the cultures

The medium used for the growth of *E. coli* strains was Luria Bertani (LB) medium (Kahn *et al.*, 1979) containing (gl^{-1}) : tryptone, 10.0; yeast extract, 5.0 and NaCl, 5.0, adjusted to pH 7.5. Rhizobial strains were maintained on Tryptone Yeast Extract Mannitol (TYM) medium (Khanuja, 1991) containing (gl^{-1}) : tryptone, 5g; yeast extract, 2g; mannitol, 4g and $CaCl_2.2H_2O$, $1mMl^{-1}$. Conjugal transfer experiments involved the use of Mannitol-Salts-Yeast Extract (MSY) medium (Khanuja and Kumar, 1989) and Rhizobial Minimal medium (RMM) (Sikka and Kumar, 1984).

Unless otherwise indicated, the concentrations of antibiotics (μg/ml) used in solid media were as follows: tetracycline (Tc), 10; ampicillin (Ap), 50; spectinomycin (Sp), 50; nalidixic acid (Nd), 10 and trimethoprim (Trp), 25.

3:2:2 Sterilization

All media and solutions were sterilized at 15 psi. Heat labile chemicals like vitamins, antibiotics and glucose were sterilized by passing through sterile Sartorius membrane filters (pore size 0.45 μm).

3:2:3 Incubation

For growth, cultures of *Azorhizobium* were incubated at 28±1°C and *E. coli* cultures at 37±1°C The broth cultures were grown in New Brunswick G-25 Refrigerated Incubator shaker and G-24 Environmental Incubator shaker, respectively.

3:3 Antibiotic / antibacterial agents resistance profile

To determine the antibiotic/antibacterial agents resistance pattern of strain IRBG 46, the culture was grown on solid TYM medium supplemented with the antibiotics mentioned in Table 4. The stock solutions of tetracycline and rifampicin were prepared in 50% ethanol and methanol, respectively. Stocks of ampicillin, spectinomycin , kanamycin, streptomycin and azide were prepared in sterile water. Stock of trimethoprim was prepared in 0.05 N HCl. Stock of nalidixic acid was prepared in 0.01 N NaOH.

3:4 Isolation of Hup⁻ mutant of *Azorhizobium caulinodans* strain IRBG 46

3:4:1 Nitrosoguanidine mutagenesis of IRBG 46

The strain IRBG 46 was grown for 24 h in 10 ml of TYM broth. To this culture, 10 ml of fresh TYM broth was added. An aliquot was kept for titre estimation. This 20 ml of culture was divided equally in 2 vials to which nitrosoguanidine (NTG) was added @ 100 μg/ml. The culture was shaken for another 3 h at 28°C at a speed not exceeding 150 rpm. The culture was then centrifuged for 15 min at 3,800 xg in a SS-34 rotor using a RC5C Sorvall refrigerated centrifuge. The supernatant was discarded into 0.2 N HCl to destroy NTG. The pellet was washed twice in sterile water and suspended in 10 ml TYM broth. Aliquot was saved for titre determination. The mutagenized cells were incubated overnight at 28°C with gentle shaking. Titre was again estimated after fixation process by dilution plating the cells on selective TYM plates containing trimethoprim and nalidixic acid.

3:4:2 Screening of the NTG mutants for Hup⁻ mutant

Screening of *Azorhizobium* IRBG 46 mutants was done by the method of Haugland *et al.* (1983) with slight modification. Large scale plating was done so as to get about 150 colonies per plate. From these plates, single colonies were picked up at random and spotted on oriented HUM, YEMA and TYM plates with the help of sterile tooth picks. In each plate 50 colonies were spotted. TYM plates were incubated at 28°C for 3 days and kept in refrigerator for future use as master plates. The HUM and YEMA plates were incubated in sealed glass jars (equipped with septa for gas injection and withdrawal) for 5 days at 28°C in an atmosphere containing 10% H_2, 5% CO_2 and 1% O_2 and the balance N_2.

Screening tray : A plexiglass rectangular tray (29x39x3 cm) was constructed with a 2 cm outer trough on all sides, to which was added water to a depth of about 1 cm. A plexiglass cover with side walls 3 cm in depth was constructed to fit inside the trough making a water seal against entry of air or loss of added gases. The cover was also equipped with two gas ports (5 mm inner diameter and located near each end of the cover) which permitted the entry and exit of gases, namely N_2 and H_2.

Procedure for screening : Derepressed colonies of *Azorhizobium* were replicated onto sterile 7 cm filter paper disks (Whatman No.541). These filter disks containing derepressed colonies were transferred to clean petriplates containing 0.8 ml of dye solution [200 mM iodoacetic acid, 200 mM malonic acid, 10 mM methylene blue, 50 mM KH_2PO_4 and 2.5 mM $MgCl_2$, (pH adjusted to 5.6 with KOH)]. After 15 min, the plates were tilted slightly and the excess solution was removed by means of a Pasteur pipette. An additional 45 min of incubation in air allowed the solution of dye and inhibitors to equilibrate with the colonies and then the dishes with filters were transferred to the screening tray described above. The sealed screening tray was flushed with N_2 for 15 min to remove most of the air and then H_2 was allowed to pass through for about 5 h.

With the screening assay, colonies of the Hup^+ *Azorhizobium* strain IRBG 46 showed visible reduction of methylene blue within 30 min to 1 h after being placed in an H_2 atmosphere, whereas colonies which exhibited no dye-reducing activity even after 5 h of incubation in H_2 were taken as Hup^- mutants.

The mutant was further verified by comparing the H_2-uptake activity and chemoautotrophic growth with that of parent strain. Only one Hup^- mutant was obtained which was named as B11.

3:5 Growth characteristics of wild type IRBG 46 and its Hup^- mutant B11

Single colonies of IRBG 46 and Hup^- mutant B11 were inoculated to 10 ml TYM broth and incubated for 24 h. The cultures were diluted so as to have an O.D. of 0.01 at 600 nm. Tubes containing 10 ml of TYM broth were inoculated with 100 μl of diluted cell suspension. Soon after inoculation, the absorbance was taken at 600 nm to get the zero hour reading. The tubes, in duplicate, were incubated at 28°C with constant shaking at 250 rpm. Absorbance was measured at regular intervals upto 30h and then growth curves were drawn for both wild type strain and its mutant.

3:6 Test for chemoautotrophic growth

In order to test the chemoautotrophic growth, both the Hup^+ IRBG 46 and its Hup^- mutant were cultured on hydrogen uptake medium (Maier *et al.*, 1978), for derepression of hydrogenase. For autotrophic growth, both the

parent strain and its mutant were streaked onto plates of Repaske medium (Repaske and Mayer, 1976) with 1.5% agar. *Cicer-Rhizobium* strain Rcd 301 (Hup^- wild type strain) was used as control.

Composition of Repaske medium

KH_2PO_4 buffer (pH 6.8)	-	0.03 M
NH_4Cl	-	0.018 M
$CaCl_2$	-	$6x10^{-5}$ M
$NaHCO_3$	-	0.012 M
K_2SO_4	-	0.001 M
Fe $(NH_4)_2$ $(SO_4)_2$	-	$2x10^{-5}$ M
$NiCl_2$	-	$5x10^{-7}$ M
$CuCl_2$	-	$4x10^{-7}$ M
$CrCl_2$	-	$2x10^{-7}$ M
$CoCl_2$	-	$1x10^{-6}$ M
$MnCl_2$	-	$2x10^{-6}$ M
Na_2MoO_4	-	$2x10^{-7}$ M
Thiamine-HCl	-	1 µg/l
Nicotinic acid	-	1 µg/l
Biotin	-	1.5 µg/l

These plates were then incubated in sealed glass jars (equipped with septa for gas injection and withdrawal) for 7 days at 28°C in an atmosphere that initially contained approximately 10% H_2, 5% CO_2, 1% O_2 and the balance N_2. Autotrophic growth was observed after 7 days.

3:7 Transfer of *hup* cosmid pHU52 into Hup^- mutant B11 of *Az. caulinodans* strain IRBG 46 and *Cicer-Rhizobium* strain Rcd 301

The triparental mating system was used for transfer of cosmid pHU52 to Hup^- mutant of *Azorhizobium* strain IRBG 46 (B11) and *Cicer-Rhizobium* strain Rcd 301. B11 and Rcd 301 were grown on TYM plates for 2 and 3 days respectively to get a log phase culture. Two to three loopfuls of this

culture were mixed with one loopful of an overnight grown culture of *E.coli* DH5α containing pHU52 on a fresh TYM plate. Overnight grown culture of *E.coli* HB101 containing pRK 2073 (5 ml) was centrifuged at 3,000 Xg and the cells were resuspended in 5 ml of mannitol salts solution (MSS). Then 0.1 ml of this suspension was added as a helper plasmid and spotted in the minimum possible space on the plate. This was subsequently dried at room temperature and incubated at 28°C for 24 h. Later a loopful of cells from the grown spot were used for dilution plating on selective RMM-Tc plates. Since *E.coli* can not grow on RMM and *Azorhizobium* mutant B11 is sensitive to tetracycline, only transconjugants carrying the cosmid pHU52 grew on RMM-Tc plates. These transconjugants were picked up for further studies of assaying the Hup activity under free-living conditions. The transconjugants obtained have been designated as B11 (pHU52) and Rcd 301 (pHU52).

3:8 Studies on expression of *hup* genes under free-living conditions

In order to study the *ex planta* expression of *hup* genes in *Azorhizobium* strain IRBG 46 and its Hup$^-$ mutant B11, the following experiment was performed.

Azorhizobium cells were cultured in 16x150 mm tubes, each containing 7 ml of the H_2-uptake medium (HUM) (Table 2) or yeast-extract mannitol (YEM) medium as 1.5% agar slants. A 100 µl suspension of log phase cells was spread on the surface of the slants. The method of derepression and assay of hydrogenase as described by Maier *et al.* (1978) was used with slight modifications. The cotton plugs in the slants were replaced by sterile serum stoppers and H_2 gas was injected aseptically into the slants after removal of an equal volume of air to give an atmosphere of 10% H_2 in air. The inoculated slants were incubated at 28°C for growth and hydrogenase induction.

After 5 days of incubation, the serum stoppers were removed and the slants left open in air for a minute and again closed with the stoppers. This whole operation was carried out under sterile conditions in a laminar flow cabinet. The slants were then provided with a new atmosphere of 5% H_2 in air after removal of an equal volume of air with a syringe. The slants were incubated at 28°C and gas samples removed after 3 h and 5 h of incubation.

Table 2. Composition of hydrogen uptake medium (Maier *et al.*, 1978)

Medium constituent	Weight/l of medium	Stock solution		Quantity of stock/l
Part A				
Sodium gluconate	0.5 g			
Sodium glutamate	0.5 g			
Yeast extract	0.1 g			
Distilled water	1.0 L			
Agar	14.0 g			
Part B				
Iron EDTA	28.0 mg	14.000 g		
Boric acid	3.0 mg	1.500 g		
$ZnSO_4.7H_2O$	2.0 mg	1.000 g		
$MnSO_4.7H_2O$	10.0 mg	5.000 g	In 250 ml H_2O	0.5 ml
$CuSO_4.5H_2O$	0.04 mg	0.020 g		
$CoCl_2.6H_2O$	0.025 mg	0.013 g		
KI	0.780 mg	0.390 g		
$Na_2MoO_4.2H_2O$	0.250 mg	1.125 g		
Part C				
$NaH_2PO_4.2H_2O$	150.0 mg	2.500 g	In 25 ml H_2O	1.5 ml
Part D				
$CaCl_2.2H_2O$	150.0 mg	0.375 g	In 25 ml H_2O	10.0 ml
$MgSO_4.7H_2O$	250.0 mg	0.625 g		
Part E				
Sucrose	0.5 g	5.000 g	In 25 ml H_2O	2.5 ml
L-arabinose	1.0 g	3.571 g		7.0 ml
Inostiol	100.0 mg	0.500 g		5.0 ml
Thiamine. HCl	10.0 mg	0.500 g		1.0 ml
Part F				
Nicotinic acid	1.0 mg	0.010 g	In 25 ml H_2O	2.5 ml
Pyridoxine. HCl	1.0 mg	0.010 g		

Note : Part B was added to Part A, mixed thoroughly and the pH was adjusted to 6.8 using 0.1 N NaOH before adding agar. It was then autoclaved at 15 psi for 15 min. Parts C and D were autoclaved separately and added at the time of slant preparation. The stock solutions in Part E were prepared separately in 25 ml water, mixed with Part F proportionately by volume and filter sterilized using a bacteriological filter (pore size 0.45 μm) and added at the time of slant preparation. Seven ml of induction medium was added to each culture tube (16 x 150 mm).

These were analyzed for their H_2 content using an AIMIL-NUCON gas chromatograph equipped with a TCD and molecular sieve 5A^0 (80-100 mesh) dual columns (200x0.32 cm). The column temperature was maintained at 60°C. Both injector and detector were maintained at 80°C. Argon was used as a carrier gas at a flow rate of 30 ml/min. The standard containing 1.03% H_2 in N_2 was used for caliberation.

The amount of H_2 consumed per slant was calculated by subtracting the amount of H_2 left at 5 h after incubation from the amount of H_2 present at 3 h after incubation. In order to account for the H_2 leakage from the tubes, uninoculated control slants were included in the experiments. Six replicates were used for each strain and the hydrogenase activity was expressed as nmol H_2 consumed per h per mg protein.

3:8:1 Estimation of soluble protein

After the H_2-uptake assay, the cells from each slant were gently scraped off and removed by repeated washing with sterile distilled water. The cell suspension was centrifuged at 12,000 Xg at 4°C for 10 min using a RC5C Sorvall refrigerated centrifuge. The cell pellet obtained was digested according to the method of Stickland (1951). The pellet was suspended in 3 ml of 0.75 M NaOH and the protein in the sample was precipitated by adding 0.6 ml of 25% TCA and 1.8 ml ethanol. After thorough mixing, the sample was centrifuged at 25,000 Xg for 30 min. The pellet obtained was again dissolved in water with the help of a minimal amount of NaOH solution and heated at 70°C for 5 min. The protein in the supernatant was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

3:9 Studies on the expression of *hup* genes in free-living transconjugants

In order to study the expression of *hup* genes of cosmid pHU52 in the transconjugants B11 (pHU52) and Rcd 301 (pHU52), the activity of uptake hydrogenase was induced in them under free-living conditions and then uptake of H_2 was measured gas chromatographically as described earlier in Section 3:8. The only difference in the conditions maintained was that the time of induction was extended to six days in an atmosphere containing 5% H_2. Subsequently, the assay of uptake hydrogenase was carried out in

an atmosphere of 1% H_2 after 3 h and 24 h of incubation. The activity of uptake hydrogenase has been expressed as nmol H_2 consumed per h per mg protein.

3:10 Studies of the factors regulating the expression of uptake hydrogenase in *Az. caulinodans* under free-living conditions

In order to check the effect of H_2 added for induction of uptake hydrogenase, two sets of HUM slants were inoculated with IRBG 46. In one set 10% H_2 in air was injected whereas in the other no H_2 was added. Strain Rcd 301 (pHU52) was used as a control. The slants were incubated at 28°C for 5 days and then assayed for uptake hydrogenase activity as described earlier in Section 3:8.

Standard HUM medium was used under various pH conditions to find out the effect of pH on growth/hydrogenase activity and to find out the optimum pH for use in further experiments.

To investigate the effect of different carbon sources on the expression of uptake hydrogenase, sodium gluconate (2.29 mM) which is required as a carbon source for the expression of uptake hydrogenase in the standard induction medium was replaced by different carbon sources (15 mM) while keeping other factors constant, as detailed in the Results.

To check the effect of combined nitrogen sources on the expression of uptake hydrogenase, sodium glutamate (2.67 mM) as sole nitrogen source in the standard medium was replaced by other nitrogen sources (10 mM) as given in the Results.

An experiment was conducted to see whether there is any reversal of repression effect by cAMP brought about by malate. Various concentrations of cAMP were used with malate as detailed in the Results.

In order to study the effect of O_2 concentration on the expression of uptake hydrogenase, the oxygen concentration was varied from 1% to 20% in N_2 atmosphere while keeping other conditions constant.

3:11 *Ex planta* nitrogenase activity in IRBG 46 and its Hup$^-$ mutant B11

To check the *ex planta* nitrogenase activity, the conditions for inoculation and incubation were kept same as described earlier for free-living uptake hydrogenase assay (Section 3:8) except that no H_2 was injected during the incubation period. The HUM slants were inoculated with 100 μl of inoculum. Tubes were fitted with sterile serum stoppers. After 3 days of growth, the serum stoppers were removed and slants were left open in air for a minute and then again stoppers were fitted. This whole operation was carried out under sterile conditions in a laminar flow chamber. One ml of air was replaced with 1 ml of acetylene with the help of a syringe. Tubes were incubated at 28°C for 10 h. After 10 h, 1 ml gas sample was removed and analyzed for their ethylene content using an AIMIL-NUCON gas chromatograph equipped with a FID and porapakT (60-80 mesh) dual columns (183 x 0.32 cm) maintained at 90°C with N_2 as carrier gas at a flow rate of 30 ml/min. Both injector and detector were maintained at 110°C. Six replicates were used for each strain and the nitrogenase activity was expressed as nmol C_2H_4 per h per mg protein.

3:11:1 Evolution of H_2 by IRBG 46 and its Hup$^-$ mutant under free-living conditions

As described above, the HUM slants which were inoculated with IRBG 46 and B11, were also used to estimate the rate of H_2 evolution under free-living conditions. After 72 h of growth, 1 ml of gas was removed from each of the slants and analyzed by gas chromatography. Another sample (1 ml) was removed after 82 h and analyzed again for the presence of H_2 using an AIMIL-NUCON gas chromatograph as described earlier.

3:12 Studies on nodulation and expression of *hup* genes of *Az. caulinodans* strain IRBG 46 under symbiotic conditions

Undamaged clean seeds of *Sesbania rostrata* selected to a reasonably uniform size were sterilized for use in nodulation experiments as described by Adebayo *et al.* (1980). They were initially treated with concentrated sulfuric

acid for 30 min. This was followed by treating them with 70% ethanol (v/v) for 5 min. and then with 0.1% acidic $HgCl_2$ for 3 min. Seeds were then rinsed several times with sterile distilled water and soaked in it for 48 h for germination. Germinated seeds were sown in plastic pots (6 inch diam.) containing acid washed sterilized sand. Plants were grown during July-September in natural conditions. Hoagland solution (Hoagland and Arnon, 1950) (see Table 3) was supplied to the pots as and when required for the growth of the plants.

Preparation of IRBG 46 inoculum : The inoculum was prepared by growing the bacterium in TGYE broth (Ladha *et al.*, 1989) containing (gl^{-1}) : tryptone, 10.0; yeast extract, 5.0 ; glucose, 5.0 and $CaCl_2.2H_2$, 0.9; at 28°C for 2 days in New Brunswick G-25 refrigerated incubator shaker. The cells were harvested by centrifugation at 8,000 Xg for 10 min at 4°C, washed twice with sterile 100 mM phosphate buffered saline (0.85% NaCl, pH 7.2) and suspended in sterile water to a cell density of 10^8 ml^{-1} and used as inoculum.

Inoculation : Roots were inoculated with the inoculum of IRBG 46 and B11 after 8 days of sowing by applying 2 ml of cell suspension per seedling. Stems were inoculated by painting cell suspension mixed with 10% gum arabic on the above ground parts of each plant after 21 days of sowing.

The plants were harvested 53 and 60 days after sowing. The roots were separated and washed free of sand. Intact nodules from roots and stems were collected separately and were used for determination of nitrogenase activity and H_2 evolution rates. The plants (leaf + stem + roots) were dried to a constant weight at 60°C. Uninoculated plants were used as control. Six replicates were used for each strain/treatment.

3:12:1 Assay of nitrogenase activity

Intact nodules were used for determining the nitrogenase (C_2H_2 reduction) activity. Nodules from roots and stem were transferred to 25.5 ml vials provided with serum stoppers. In each vial 3.0 ml of air was replaced by 3.0 ml of C_2H_2 and incubated at 28°C for 30 min. After incubation, 1 ml gas sample was removed from each vial and analyzed for C_2H_4 content as described in section 3:11. Nitrogenase activity has been expressed as nmol C_2H_4 formed per h per g nodule dry wt. The standard containing 1094 vpm

Table 3. Composition of Hoagland solution (Hoagland and Arnon, 1950)

Salt	Concentration	Qty. (ml/l)
	Solution A (macronutrient)	
K_2SO_4	0.500 M	5
$MgSO_4.7H_2O$	1.000 M	2
$Ca(H_2PO_4)_2$	0.050 M	10
$CaSO_4$	0.010 M	200
	Solution B (micronutrient), g / 500 ml	
$H_3BO_3.H_2O$	1.430	
$MnCl_2.4H_2O$	0.905	1
$ZnSO_4.7H_2O$	0.110	
$CuSO_4.5H_2O$	0.040	
$Na_2MoO_4.H_2O$	0.010	
	Solution C	
Iron citrate	0.5%	1

The pH was adjusted to 6.5 using 0.1 N NaOH. The medium was then autoclaved at 15 psi for 15 min. The sterilized medium was stored, sometimes upto 3 to 4 days. Once the container was opened, the left out medium was discarded.

C_2H_4 in a gas mixture of acetylene, ethylene, propylene, methane and hydrogen was used.

3:12:2 Determination of H_2 evolution rate of nodules

The same nodules which were earlier used for determining nitrogenase activity were also used for estimation of H_2 evolved by nodule. The serum stoppers were removed from the vials containing nodules and flushed with N_2 gas to remove C_2H_2 and C_2H_4. The vials were left open for another 5 min and then serum stoppers were replaced back. The vials were incubated at 28°C for 1 h. One ml gas sample was removed from each vial and H_2 evolved was estimated as described earlier. The standard contained 1006 vpm H_2 in a gas mixture containing acetylene, ethylene, propylene, methane and hydrogen. H_2 evolution rate has been expressed as nmol H_2 evolved in air per h per g nodule dry weight.

3:12:3 Determination of relative efficiency (RE)

The expression of *hup* genes in nodules was studied by measuring the relative efficiency (RE) of electron transfer to N_2 via nitrogenase as described by Schubert and Evans (1976), at 58 days of plant growth.

$$\text{Relative efficiency (RE)} = 1 - \frac{\text{Rate of } H_2 \text{ evolution in air}}{\text{Rate of } C_2H_2 \text{ reduction in air}}$$

If RE is greater than 0.8, then the rhizobial strains are considered to have uptake hydrogenase activity.

3:13 Estimation of Ribulose 1,5-bisphosphate carboxylase under free-living conditions in IRBG 46 and B11

The procedure used for the facile measurement in the intact cells of RuBP carboxylase was as described by Tabita *et al.* (1978). This procedure involves a simple toluene treatment to render cells permeable to the necessary substrates, effectors and co-factors.

3:13:1 Cell-preparation

Overnight grown cultures of IRBG 46 and B11 were spread plated on petri plates containing HUM medium (Maier *et al.*, 1978). These plates were

incubated at 28°C for 5 days in a jar containing 10% H_2, 5% CO_2, 5% O_2 and 80% N_2. The cells were then removed from the plates by washing with sterile water. These cells were washed twice in 0.02 M Tris-Cl buffer (pH 7.8), containing 50 mM $NaHCO_3$, 10mM $MgCl_2$, 1mM EDTA and 1mM DTT at 4°C. After washing, the cells were resuspended in the same buffer at $O.D_{660}$ ranging from 1.0 to 2.0. Over each volume of completely suspended cells, one-half volume of toluene was layered. The cell suspension-toluene mixture was gently mixed for about 3 min and then allowed to stand in an ice-bucket for 10 min. The toluene layer was then carefully removed with a Pasteur pipette, taking care that any bubbles of toluene clinging to the sides of the tube were also removed. Aliquots of the toluene treated cell suspension was then added to RuBP carboxylase reaction mixture.

3:13:2 Assay of RuBP carboxylase

Assay for RuBP carboxylase was done as described by Mayer *et al.* (1976) with some modification. RuBP-dependent incorporation of $^{14}CO_2$ into acid stable product was measured at 30°C. The reaction mixture in a final volume of 0.25 ml contained : 150 mM Tris-Cl buffer (pH 7.8), 10 mM $MgCl_2$, 50 mM $NaH^{14}CO_3$ (specific radioactivity 0.05 μCi/mole), 50 μl toluene treated cell suspension and 1.5 mM RuBP. Before adding RuBP, reaction mixture was preincubated in a water bath at 30°C for 10 min. RuBP was then added to start the reaction, which was allowed to proceed for 30 min at 30°C. The reaction was terminated by the addition of 0.1 ml of 6N HCl and the mixture shaken for 60 min to liberate unused $^{14}CO_2$. A 0.1 ml sample was placed in a scintillation vial containing 10 ml of liquid scintillator solution in dioxane and its radioactivity determined in a liquid scintillation counter. With each experiment, substrate (RuBP) and enzyme blanks were also used. The sample values were corrected for blank values. The results were expressed as dpm per min per mg protein.

3:14 Localization of *hup* genes in *Az. caulinodans* strain IRBG 46

In order to determine whether the *hup* genes are present on the megaplasmid or on the chromosome of *Az. caulinodans*, megaplasmid and total genomic DNA was hybridised with labelled 12.9 kb *hup* fragment of cosmid pHU52.

3:14:1 Isolation of genomic DNA

Az. caulinodans strain IRBG 46 and its Hup^- mutant B11 were grown for 24 h in 100 ml of TYM broth. The cultures were centrifuged at 3,800 Xg in a GS-3 rotor using a Sorvall RC5C refrigerated centrifuge. The pellet was resuspended gently in 9.5 ml TE ($T_{10}E_1$) buffer, 0.5 ml of 10% SDS and 50 μl of proteinase K (20 mg/ml). After thorough mixing, this was incubated at 37°C for 1 h. After incubation, 1.8 ml of 5 M NaCl was added and mixed well. To this, 1.5 ml CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) was added, mixed well and incubated for 20 min at 65°C. Equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed by inversion for 5 min to emulsify. The emulsified mixture was centrifuged at 5,000 Xg for 10 min to separate layers. The aqueous layer was transferred to a fresh tube using a Pasteur pipette. The extraction step was repeated again once. To the aqueous layer, 0.6 volume of isopropanol was added and mixed until a stringy white DNA pellet precipitated out of solution and condensed into a tight mass. The nucleic acids which precipitated as a stringy glob were spooled out, kept for overnight dissolution at 4°C and purified using cesium chloride equilibrium density gradient centrifugation.

For purification, CsCl was added at the rate of 1 g ml^{-1} DNA solution, and 200 μl ethidium bromide (10 mg/ml) was also added. The DNA sample was loaded into Beckman 5 ml Quickseal polypropylene tubes which were sealed and then spun at 3,50,000 Xg at 20°C in a VTi 65 rotor for 18 h. The genomic DNA band was carefully removed with the help of a sterile 18 gauge needle into tubes wrapped in aluminium foil to avoid nicking of DNA. Ethidium bromide in the DNA solution was removed by repeated extraction with an equal volume of n-butanol saturated with water. When all the pink colour disappeared, the lower aqueous phase containing DNA sample was dialyzed for 24 h against several changes of TE buffer (pH 8.0) to remove CsCl. The $O.D._{260}$ of the final solution of DNA was taken to calculate the concentration of DNA. This DNA was stored in aliquots of 100 μl at -20°C.

3:14:2 Isolation of megaplasmid

The procedure described by Barnes (1977) for rapid disruption of bacterial colonies to test for the presence of plasmid was modified and used for isolating the megaplasmid. Bacterial colonies were grown to a large size

(1-2 mm) on agar medium. Using a sterile loop, a colony was transferred to an Eppendorf tube containing 25 µl of 50 mM NaOH, 0.5% SDS, 5 mM EDTA and 3 µl of 10X gel loading dye (cracking buffer). The colonies were dispensed by stirring the solution gently with a tooth pick. The tubes were incubated at 65°C for 60 min. Then 5 µl of 25% Ficoll 400 was added, mixed and the tube centrifuged at room temperature for 10 min. The contents were loaded onto a 0.7% agarose gel. After electrophoresis at 50 V for 20 h, the gel was stained with ethidium bromide and visualised under Fotodyne UV transilluminator.

Megaplasmid analysis of strain IRBG 46 was done according to the procedure of Rosenberg *et al.* (1982) with some modifications. To a 3 ml culture pellet, 25 µl lysis mixture consisting of $T_{50}E_{20}$ (pH 8.0), lysozyme (5 mg/ml) and RNase A (1 mg/ml) was added and the pellet resuspended in it. The bacterial suspension was mixed using a sterile tooth pick and kept at 30°C for 25-30 min. To this, 25 µl of 20% Ficoll 400 and 50 µl of 2% SDS were added, mixed and again incubated for 10 min at 30°C. The samples were centrifuged at room temperature at 10,000 Xg for 20 min. The supernatant was loaded into wells of 0.7% agarose gel. Electrophoresis was carried out at 50 V for 20 h. After ethidium bromide staining, the gel was visualised under Fotodyne UV transilluminator.

3:14:3 Isolation of plasmid DNA

The method used for plasmid isolation was essentially that of Sambrook *et al.* (1989) with a few modifications. An *E. coli* strain DH5α carrying the cosmid pHU52 was grown overnight at 37°C in 200 ml LB broth containing tetracycline (25 µg/ml). The cells were spun at 3,800 Xg for 10 min in a GS-3 rotor using Sorvall RC5C refrigerated centrifuge. The cell pellet was resuspended in 2 ml freshly prepared lysis buffer [15% sucrose (w/v), 25mM Tris-Cl (pH 8.0), 10mM EDTA, 5 mg/ml lysozyme]. This suspension was transferred to a 40 ml oakridge tube and incubated on ice for 10 min. To this, 4 ml of freshly prepared NaOH/SDS solution (0.2 N NaOH and 1% SDS) was added, inverted to mix and kept on ice for 10 min. Then 3 ml of ice cold 3M sodium acetate (pH 5.2) was added to the tube and the contents mixed thoroughly before keeping back on ice for 30 min. The sample was centrifuged in a Sorvall SS-34 rotor at 17,450 Xg at 4°C for 15 min. The supernatant was

decanted carefully into clean oakridge tube. To the supernatant, 20 μl of RNase (10 mg/ml) was added and tube was incubated for 30 min at 37°C. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed well by inversion and centrifuged at 7,000 Xg for 5 min to separate layers. To the aqueous layer, once again equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed and centrifuged to separate layers. To the aqueous layer, 2 volumes of cold ethanol was added, left at -20°C for 1 h and centrifuged at 17,500 Xg at 4°C for 20 min. The supernatant was discarded and pellet was dried by inverting the tubes on paper towel for 15 min.

The DNA pellet was dissolved in 640 μl of water. To this 160 μl of 4M NaCl and 800 μl polyethylene glycol (PEG) (13%, w/v) were added and mixed by inversion. The tube was kept on ice for 60 min, centrifuged at 17,500 Xg in a microfuge for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, resuspended in TE buffer and again ethanol precipitated at -20°C overnight. DNA pellet was washed with 70% ethanol and dissolved in 100 μl of TE (pH 8.0).

3:14:4 Dot-blot experiment

The total genomic DNA (2 μg) isolated as above, was denatured by adding NaOH to a final concentration of 0.2N and incubating at room temperature for 30 min. The denatured DNA was transferred to nylon membrane by using a dot-blot kit. Three pieces of Whatman 3 MM filter paper and nylon membrane were cut to the size of the apparatus. The prewetted membrane was placed on the Whatman filter papers which were placed on the apparatus. The lid was put on the apparatus and latched in place. The apparatus was then connected to a vacuum source. The DNA sample was added to the well. Vacuum was then turned-off and nylon membrane was removed. It was dried at room temperature and baked in a vacuum oven at 80°C for 1 hour.

3:14:5 Southern transfer of megaplasmid DNA

Megaplasmid isolated and run on a 0.7% agarose gel was denatured, transferred to Biodyne nylon membrane and immobilized. The DNA was transferred to the nylon membrane as follows.

After electrophoresis, gel was washed in a glass tray with gentle agitation at room temperature as given below:

1. 1x15 min in depurination solution (0.25 N HCl)
2. 2x25 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH)
3. 2x25 min in neutralization solution (1 M Tris-Cl (pH 8.0), 1.5 M NaCl)

The gel containing denatured, neutralized DNA was inverted on a glass plate over which a nylon membrane, Whatman paper and a stack of rough blotting paper (all cut to size of the gel) with a weight on it was placed, in that order. The transfer was allowed to continue overnight (14-16 h). After removing the paper stack, the filter was peeled off from the gel and positions of the wells were marked using a soft pencil. The filter was vacuum dried at 80°C and stored in a dessicator for later use.

The membrane filter was then hybridised with α-^{32}P labelled DNA and autoradiography was used to locate the position of any band complementary to radioactive probe (Southern, 1975; Sambrook *et al.*, 1989).

3:14:6 Restriction of cosmid pHU52 DNA

pHU52 DNA was restricted with *Eco* RI in a reaction mixture containing: 15 μl DNA (10 μg), 10 μl high salt buffer, 2 μl *Eco* RI (20 U/ml), and water to make up the volume to 100 μl. The reaction mixture was incubated at 37°C for 3 h. The restricted sample was run on a 0.7% agarose gel along with λ*Hin*d III DNA marker.

3:14:7 Recovery of *hup* gene fragments from agarose gel by freeze thaw method for the preparation of probe

The agarose gel was cut so as to remove the 12.9 kb *hup* gene fragment of pHU52. The gel pieces were transferred to microfuge vial and crushed by passing it through a 1 ml syringe. Equal volume of buffered phenol was added to thoroughly crushed gel pieces and mixed by vortexing. This mixture was frozen at -70°C for a minimum period of 1 h. The sample was then centrifuged at 25,000 Xg for 20 min at 4°C. To the aqueous layer, equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed and centrifuged to separate layers. The aqueous layer was removed and DNA was precipitated

with two volumes of chilled ethanol after adding 0.1 volume of sodium acetate overnight at -20°C and centrifuged at 17,500 Xg for 10 min to pellet DNA. The pellet was washed once with 70% alcohol, dried and dissolved in TE buffer.

3:14:8 Preparation of *hup* gene probe by nick-translation

For the preparation of *hup* probe, 12.9 kb fragment of pHU52 isolated as above, was nick-translated using the Promega nick-translation kit. The nick translation reaction was set up as follows:

Nucleotide mix	-	10 μl
Nick translation 10 X buffer	-	5 μl
(α-^{32}P) dCTP (Sp.act., 3000 Ci/mmole)	-	5 μl
Sample DNA	-	1 μg
Optimized enzyme mix	-	5 μl
Sterile water to a final volume 50 μl		

The nucleotide mix was prepared by mixing equal volumes of the unlabelled dNTPs (1.5 mM stock solutions) except dCTP.

The above mixture was incubated in a water bath at 15°C for 90 min. The reaction was stopped by adding 5 μl of 0.5 M EDTA (pH 8.0). The probe was denatured by adding 0.2 M NaOH and the mixture was then incubated for 30 min at room temperature. The denatured probe was then used for Southern hybridization.

3:14:9 Prehybridization

The membranes (dot blot of genomic DNA and southern blot of megaplasmid DNA) were placed in a polythene bag and the prehybridization solution [1mM EDTA, 0.5M NaH_2PO_4 (pH 7.2), 7% SDS] was added @ 150 μl/cm^2. The air bubbles were carefully removed and the sealed bag was immersed in water bath at 65°C for 30 min.

3:14:10 Hybridization

After prehybridization was over, through a small cut in the bag, the excess prehybridization solution was discarded to such an extent that the

remaining solution was just enough to wet the filter. The denatured probe was added, bag resealed and incubated for 16-18 h at 65° in a shaking water bath.

3:14:11 Washing of the filter

After the hybridization was over, the solution was drained out. The filter was washed twice with the wash solution I [1 mM EDTA; 40 mM $NaHPO_4$ (pH 7.2); 5% SDS] at 65°C for 30 min each with shaking. The filter was then washed twice with wash solution II [1 mM EDTA; 40 mM $NaHPO_4$ (pH 7.2); 1% SDS] at 65°C for 30 min each in a shaking water bath. The wet filter was used for autoradiography.

3:14:12 Autoradiography

The blot was covered with saran wrap and exposed to Indu X-ray film in the dark. The cassette was kept at -70°C for 3-4 days. The film was developed in Indu developer for 5 min at room temperature in dark followed by washing in water briefly and then fixed in Indu fixer for 5 min. The film was washed in running water and then air dried.

3:15 Hybridization pattern of *hup* gene from *Az. caulinodans* strain IRBG 46 and its Hup$^-$ mutant B11

3:15:1 Restriction of genomic DNA

Genomic DNA (12 μg) each of IRBG 46 and B11 isolated as discussed previously were restricted with each of the following restriction enzymes in separate reactions - *Eco* RI, *Hind* III and *Eco* RI + *Hind* III. Five units of each restriction enzyme per μg of DNA were added along with appropriate buffer. The reaction mixture was mixed well and incubated at 37°C overnight in 1.5 ml Eppendorf tubes. To analyze the digested DNA, 0.1 volume of 10X gel loading dye was added to it, mixed well and loaded on a 0.7% agarose gel. Electrophoresis was conducted at 5V/cm constant voltage. After completion of run, gel was stained in ethidium bromide (1 μg/ml sterile water) for 10 min, washed twice with sterile water and viewed under Fotodyne UV Transilluminator and photographed using polaroid camera.

3:15:2 Southern hybridization

DNA fragments that had been separated according to size by agarose gel electrophoresis were denatured and transferred to Biodyne nylon membrane and immobilised as described in the previous section. This was hybridized with radiolabelled 12.9 kb *hup* gene fragment of pHU52 and autoradiographed as discussed earlier.

Chapter 4

RESULTS

Nitrogenase from all known sources catalyzes an ATP-dependent reduction of not only N_2 to NH_3 but also proton to H_2. This evolution of H_2 results in an inefficient use of the energy provided for the N_2-fixing process. Some N_2-fixing organisms are capable of oxidizing the H_2 evolved by nitrogenase via hydrogen uptake (Hup) system. A minority of strains of *Rhizobium* possess the capability for synthesis of an H_2-recycling system that oxidizes the H_2 produced during N_2 fixation, thus recapturing some of the energy expended during H_2 evolution. In view of the potential benefits of H_2 oxidation in agriculture, it is worthwhile to have a basic understanding of the Hup system especially of *Azorhizobium caulinodans*, because *Az. caulinodans*, which not only forms root nodules but also stem nodules on *Sesbania rostrata*, has got an ability to fix N_2 *ex planta* and whose Hup system is not repressed by high organic carbon. In the present investigation, studies have been made on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46. The experimental findings are reported under the following headings:

1. Antibiotics/antibacterial agent resistance profile.
2. Isolation of Hup^- mutant.
3. Growth characteristics of mutant B11
4. Chemoautotrophic growth
5. Transfer and expression of *hup* gene cosmid pHU52 into Hup^- mutant B11.
6. Studies on the expression of *hup* genes under free-living conditions.
7. Factors affecting the expression of uptake hydrogenase in IRBG 46 under free-living conditions.
8. *Ex planta* N_2ase activity in IRBG 46 and its Hup^- mutant B11.
9. Symbiotic efficiency of IRBG 46 and B11.
10. Comparison of RuBP carboxylase activity in IRBG 46 and B11.
11. Localization of *hup* genes in *Az. caulinodans*.

4:1 Antibiotics/antibacterial agent resistance profile

Since not much was known about the characteristics of *Az. caulinodans* strain IRBG 46, a study was made to find out antibiotic/antibacterial agent resistance profile. The growth of the strain IRBG 46 was tested on TYM agar

medium with different concentrations (5-50 μg/ml) of various antibiotics/ antibacterial agents namely gentamycin (Gm), tetracycline (Tc), kanamycin (Km), streptomycin (Sm), ampicillin (Ap), nalidixic acid (Nd), rifampicin, azide and trimethoprim. The growth of the strain on the respective plates was observed after two days of incubation at 28°C.

The results presented in Table 4 show that the strain is resistant to Ap and trimethoprim upto a concentration of 50 μg/ml, to Nd upto 25 μg/ml and to azide upto 10 μg/ml. However, the strain was sensitive to Gm, Tc, Km, Sm and rifampicin.

4:2 Isolation of Hup⁻ mutant

Hup⁻ mutants have been useful for the study of the regulation and genetics of the H_2 oxidation system as well as for the assessment of the importance of H_2 oxidation to symbiotic N_2 fixation. Keeping this in view, mutation was carried out in the strain IRBG 46 using nitrosoguanidine to isolate Hup⁻ mutant(s).

4:2:1 Nitrosoguanidine (NTG) mutagenesis

An overnight grown culture of IRBG 46 was treated with NTG as described in 'Materials and Methods'. Titre was estimated at all the steps involved. Survival percentage was calculated as shown in Table 5. Only 0.0435% of the cells survived the NTG mutagenesis.

4:2:2 Screening of the NTG mutants for Hup⁻ character

About 1300 mutants were screened by the method of Haugland *et al.* (1983) to select Hup⁻ mutant(s). The method was based on the inability of the bacterial colonies to reduce methylene blue in the presence of H_2. By this technique, only one Hup⁻ mutant could be obtained which was designated as B11.

4:3 Growth characteristics of mutant B11

To check whether NTG mutagenesis has any effect on the growth pattern of the mutant, growth curves for the wild type strain IRBG 46 and its putative Hup⁻ mutant B11 were prepared by growing them in TYM broth. The growth curves depicted in Fig.1 show that there is no effect of mutation on growth of the culture. Both the wild type and putative Hup⁻ mutant are fast

Table 4. Effect of various antibiotics and antibacterial agents on the growth of *Az. caulinodans* strain IRBG 46

Antibiotic	Concentration (μg/ml)				
	5	10	15	25	50
Gentamycin	+	-	-	-	-
Tetracycline	-	-	-	-	-
Kanamycin	-	-	-	-	-
Ampicillin	++	++	++	++	++
Streptomycin	-	-	-	-	-
Nalidixic acid	++	++	+	+	-
Rifampicin	-	-	-	-	-
Azide	++	+	-	-	-
Trimethoprim	++	++	++	++	++

'++' indicates good growth

'+' indicates faint growth

'-' indicates no growth.

Table 5. Survival percentage of the cells after nitrosoguanidine mutagenesis

	Time (h)	Dilution	No. of colonies	cfu/ml	
Overnight grown culture	0	10^{-6}	1300	1.3×10^{9}	
		10^{-7}	215	2.15×10^{9}	
Overnight grown culture diluted to double volume		10^{-6}	317	3.17×10^{8}	3.54×10^{8}
		10^{-7}	39	3.9×10^{8}	
Fixation	18	10^{-2}	1480	1.48×10^{5}	1.54×10^{5}
		10^{-3}	160	1.6×10^{5}	

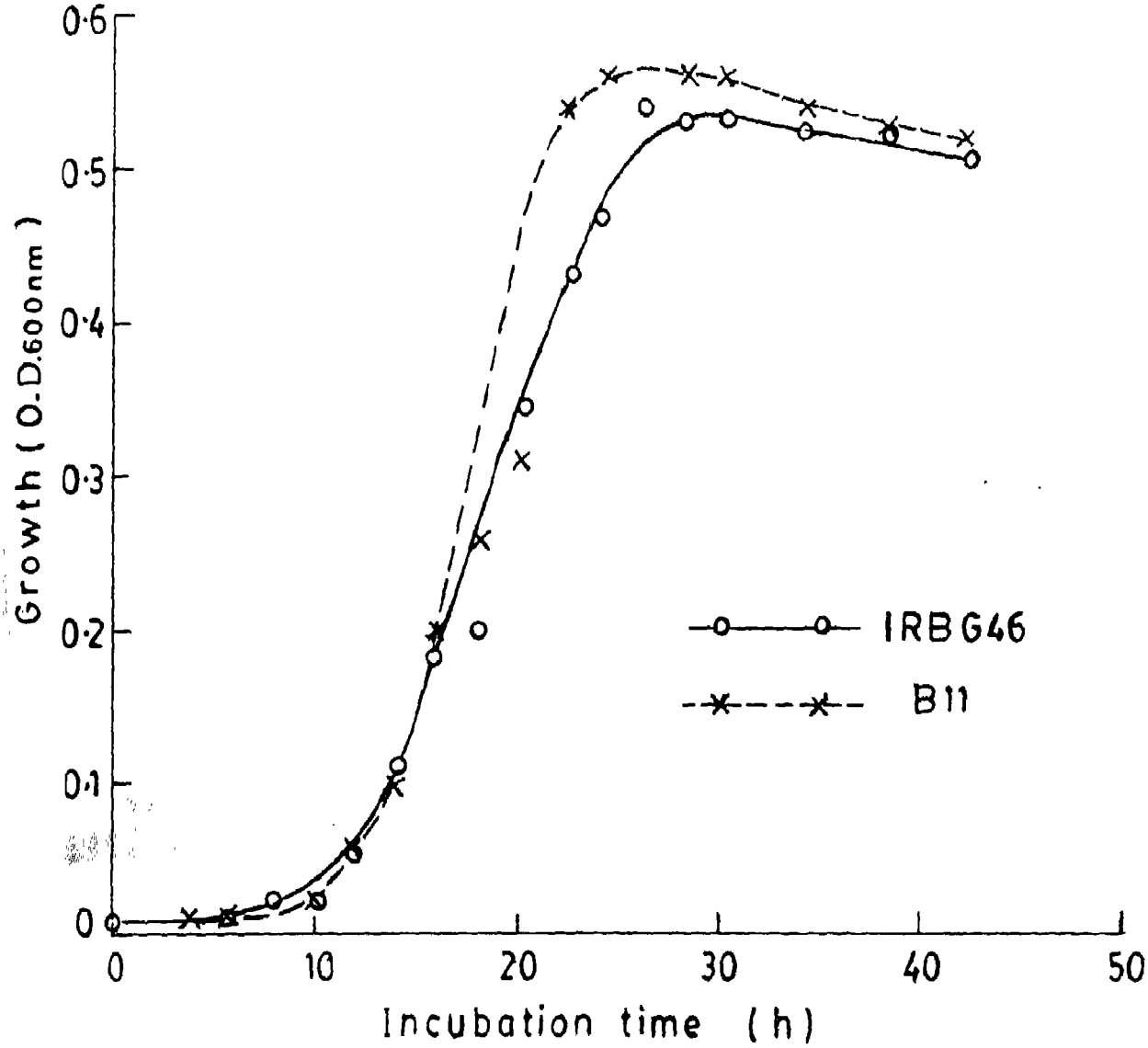
$$\text{Survival \%} = \frac{1.54 \times 10^{5}}{3.54 \times 10^{8}} \times 100$$

$$= 0.0435$$

$$\text{Killing \%} = 100 - 0.0435$$

$$= 99.96$$

Fig. 1 Growth curves of *Az. caulinodans* strain IRBG 46 and its Hup mutant B11


0·6
0·5
0·4
0·3
0·2
0·1
0
Growth (O.D.600nm)
0
10
20
30
40
50
Incubation time (h)
IRBG46
B11

growing and the log phase lies between 10-26 h. For all the subsequent studies ~ 24 h growth in TYM broth was taken for inoculum preparation. Inoculum for all the studies was kept more or less constant.

4:4 Chemoautotrophic growth

Both the wild type strain and its mutant B11 were tested for their ability to grow chemoautotrophically. The *Cicer-Rhizobium* strain Rcd301 which is known to be Hup$^-$ was taken as control. These strains were grown on Repaske medium under the appropriate conditions in the presence of H_2 and CO_2. After 7 days of incubation, the strain IRBG 46 was found to grow very well, whereas B11 showed very less growth and Rcd301 showed no growth at all. The results are shown in Plate I. This study indicates that mutant B11 like Hup$^-$ control Rcd 301, is unable to utilise H_2 as the energy source.

4:5 Transfer and expression of *hup* cosmid pHU52 into B11

Cosmid pHU52, which contains *hup* genes of *Bradyrhizobium japonicum*, was transferred into Hup$^-$ mutant B11 by triparental mating to check whether it can restore the Hup activity. The transconjugants obtained were pooled together and have been designated as B11 (pHU52). In similar way, a transconjugant of Rcd301, Rcd301 (pHU52) was obtained and used as control. These transconjugants were derepressed for uptake hydrogenase under free-living conditions. The results are presented in Table 6.

It can be seen from the results that the level of expression of the enzyme activity was more or less comparable in both the transconjugants; for Rcd301 (pHU52), the activity being 361 nmol H_2/h/mg protein and for B11 (pHU52) the activity being 411. The expression of uptake hydrogenase in wild type strain IRBG 46 was much higher (27-fold) compared to the transconjugant, B11 (pHU52). It is thus evident that there was very little restoration of Hup activity in Hup$^-$ mutant B11 when the *hup* cosmid pHU52 was transferred into it.

Plate I

Chemoautotrophic growth capability of *Az. caulinodans* strain IRBG 46 and its Hup^- mutant B11.

a. Hup^+ strain IRBG 46

b. Hup^- mutant B11

c. Wild type Hup^- strain Rcd 301

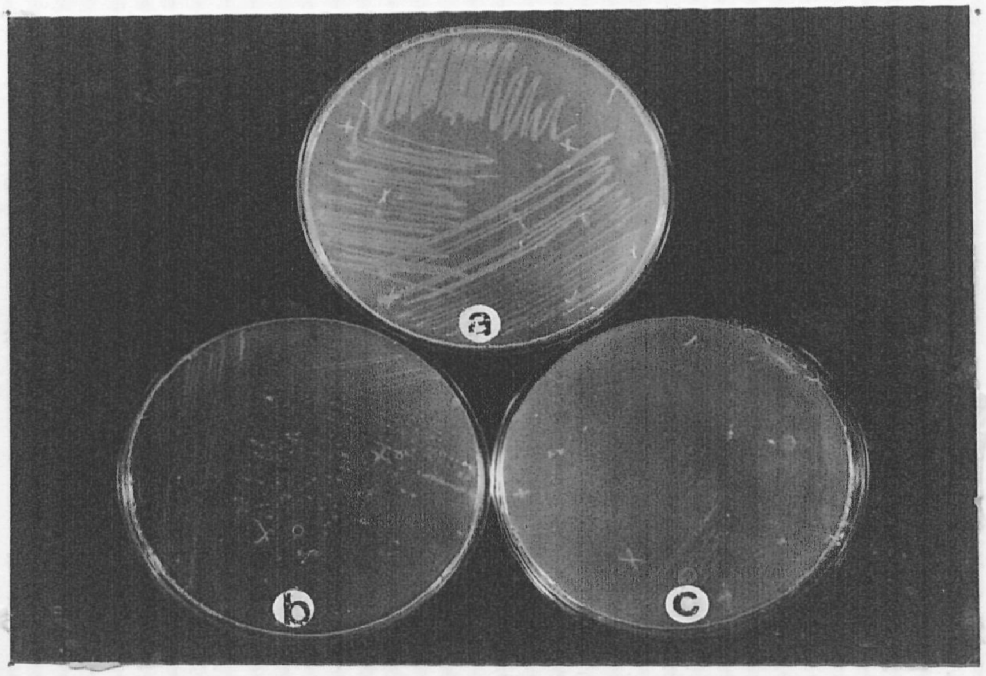

a
b
c

Table 6. Expression of uptake hydrogenase in the transconjugants B11 (pHU52) and Rcd 301 (pHU52) in free-living conditions

Strains/ Transconjugants	uptake hydrogenase activity (nmol H_2/h/slant)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/h/mg protein)
IRBG 46	7812	702	11,128
Rcd 301	nd	-	-
Rcd 301 (pHU52)	78	216	361
B11	nd	-	-
B11 (pHU52)	104	253	411

nd = not detected

4:6 Studies on the expression of *hup* genes under free-living conditions

4:6:1 Expression of *hup* genes in low and high carbon media

The Hup$^-$ character of the mutant B11 was confirmed by gas chromatographic method. *Az. caulinodans* strain IRBG 46 and its mutant B11 were assayed for *hup* gene expression under free-living conditions. Two different media viz. YEMA (high organic carbon) and HUM (low organic carbon) were used for derepression of uptake hydrogenase. The results presented in Table 7 show that there was an expression of uptake hydrogenase activity in IRBG 46 on both low as well as high organic carbon media. However, B11 did not show any uptake hydrogenase activity on either of the media. In case of strain IRBG 46, the specific activity was 50% higher on HUM compared to YEMA medium.

4:6:2 Requirement of H_2 for induction of uptake hydrogenase in IRBG 46

To find out whether there is a constitutive expression of *hup* genes in IRBG 46, one set of HUM slants, inoculated with IRBG 46 was incubated in the absence of any added H_2. The Hup$^+$ transconjugant, Rcd301 (pHU52) was kept as control.

The results presented in Table 8 show that when the bacterial strains were incubated in the absence of H_2, there was negligible uptake hydrogenase activity (31 nmol H_2/h/mg protein) in case of Rcd301 (pHU52) but very high activity (6483 nmol H_2/h/mg protein) was observed in strain IRBG 46. When the strains were incubated in the presence of H_2, the specific activity was increased to 11.3-fold in Rcd301 (pHU52), whereas in strain IRBG 46, it increased by only 1.3-fold.

These results thus show that IRBG 46 does not require the addition of H_2 to induce *hup* genes, whereas same was not true in case of Rcd301 (pHU52).

Table 7. **Uptake hydrogenase activity of *Az. caulinodans* strain IRBG 46 and its mutant B11 in two different media under free-living conditions**

Strain	Medium			
	YEMA		HUM	
	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)
Az. caulinodans				
IRBG 46	584	4670 ± 1416	568	6963 ± 833
B11	602	nd	585	nd
Cicer- Rhizobium				
Rcd 301	500	nd	522	nd

nd = not detected

Table 8. Expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46 in the presence/absence of H_2 during incubation period

Strain	In the absence of H_2		In the presence of H_2	
	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)
Rcd 301 (pHU52)	254	31 ± 6.5	248	352 ± 25
IRBG 46	646	6483 ± 499	651	8480 ± 781

The values are mean ± SD of six replicates.

4:7 Factors regulating the expression of uptake hydrogenase in *Az. caulinodans* IRBG 46 under free-living conditions

4:7:1 Effect of pH

To find out the optimum pH, the effect of different pH was seen on the expression of uptake hydrogenase enzyme. The results presented in Table 9 show that the optimum pH for growth as well as expression of the enzyme was 6.8; at this pH, the sp. activity being 11,128 nmol H_2/h/mg protein. At pH 6.0, the sp. activity decreased to 8464 nmol H_2/h/mg protein. Similarly, at pH 8.0 also, the sp. activity decreased to 7584 nmol H_2/h/mg protein. This study thus suggests that slight decrease or increase in pH compared to optimum pH of 6.8, might affect growth as well as expression of the enzyme activity drastically. The optimum pH of 6.8 was used in the subsequent studies.

4:7:2 Effect of carbon sources

A study was carried out to find out the effect of different carbon sources on the expression of uptake hydrogenase and the results have been summarised in Table 10. For the study, sodium gluconate (2.29 mM), which is required as a carbon source for the expression of uptake hydrogenase in the standard medium (HUM), has been replaced by different carbon sources (15 mM) while keeping other factors constant.

The results presented in Table 10 show that complete removal of sodium gluconate from the standard medium lowered the expression of the enzyme by 53%, although the growth was not much affected. Carbon sources like Na-citrate and malate did not allow the expression of uptake hydrogenase to take place. Glucose, gluconate and Na-succinate did not affect the growth but reduced the expression by nearly 50%.

Among the other carbon sources tested, mannitol and arabinose did not much affect the expression of enzyme, while sucrose and fructose increased the expression by 39% and 34%, respectively. No growth was observed on oxaloacetate and α - ketoglutarate when used at a concentration of 15 mM.

Table 9. Effect of pH on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46 under free-living conditions

pH	Uptake hydrogenase activity (nmol H_2/h/slant)	Growth (μg protein/slant)	Sp. uptake hydrogenase activity (nmol H_2/h/mg protein)
4.0	1861	264	7049
5.0	2390	288	8299
6.0	2590	306	8464
6.8	7812	702	11,128
8.0	2101	277	7584
9.0	2130	372	5726

Table 10. Effect of various carbon sources on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46 in free-living conditions

Carbon* source	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
SM	838	9921	100
SM-gluconate	664	4640	47
Glucose	785	5191	52
Gluconate	707	5218	53
Mannitol	381	9858	99
Na-citrate	523	1015	10
Glycerol	748	4117	41
Na-succinate	844	5226	53
Sucrose	504	13792	139
Fructose	488	13273	134
Arabinose	554	9022	91
Ribose	435	7834	79
Malate	842	—	0
Oxaloacetate	nd	—	0
α-ketoglutarate	nd	—	0

*Sodium gluconate in the standard hydrogen uptake medium (SM) was replaced by 15 mM appropriate carbon source.

4:7:3 Effect of glucose concentration

As replacement of sodium gluconate by glucose (15 mM) lowered the expression of uptake hydrogenase to a great extent (see Table 10), another study was conducted to find out the effect of increasing glucose concentration when added to standard HUM, without replacing gluconate, on the expression of uptake hydrogenase. The results are summarised in Table 11.

It is evident from the results that with the increase in glucose concentration from 5 to 25 mM, inhibition on the expression of uptake hydrogenase also increased from 32% to 56%. However, the inhibition was more steep at 5 mM and 10 mM and beyond that the relative activity remained almost constant. There was not much effect of glucose on growth of the culture.

4:7:4 Effect of succinate concentration

As replacement of sodium gluconate (2.29 mM) by 15 mM succinate also lowered the expression of uptake hydrogenase (see Table 10), another study was conducted to check the effect of different concentrations of succinate.

It is clear from the data presented in Table 12 that with the increase in succinate concentration, there was a decrease in uptake hydrogenase activity. At 10 mM concentration, the inhibition on the expression was 50%, whereas at 20 and 30 mM concentrations, it was 65% and 72%, respectively. On the contrary, the growth was not affected by different concentrations of succinate.

4:7:5 Effect of combined N-sources

In this study, sodium glutamate (2.67 mM) as the sole nitrogen source was replaced by different nitrogen sources (10 mM) and their effect on the expression of uptake hydrogenase was studied. The results are presented in Table 13.

It can be seen from the results that removal of sodium glutamate from HUM resulted in decrease of growth as well as sp. activity of the enzyme. All N-sources except KNO_3 and NH_4NO_3 either did not have any effect or had slightly stimulatory effect on the expression of the enzyme; KNO_3 had an inhibitory effect to the extent of 43% on the expression of the enzyme.

Table 11. Effect of different concentrations of glucose on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46

Glucose addition to HUM (mM)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
Nil	746	6348	100
5	648	4345	68
10	825	3467	55
15	845	3039	48
20	791	2881	45
25	850	2759	44

Table 12. Effect of different concentrations of succinate on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46

Succinate addition to HUM (mM)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
Nil	775	8984	100
10	720	4578	51
20	722	3116	35
30	732	2475	28

Table 13. Effect of various combined nitrogen sources on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46 under free-living conditions

N-source*	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
SM	758	8580	100
SM-glutamate	590	6155	72
KNO_3	308	4915	57
NH_4Cl	547	8340	98
NH_4NO_3	nd		
Na-glutamate	590	9257	108
Urea	588	9340	109

* Sodium glutamate (2.67 mM) in the standard hydrogen uptake medium (SM) was replaced by 10 mM appropriate nitrogen source.

With respect to the effect of N-sources on growth, NH_4NO_3 did not allow the growth to take place, KNO_3 reduced the growth by 60%, whereas rest of the N-sources reduced the growth by about 20%.

4:7:6 Effect of different concentrations of NH_4NO_3

Since NH_4NO_3 at 10 mM concentration completely inhibited the growth, lower concentrations were tested to find out their effect on growth as well as expression of uptake hydrogenase. NH_4NO_3 was added at 2.5, 5, 7.5 and 10 mM concentration to the standard HUM without replacing sodium glutamate. The results are presented in Table 14.

As evident from the results, lower concentrations of NH_4NO_3 were also inhibitory with respect to growth as well as activity. There was 80% inhibition on the expression of enzyme at 2.5 mM, whereas no activity was observed at concentrations higher than 5 mM. At these higher concentrations, there was an inhibition of growth also. At 2.5 mM NH_4NO_3, the growth was inhibited by 63%, whereas at 10 mM concentration, the inhibition was 78%.

4:7:7 Effect of oxygen concentration

Since the partial pressure of oxygen in soil medium as well as within the bacteroid system may be one of the important factors regulating the expression of uptake hydrogenase, a study was undertaken to investigate the effect of oxygen concentration on the expression of uptake hydrogenase. The results are summarised in Table 15.

For this study, oxygen concentration was varied from 1% to 20% in N_2 atmosphere while keeping other conditions constant during incubation for induction. Hydrogen-uptake rates were determined at atmospheric level of O_2. It can be seen from Table 15 that at 1% O_2, the activity was reduced by 77% although the growth was reduced by only 28% compared to normal atmospheric O_2 concentration (~20%). At 10% and 15% O_2 concentration, the growth was not much affected and also the activity remained more or less constant.

This study thus suggests that with the increase in O_2 concentration from 1% to 10% during incubation period, there was an increase in the expression of the enzyme activity and further increase in O_2 concentration did not affect the expression.

Table 14. Effect of different concentrations of NH_4NO_3 on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46

Addition to the standard medium (mM)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
Nil (control)	673	7102	100
KNO_3 (10)	324	3481	49
NH_4Cl (10)	458	7000	99
NH_4NO_3 (2.5)	252	1383	20
NH_4NO_3 (5.0)	255	720	10
NH_4NO_3 (7.5)	207	nd	0
NH_4NO_3 (10.0)	150	nd	0

nd = not detected

Table 15. Effect of different oxygen concentrations on the expression of uptake hydrogenase in *Az. caulinodans* strain IRBG 46

O_2 conc. in N_2 atmosphere (%)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
~20	720	8031	100
15	741	7712	96
10	703	7681	96
5	667	6142	77
1	515	1814	23

4:7:8 Effect of cyclic AMP

From the earlier studies (see Table 10), it has been observed that the addition of malate (15 mM) to the standard hydrogenase induction medium resulted in complete inhibition of the expression of uptake hydrogenase. Therefore, an experiment was conducted to see if cyclic AMP can reverse this repression effect brought about by malate on the expression of hydrogenase.

As seen from Table 16, when malate was added to HUM at a concentration of 15 mM, it resulted in nearly 90% inhibition on the expression of uptake hydrogenase. Addition of increasing concentration (0.25-1.0 mM) of cAMP to malate-containing HUM, stimulated the expression only to a very small extent; the stimulation at 1.0 mM being 11%.

4:8 Expression of *ex planta* nitrogenase activity in *Az. caulinodans* strain IRBG 46 and its Hup$^-$ mutant B11

Since addition of H_2 was not required for the induction of uptake hydrogenase in IRBG 46 when grown on HUM (see Table 8), one possibility could be that this medium also induces *ex planta* nitrogenase activity which is known to evolve H_2 and the H_2 thus evolved acts as inducer of *hup* genes. Keeping this probability in view, IRBG 46 and its Hup$^-$ mutant B11 were tested for *ex planta* expression of nitrogenase under the conditions in which derepression of *hup* genes was carried out. The results are presented in Table 17.

The rate of acetylene reduction in case of IRBG 46 was 23 nmol C_2H_4/h/mg protein, whereas in B11, it was 27 nmol C_2H_4/h/mg protein. When IRBG 46 and B11 were tested for H_2 evolution, only mutant B11 showed H_2 evolution @ 9 nmol/h/mg protein and no H_2 evolution could be detected in case of IRBG 46. The relative efficiency (RE) of electron transfer to nitrogen was calculated as given by Schubert and Evans (1976). In case of B11, RE was 0.67, whereas IRBG 46 had a RE of 1. This study thus shows that under the condition in which uptake hydrogenase is induced, nitrogenase is also expressed and H_2 evolved by it may help in the induction of uptake hydrogenase. As the RE for B11 is less than 0.8, it further confirms Hup$^-$ character of mutant B11.

Table 16. Effect of cyclic AMP on the expression of uptake hydrogenase in free-living *Az. caulinodans* strain IRBG 46 when grown on malate containing medium

Addition(s) to HUM (mM)	Growth (μg protein/ slant)	Sp. uptake hydrogenase activity (nmol H_2/ h/mg protein)	Relative activity
None	756	7784	100
Malate (15)	740	688	9
Malate (15) +cAMP (0.25)	690	934	12
Malate (15) +cAMP (0.5)	810	1045	13
Malate (15) +cAMP (0.75)	900	1241	16
Malate (15) +cAMP (1.0)	740	1557	20

Table 17. Acetylene reduction activity, hydrogen evolution rate and relative efficiency in *Az. caulinodans* strain IRBG 46 and its Hup⁻ mutant B11 under free-living conditions

Strain/ mutant	Growth (μg protein/ slant)	N_2ase activity (nmol C_2H_4/h/mg protein)	H_2 evolution (nmol/h/mg protein)	RE*
IRBG 46	536	23 ± 1.5	nd	1.00
B11	629	27 ± 3.3	9 ± 1.6	0.67

Values are the mean ± SD of six replications.

$$* \text{ RE (Relative efficiency)} = 1 - \frac{\text{Rate of } H_2 \text{ evolution in air}}{\text{Rate of } C_2H_2 \text{ reduction in air}}$$

4:9 Studies on H_2-recycling ability of *Az. caulinodans* strain IRBG 46 and its Hup$^-$ mutant B11 under symbiotic conditions

The expression of *hup* genes in *Sesbania rostrata* stem and root nodules formed by *Az. caulinodans* strain IRBG 46 and its Hup$^-$ mutant B11 was studied by measuring the relative efficiency (RE) of electron transfer to N_2 via nitrogenase. RE is an estimate of H_2-recycling ability; when RE is more than 0.8, the rhizobial strain is considered to be Hup$^+$.

The study was carried out at 53 and 60 days after sowing (DAS). Root and stem nodules were taken separately and assayed for H_2 evolution and nitrogenase (acetylene reduction) activity. The relative efficiency was then calculated. The results for root nodules, stem nodules and whole plant are presented in Tables 18 a, b and c, respectively.

a. **Root nodules:** At 53 DAS, the rate of acetylene reduction in root nodules in case of IRBG 46 was 45.81 μmol C_2H_4/h/g nodule dry wt., whereas in case of B11, it was 41.76 μmol C_2H_4/h/g nodule dry wt (Table 18a). B11 showed a H_2 evolution rate of 12.70 μmol/h/g nodule dry wt. However, no H_2 evolution could be detected in case of IRBG 46. Similarly, at 60 DAS, nitrogeanse activity was 63.40 μmol and 45.79 μmol C_2H_4/h/g nodule dry wt for IRBG 46 and B11, respectively. Again no H_2 evolution was observed in case of IRBG 46, whereas rate of H_2 evolution for B11 was 12.42 μmol/h/g nodule dry wt. RE was found to be 1.0 in case of IRBG 46 for both the stages and for B11, it was 0.70 and 0.73 at 53 and 60 DAS, respectively.

b. **Stem nodules:** At 53 DAS, the rate of acetylene reduction in stem nodules was 29.12 μmol and 31.61 μmol C_2H_4/h/g nodule dry wt for IRBG 46 and B11, respectively (Table 18b). Again no H_2 evolution was observed in case of strain IRBG 46, whereas rate of H_2 evolution for B11 was 10.16 μmol/h/g nodule dry wt. At 60 DAS, acetylene reduction rate was 39.35 μmol and 25.38 μmol C_2H_4/ h/g nodule dry wt for IRBG 46 and B11 respectively. Rate of H_2 evolution for B11 was 7.60 μmol/h/g nodule dry wt. However, no H_2 evolution was observed in case of IRBG 46. RE for IRBG 46 was 1.0, whereas for B11, it was 0.68 and 0.70 at 53 and 60 DAS, respectively.

c. **Total plant** : The overall nitrogenase activity per plant nodule was calculated by summing up together the nitrogenase activity obtained for roots

Table 18a. Acetylene reduction activity, hydrogen evolution rate and relative effeciency in root nodules of *Sesbania rostrata* infected with *Az. caulinodans* strain IRBG 46 and its Hup⁻ mutant B11

DAS	Strain/ mutant	Root nodule dry wt. (mg/plant)	Nitrogenase activity (μmol C_2H_4 /h)		Hydrogen evolution (μmol /h)		RE*
			per g nodule dry wt.	per plant nodule	per g nodule dry wt.	per plant nodule	
53	IRBG 46	43	45.81 ± 2.60	1.97 ± 0.30	nd	nd	1.0
	B11	74	41.76 ± 8.08	3.09 ± 1.32	12.70 ± 1.50	0.94 ± 0.20	0.70
60	IRBG 46	94	63.40 ± 3.61	5.96 ± 1.5	nd	nd	1.0
	B11	95	45.79 ± 2.82	4.35 ± 1.13	12.42 ± 1.10	1.18 ± 0.16	0.73

Note : The values are mean ± SD of three replicates. Each replicate consisted of three plants.

$$*\text{RE (Relative efficiency)} = 1 - \frac{\text{Rate of } H_2 \text{ evolution in air}}{\text{Rate of } C_2H_2 \text{ reduction in air}}$$

Table 18b. Acetylene reduction activity, hydrogen evolution rate and relative effeciency in stem nodules of *Sesbania rostrata* infected with *Az. caulinodans* strain IRBG 46 and its Hup⁻ mutant B11

DAS	Strain/ mutant	Root nodule dry wt. (mg/plant)	Nitrogenase activity (μmol C_2H_4 /h)		Hydrogen evolution (μmol /h)		RE*
			per g nodule dry wt.	per plant nodule	per g nodule dry wt.	per plant nodule	
53	IRBG 46	57	29.12 ± 1.34	1.66 ± 0.51	nd	nd	1.0
	B11	62	31.61 ± 5.35	1.96 ± 0.20	10.16 ± 2.16	0.63 ± 0.07	0.68
60	IRBG 46	92	39.35 ± 2.01	3.62 ± 1.09	nd	nd	1.0
	B11	104	25.38 ± 3.68	2.64 ± 0.50	7.60 ± 2.34	0.79 ± 0.31	0.70

Note : The values are mean ± SD of three replicates. Each replicate consisted of three plants.

$$*\text{RE (Relative efficiency)} = 1 - \frac{\text{Rate of } H_2 \text{ evolution in air}}{\text{Rate of } C_2H_2 \text{ reduction in air}}$$

Table 18c. Acetylene reduction activity, hydrogen evolution rate and relative effeciency *of Sesbania rostrata* plants infected with *Az. caulinodans*

DAS	Strain/ mutant	Nodule dry wt - Root + stem (mg/plant)	Nitrogenase activity (μmol C_2H_4 /h/plant)	Hydrogen evolution (μmol/h/plant)	RE*
53	IRBG 46	100	3.63 ± 0.90	nd	1.00
	B11	136	5.05 ± 1.39	1.57 ± 0.24	0.69
60	IRBG 46	186	9.58 ± 3.66	nd	1.00
	B11	199	7.00 ± 1.58	1.97 ± 0.30	0.72

Note : The values are mean ± SD of three replicates. Each replicate consisted of three plants.

$$*\text{RE (Relative efficiency)} = 1 - \frac{\text{Rate of } H_2 \text{ evolution in air}}{\text{Rate of } C_2H_2 \text{ reduction in air}}$$

and stem nodules separately. Similarly, H_2 evolution rate per plant nodule was also calculated from the data presented in Tables 18a and 18b. The results are presented in Table 18c. It can be seen from the Table that total nitrogenase activity at 53 DAS was 3.63 μmol and 5.05 μmol C_2H_4/h/plant for IRBG 46 and B11, respectively. RE for strain IRBG 46 was 1.0 and for B11, it was 0.69. Similarly at 60 DAS, total nitrogenase activity was 9.58 μmol and 7.00 μmol C_2H_4/h/plant for IRBG 46 and B11 respectively. RE for IRBG 46 was 1.0 whereas for B11, it was 0.72. These results thus demonstrate that the plants inoculated with IRBG 46 are highly efficient in recycling the H_2 produced via nitrogenase.

4:10 Localization of *hup* genes in *Az. caulinodans* strain IRBG 46

In our efforts to localize *hup* genes, analysis for the presence of megaplasmid, if any, was done according to modified procedure of Barnes (1977). Plate IIA (Lanes 1,2) shows the presence of a megaplasmid in strain IRBG 46 of a size equal to that of megaplasmid plJ1008 from *R. leguminosarum* strain B 164 (Lanes 3,4) which is ~280 kb in size. When the analysis of megaplasmid was done by the method of Rosenberg *et al.* (1982), two bands were obtained in IRBG 46 (Plate IIIA, Lanes 1,2). The upper band was comparable in size with the megaplasmid plJ1008 (Lanes 3,4,5) while the second band perhaps represents another plasmid of a smaller size.

Since a megaplasmid has been found with reproducibility in IRBG 46, it was worthwhile to know whether the *hup* genes are present on it or on the chromosome. For this study, a dot-blot of genomic DNA and a Southern blot of megaplasmid were hybridised using radiolabelled 12.9 kb *Eco* RI *hup* gene fragment of cosmid pHU52 as a probe. The heterologous probe showed good homology with the megaplasmid DNA (Plates IIB and IIIB) as well as total genomic DNA (Plate IV). This study thus shows that there could be multicopies of *hup* genes in *Az. caulinodans* strain IRBG 46. Additional studies dealing with isolation and characterization of these genes will be rewarding in future.

In order to find out the hybridization pattern, the genomic DNA of IRBG 46 and its Hup$^-$ mutant B11 were restricted with enzymes, *Eco* RI, *Hind* III and a double digest using *Eco* RI + *Hind* III. The restriction pattern is shown in plate VA. The gel containing the restricted DNA was blotted on

Plate II

A. Megaplasmid analysis of *Az. caulinodans* strain IRBG 46 by agarose gel (0.7%) electrophoresis. Colonies were disrupted using cracking buffer.

B. Southern hybridization of megaplasmid using nick translated 12.9 kb *Eco* RI fragment of pHU52 as probe.

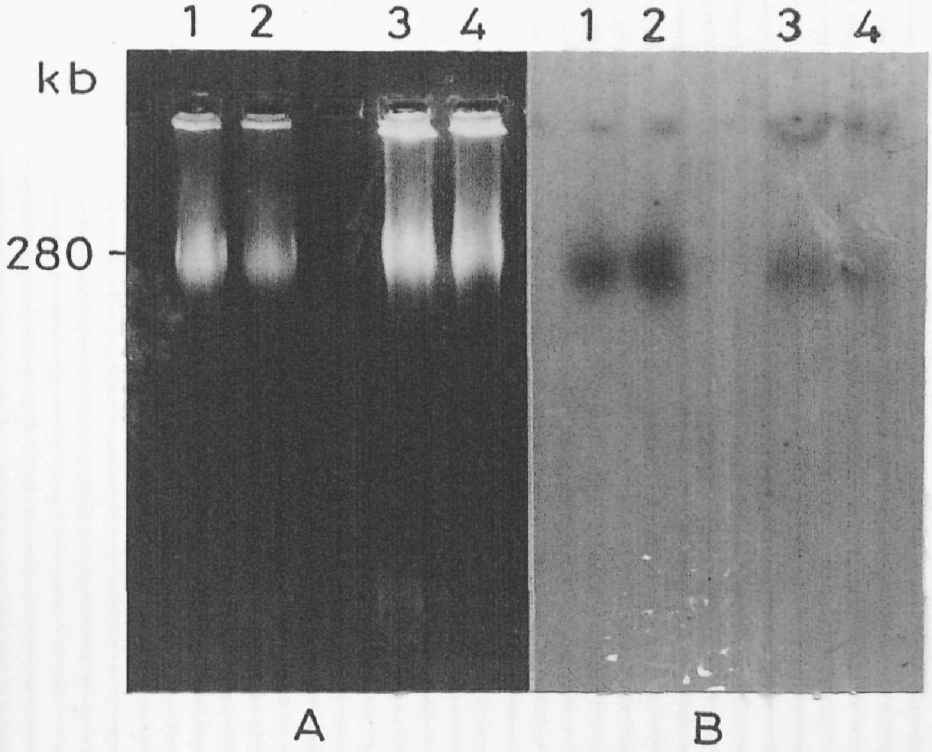
Lanes 1,2 : Megaplasmid of IRBG 46
Lanes 3,4 : Megaplasmid marker (plJ1008)

Plate III

A. Megaplasmid analysis of *Az. caulinodans* strain IRBG 46. The megaplasmid was isolated by *in situ* lysis and electrophoresed on a 0.7% agarose gel.

B. Southern hybridization of megaplasmid using nick translated 12.9 kb *Eco* RI fragment of pHU52 as probe.

Lanes 1,2 : Megaplasmid of IRBG 46
Lanes 3,4,5 : Megaplasmid marker (plJ1008)


1 2 3 4 1 2 3 4
kb
280
A
B

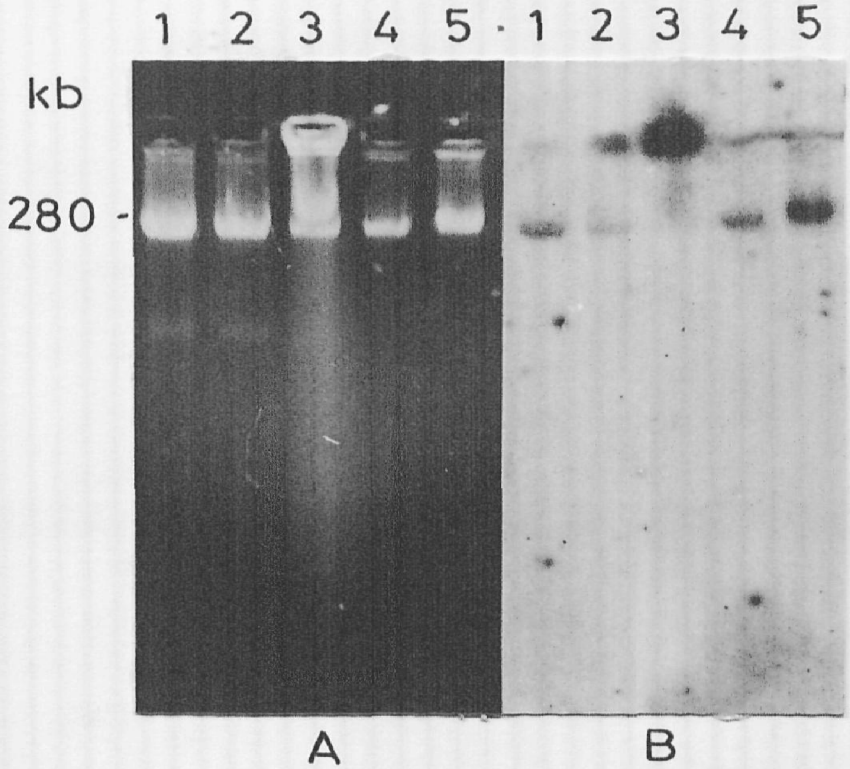

1 2 3 4 5 1 2 3 4 5
kb
280
A
B

Plate IV

Dot-blot hybridization of total DNA from *Az. caulinodans* strain IRBG 46 using nick translated12.9 kb *Eco* RI fragment of pHU52 as probe.

a. Positive control (pHU52)
b. *Az. caulinodans* strain IRBG 46
c. Negative control (pRK290)

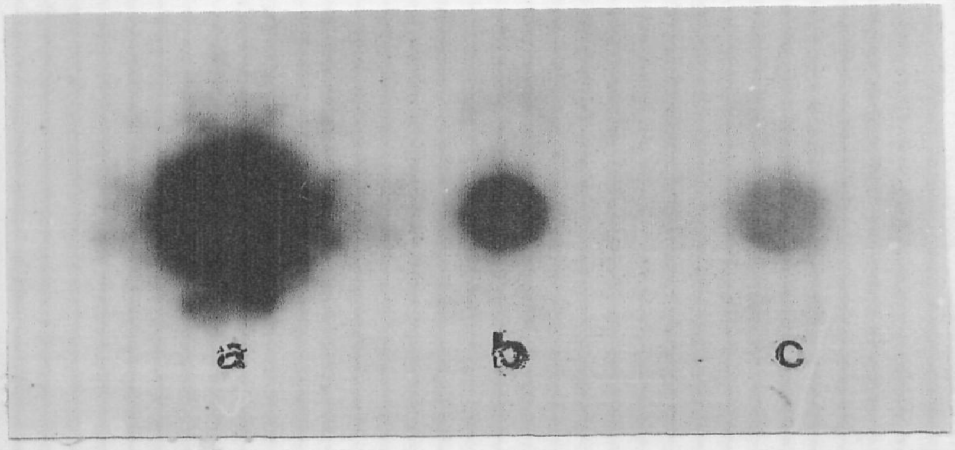

a
b
c

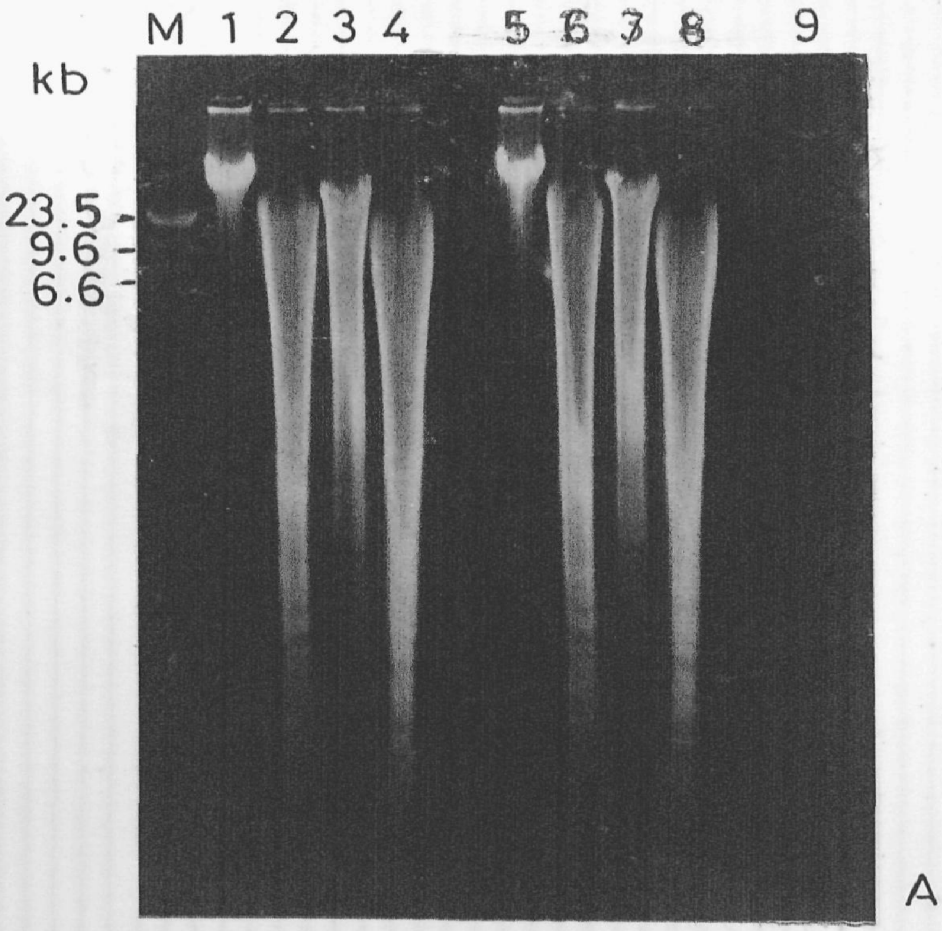
Plate V

Restriction analysis and Southern hybridization patterns of total DNA from *Az. caulinodans* strain IRBG 46 and its Hup$^-$ mutant B11.

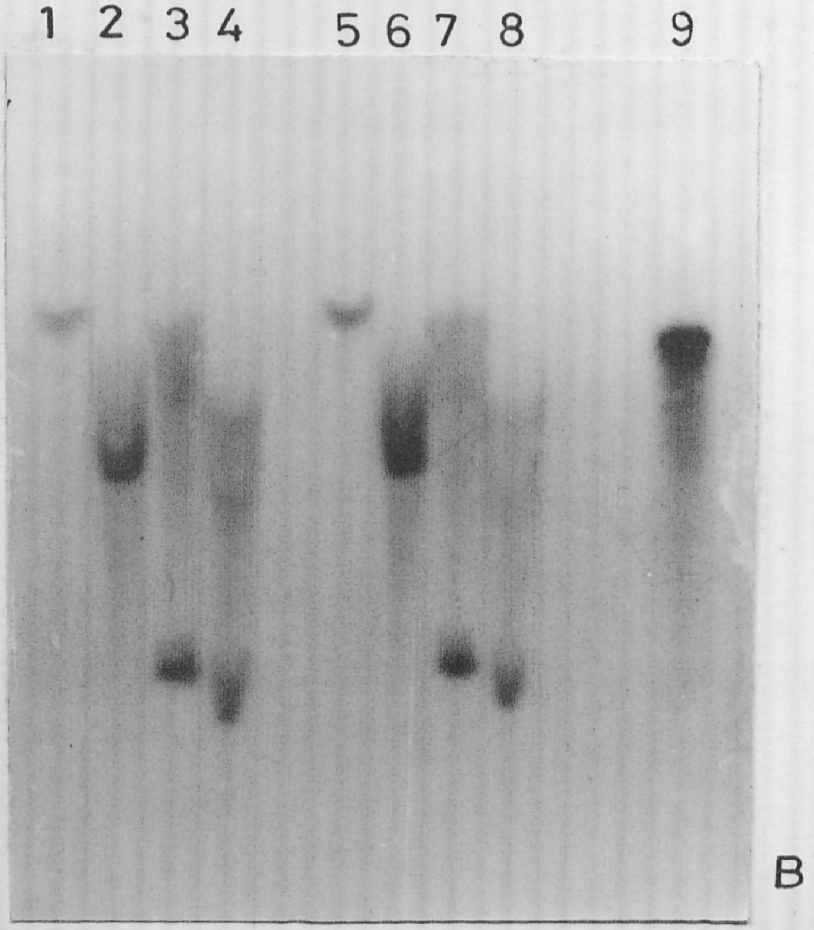
A. Restriction analysis on 0.7% agarose gel.

   λ *Hind* III marker (lane M), uncut IRBG 46 DNA (lane 1), IRBG 46 DNA restricted with enzymes : *Eco* RI (lane 2), *Hind* III (lane 3) and *Eco* RI + *Hind* III (lane 4). Uncut total DNA of Hup$^-$ mutant B11 (lane 5), B11 DNA restricted with enzymes : *Eco* RI (lane 6), *Hind* III (lane 7) and *Eco* RI + *Hind* III (lane 8). Positive control pHU52 not visible in the gel (lane 9)

B. Southern hybridization of the above gel using 12.9 kb *Eco* RI fragment of pHU52 as probe



A



B

a nylon membrane and hybridized using radiolabelled 12.9 kb *Eco* RI *hup* gene fragment of cosmid pHU52. The results are shown in plate VB.

In both the parent and mutant, *Eco* RI restricted DNA hybridized strongly with a band of 5.4 kb size (lanes 2,6). There were 3 more bands corresponding to approximate sizes of 6.8, 4.8 and 4.3 kb respectively. In case of *Hind* III restricted DNA, hybridization occured with a band of approximately 1.9 kb strongly (lanes 3,7) and two more bands of lesser intensity with approximate size of 13.5 kb and 9.6 kb. Restriction with *Eco* RI + *Hind* III (lanes 4,8) resulted in a band of 1.4 kb which hybridized strongly and two more bands corresponding to 9.6 kb and 4.8 kb which were very faint and diffused in both the parent strain and the mutant. Thus, it appears that hybridization pattern of the Hup$^-$ mutant B11 is similar to the parent strain IRBG 46.

4:11 RuBP carboxylase and uptake hydrogenase activities in IRBG 46 and B11

Az. caulinodans strain IRBG 46 and its Hup$^-$ mutant B11 were tested for RuBP carboxylase and H_2-uptake activities during growth conditions which induced the formation of hydrogenase system. Assay of RuBP carboxylase was done by measuring the RuBP dependent incorporation of $^{14}CO_2$ into acid stable product. The results are presented in Table 19.

In IRBG 46, RuBP carboxylase activity was 141 dpm/min/mg protein while that of uptake hydrogenase was 159 nmol H_2/min/mg protein. On the contrary mutant B11 showed very low RuBP carboxylase activity of 54 dpm/min/mg protein and no activity of uptake hydrogenase. These results thus suggest that there could be a relationship between the expression of RuBP carboxylase and uptake hydrogenase.

Table 19. **RuBP carboxylase and uptake hydrogenase activities of *Az. caulinodans* strains IRBG 46 and its Hup$^-$ mutant B11 in free-living conditions**

Strain/ mutant	Sp. uptake hydrogenase activity (nmol H_2/min/mg protein)	Sp. RuBP carboxylase activity (dpm/min/mg protein)
IRBG 46	159	141
B11	nd	54

nd = not detected

Chapter 5

DISCUSSION

Legume-*Rhizobium* symbiotic system utilizes energy produced from photosynthates to fix atmospheric dinitrogen gas. One characteristic of all the N_2-fixing organisms besides fixing N_2 is to catalyze reduction of protons to H_2 whereby a significant amount of energy is lost during the N_2-fixing process. The loss of energy as ATP and reducing power in soybean and other legume root nodules attributed to nitrogenase catalyzed H_2 evolution may be as high as 30-50% of the total energy flux through nitrogenase (Evans *et al.*, 1981).

Many N_2-fixing organisms are able to recycle the H_2 evolved by nitrogenase using a membrane bound uptake hydrogenase, thereby recovering some of the energy lost in the formation of H_2. Since H_2-recycling system has been detected in relatively few strains of rhizobia, study of additional strains for the presence of H_2-uptake (Hup) system and its determinants is needed. Considerable efforts towards the understanding of the organization of *hup* genes in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* have been made. But the efforts leading to an understanding of *hup* genes organization in *Azorhizobium caulinodans*, which forms nodules on roots as well as stem of *Sesbania rostrata*, are still at a preliminary stage.

The *hup* genes of *B. japonicum* can not be derepressed in the presence of high organic carbon (Maier *et al.*, 1979). In contrast to this, *hup* genes of *Az. caulinodans* are known to be derepressed in *ex planta* conditions in the presence of high organic carbon substrates (de Vries *et al.*, 1984). Thus, the relevance of the differences in the regulation of the Hup system could be demonstrated by isolation and characterization of genetic-determinants that are involved in the H_2-recycling process. It has been postulated that quantity and type of carbon substrates translocated to root nodules, partial pressure of O_2 within the nodules and the extent of H_2 evolution from the N_2-fixing process within bacteroids are the primary factors that may regulate the derepression of hydrogenase in the bacteroids, which possess the genetic potential for hydrogenase synthesis (Eisbrenner and Evans, 1983).

Since not much is known about the Hup system of *Azorhizobium* which is derepressed even in the presence of organic carbon substrates, the present investigation has been undertaken in *Az. caulinodans* strain IRBG 46 to study the factors affecting/regulating the expression of uptake hydrogenase and also to find out the relationship of the enzyme uptake hydrogenase with nitrogenase and RuBP carboxylase.

Developing Hup$^-$ mutant of *Az. caulinodans*

Mutants have been used earlier to study the biochemistry, regulation and genetics of hydrogen oxidation. Hup$^-$ mutants have been isolated using chemical mutagens like EMS in *R. japonicum* (Maier, 1981), NTG in *Azotobacter chroococcum* (Yates and Robson, 1985) and biological mutagens like transposon Tn5 in *R. japonicum* (Haugland *et al.*, 1984). These mutants have helped in understanding the structure and regulation of the genes involved. Hup$^-$ mutants have also been used in studying the regulation by O_2 which plays a key role in the symbiotic properties in *Rhizobium* spp.

As nitrosoguanidine (NTG) has been used earlier for isolating mutants of *A. chroococcum* defective in hydrogenase activity, in the present study also, this mutagen was used to isolate Hup$^-$ mutants of the strain IRBG 46. By following methylene blue reduction assay, only one Hup$^-$ mutant could be obtained on screening 1300 NTG mutants which was designated as B11. The Hup$^-$ character of the mutant thus obtained was further established by following uptake hydrogenase assay and chemoautotrophic growth.

The mutant B11 was complemented for hydrogenase activity by transferring cosmid pHU52 containing *R. japonicum* uptake hydrogenase (*hup*) genes. The transconjugant B11 (pHU52) showed very low activity (412 nmol H_2/h/mg protein) as compared to the parent strain IRBG 46 (11,128 nmol H_2/h/mg protein). There could be two possibilities for low Hup activity in the transconjugant : one is that the *hup* genes of *R. japonicum* and *Azorhizobium* may be structurally different with the result that complementation does not take place. Whatever Hup activity is observed could be a contribution of *hup* genes from cosmid pHU52 itself. Another possibility is that the low Hup activity could be due to the mutation occurring in regulatory gene(s) resulting in permanent repression of Hup system. These possibilities must await further characterization of the *hup* genes from *Az. caulinodans*. Yates and Robson (1985) also did not obtain any complementation when *hup* cosmid pHU1 was transferred to Hup$^-$ mutant strain MCD-124 of *Azotobacter chroococcum*.

Expression of RuBP carboxylase and uptake hydrogenase activity

The expression of RuBP carboxylase has been found to be linked with H_2 oxidation activity in free-living *R. japonicum*. Both the activities were found to be repressed in the presence of organic-C and were induced when cells were incubated in carbon-poor medium in an atmosphere containing H_2 and O_2 (Simpson *et al.*, 1979). In the present study also, *Azorhizobium caulinodans* strain IRBG 46 and its Hup^- mutant B11 were examined for RuBP carboxylase and H_2-uptake activities during growth conditions which induced formation of the hydrogenase system.

The Hup^- mutant B11 lacking H_2-oxidizing ability showed low RuBP carboxylase activity. On the other hand, the wild type Hup^+ strain expressed both uptake hydrogenase and RuBP carboxylase activities. Since the medium (HUM) contained very less amount of oxidizable organic substrates and the mutant lacked the ability to oxidize H_2, the mutant was probably energy deficient. Therefore, the low RuBP carboxylase activity could be due to lack of ATP to support synthesis of RuBP carboxylase rather than a specific defect in RuBP carboxylase related gene. Another reason for low RuBP carboxylase activity in Hup^- mutant B11 could be a mutation taking place in common regulatory gene(s), affecting expression of both Rubisco and *hup* genes. This study suggests a co-ordinate regulatory relationship between the two systems, as has been reported in *R. japonicum* (Simpson *et al.*, 1979).

The mutant B11 was also unable to grow chemoautotrophically when grown on Repaske medium in an atmosphere of 10% H_2, 5% CO_2, 1% O_2 and rest N_2. This inability of mutant strain to grow chemoautotrophically with H_2 and CO_2 can be correlated to a deficiency in the ability to oxidize H_2 to generate energy and also to incorporate CO_2.

Expression of *hup* genes in *Az. caulinodans*

For studying the expression of uptake hydrogenase activity, it is necessary that first the *hup* genes are derepressed under *ex planta* conditions so that the growth conditions can be easily manipulated. In this regard de Vries *et al.* (1984) provided the first report that in *Azorhizobium* ORS 571, the presence of an organic carbon source does not repress or inhibit hydrogenase synthesis/activity.

In the present study also, when the *Az. caulinodans* strain IRBG 46 was grown on high carbon medium like YEMA, high Hup activity (4670 nmol H_2/h/mg protein) was observed. Saini *et al.* (1987) also demonstrated Hup activity on high organic carbon media (YEMA and nitrogenase induction medium) in all 8 Hup^+ strains of *Rhizobium* sp. (*Sesbania*) studied. However, the Hup activity in IRBG 46 on YEMA was 8 to 15-fold higher as compared to the activity reported by Saini *et al.* (1987).

When the low carbon medium (HUM) was used, the Hup activity increased by 50% as compared to YEMA (see Table 7). The derepression of Hup activity on high carbon medium in this strain could possibly be due to the reason that the *hup* genes in this strain constitute an hydrogenase operon which is less sensitive to high organic carbon repression (Saini *et al.*, 1987). Another possibility could be that due to high respiratory activity, organic carbon substrates are utilised at a faster rate lowering the concentration of the substrate to an extent which allows derepression of *hup* genes.

Nitrogenase-hydrogenase relationships in *Az. caulinodans*

Nitrogenase and uptake hydrogenase activities have been found to occur concurrently at high levels in bacteroids of soybean nodules (Evans *et al.*, 1981). Low levels of Hup activities were reported in free-living N_2-fixing *R. japonicum* cultures. Maier *et al.* (1979) concluded that H_2, either added or produced by nitrogenase, was required to induce Hup activity.

In the present study with strain IRBG 46, it was found that expression of hydrogenase activity does not require addition of H_2 for derepression of *hup* genes. The hydrogenase expression in the samples which were derepressed in 10% H_2 was just 1.3-fold more than the expression obtained in the samples incubated without added H_2. Friedrich *et al.* (1984) also reported that in some H_2-oxidizing bacteria, H_2 is needed to induce hydrogenase, whereas in other hydrogenase synthesis occurs without added H_2.

Also, *ex planta* nitrogenase activity was observed in strain IRBG 46 and its Hup^- mutant B11 when tested on the medium used for derepression of *hup* genes under atmospheric level of oxygen. Earlier studies by Agarwal and Keister (1983) have shown that in *R. japonicum* nitrogenase activity is expressed in free-living conditions in microaerobic conditions when liquid

cultures were used. However, Pagan *et al.* (1975) observed nitrogenase activity in *Rhizobium* strain 32H1 on solid media under atmospheric oxygen levels. Hydrogen evolution is an inherent property of enzyme nitrogenase. In Hup$^-$ mutant B11, H_2 evolution was detected in free-living state. However, in case of strain IRBG 46, no H_2 evolution could be detected, as H_2 evolved by nitrogenase system is recycled by the efficient Hup system present in it. Thus in strain IRBG 46, H_2 produced via nitrogenase might be stimulating the expression of uptake hydrogenase. This explains why addition of H_2 was not required for derepression of *hup* genes in this strain of *Az. caulinodans*. Similar results were reported in *R. japonicum* by Graham *et al.* (1984) who observed H_2-oxidation activity in the nitrogenase induction medium without addition of H_2.

Since Hup$^-$ mutant B11 was found to contain nitrogenase activity, but lacks H_2-uptake activity it shows that these two activities are controlled by two different set of genes. Even in the absence of H_2-recycling ability nitrogenase expression in the mutant B11, was as high as obtained in its parent strain IRBG 46 suggesting thereby high tolerance to O_2 even in N_2-fixing mutant B11.

In order to evaluate the extent of energy loss as H_2 by Hup$^-$ mutant, a relative efficiency (RE) estimate as described by Schubert and Evans (1976) has been utilized in the present study. The RE estimate measures the fraction of total nitrogenase electron flux that is used in N_2 reduction. The RE value (see Table 18c) suggests that energy lost by B11 nodules in the evolution of H_2 is 31% and 28% at 53 and 60 DAS, respectively; the RE being 0.69 and 0.72, respectively. On the other side, RE in case of IRBG was 1.0 at both the stages tested showing thereby that all the H_2 evolved by nodule nitrogenase is recycled by efficient Hup system. Thimmaiah and Lodha (1986) also reported that rhizobial strains with an active Hup system were relatively more efficient than H_2-evolving Hup$^-$ strain in mungbean. More recently, in 1990, Vasudev *et al.* showed that transfer of *B. japonicum hup* genes into *Cicer*-rhizobia increased the relative efficiency of nitrogen fixation from 0.73 to 0.81 and 0.70 to 0.83 in two different strains.

When tested under free-living conditions, energy lost as H_2 by Hup$^-$ mutant B11 was 33%; the RE being 0.67. In case of strain IRBG 46 wherein *hup* genes are being expressed, there was no loss of energy in the form of

H_2 as shown by RE value of 1.0. Thus it can be said that H_2 evolution in the absence of a functional Hup system is a cause for energy loss in free-living as well as under symbiotic conditions. Moreover, this is the first ever report showing a comparable relative efficiency under symbiotic as well as *ex planta* conditions for both Hup^- as well as Hup^+ strains of *Az. caulinodans*.

Factors affecting the expression of uptake hydrogenase

It has been reported that low concentration of carbon substrates and low partial pressure of O_2 are required for the expression of high Hup activity in free-living cultures of *R. japonicum* (Maier *et al.*, 1978). A number of factors, for example, different C and N-sources, that regulate the expression of Hup system in free-living cultures of *R. japonicum* have also been investigated earlier by Maier *et al.* (1979). However, not much information is available on this aspect in *Az. caulinodans*. Therefore, during the present study, factors regulating the expression of uptake hydrogenase in free-living *Az. caulinodans* have been investigated as discussed below.

Effect of pH : The expression of uptake hydrogenase in strain IRBG 46 has been found to be significantly influenced by pH of the induction medium. The optimum pH for growth as well as expression of the enzyme was found to be 6.8. A slight shift in pH significantly decreased the Hup activity. It has also been found in *Bradyrhizobium japonicum* SR that acidification of induction medium with low buffering capacity (resulting from carbon substrate metabolism) inhibited Hup activity, whereas cell suspension in medium with adequate buffering capacity stimulated Hup expression (van Berkum and Maier, 1988).

Effect of oxygen : Since the partial pressure of oxygen in soil medium as well as within the bacteroid system may be one of the important factors which regulate the expression of uptake hydrogenase, a study was undertaken to investigate the effect of oxygen concentration on the expression of uptake hydrogenase.

The expression of uptake hydrogenase in strain IRBG 46 was found to be high at atmospheric level of oxygen as compared to 1% O_2 concentration. Similarly Hup^c mutants of *R. japonicum* were found to be insensitive to O_2 repression of uptake hydrogenase and these mutants produced upto five times more H_2-activating hydrogenase enzyme. In contrast to these results, an

increase in the inhibition on the expression of uptake hydrogenase with the increase in O_2 concentration has been observed in *R. japonicum* (Maier *et al.*, 1979) and mungbean *Rhizobium* (Thimmaiah *et al.*, 1986). However, in both IRBG 46 and mungbean *Rhizobium* there was a similarity with respect to increase in growth with increasing levels of oxygen.

Effect of carbon sources : It is known that sucrose is the major carbohydrate translocated from the leaves to the nodules where it is hydrolysed by nodule cytosol invertase into glucose and fructose which are utilised by the bacteroids. In this regard, in the present study, it is of interest to note that while fructose has got stimulatory effect (34%), glucose has an antagonistic effect on the expression of uptake hydrogenase in IRBG 46. However, sucrose was found to enhance the expression by 39%. When mannitol was used as a carbon source, the growth was reduced by 45% but there was no effect on the specific activity of the enzyme. Thimmaiah *et al.* (1986) also did not find any effect of mannitol on uptake hydrogenase expression in mungbean *Rhizobium*.

When glucose, Na-succinate, gluconate or glycerol (all at 15 mM) replaced Na-gluconate (2.24 mM) as a carbon source, growth of the strain IRBG 46 was supported well, but at the same time expression of uptake hydrogenase was inhibited by about 50%. When Na-gluconate in HUM was replaced by malate it showed the growth equivalent to that observed in standard HUM medium, but the expression was completely inhibited. The growth was inhibited completely in the presence of oxaloacetate or α-ketoglutarate.

The complete repression of hydrogenase expression by sodium gluconate, sodium citrate and glycerol (all at 15 mM) was also reported for *R. japonicum* strain USDA 122 (Maier *et al.*, 1979). The repression was also observed in mungbean *Rhizobium* (Thimmaiah *et al.*, 1986) when sodium gluconate, sodium citrate or glycerol (15 mM) were used as carbon sources. Lim and Shanmugan (1979), on the contrary, reported that 0.4% of these carbon sources did not completely repress the expression of hydrogenase in *R. japonicum* strain 110.

In the present study, it was observed that with increasing concentration of glucose from 5 mM to 25 mM, the inhibition on the expression of hydrogenase also increased from 32% to 56%. Also increasing concentration

of succinate (10 mM to 30 mM) increased the inhibition on the expression of enzyme from 49% to 72%. Similar results were obtained for Hup^c mutants of *R. japonicum,* When succinate concentration in the medium was increased from 1 mM to 10 mM, the per cent activity decreased from 76 to 48 (Merberg *et al.*, 1983). It can be speculated that glucose and succinate function as catabolite repressors in *Az. caulinodans* strain IRBG 46.

On the contrary, lack of carbon substrate repression of uptake hydrogenase activity has been reported in *Bradyrhizobium japonicum* SR (van Berkum and Maier, 1988). In this strain, the cell suspensions provided with adequate O_2 and buffering capacity showed the expression of uptake hydrogenase in the presence of carbon substrate. In the present study, the differences observed in the expression of hydrogenase with different carbon sources could also be due to differential changes in pH during metabolism of these substrates. This is supported by the observation in the present study that the expression of uptake hydrogenase in *Az. caulinodans* is highly sensitive to changes in pH.

Thus, it can be suggested that the expression of uptake hydrogenase in free-living *Azorhizobium* strain IRBG 46 depends on the type of carbon source present in the medium. Further, it appears that regulation of hydrogenase expression is complex and may prove to be important in the control of activity within the bacteroids where hydrogenase may play an important physiological role in nodule metabolism.

Effect of cyclic nucleotides : Cyclic nucleotides seem to play a role in the expression of both hydrogenase and nitrogenase in *R. japonicum.* Lim and Shanmugan (1979) showed that in *R. japonicum,* addition of cyclic AMP to a medium containing malate, overcame the repression of hydrogenase by malate.

In the present study, addition of cAMP (1.0 mM) failed to alleviate the repression caused by malate. There was very little (11%) increase in the enzyme activity when 1.0 mM cAMP was added to the standard induction medium containing malate (15 mM). This small increase could be because of the presence of cAMP alone rather than cAMP lowering the repression effect caused by malate. In mungbean *Rhizobium* when cyclic nucleotides alone were added to the standard induction medium, an increase in the

expression of enzyme was observed (Thimmaiah *et al.*, 1986). In Hup^c mutants of *R. japonicum* it was reported by Merberg *et al.* (1983) that the constitutive expression of hydrogenase was probably not due to a change in the levels of the two cyclic nucleotides, cAMP and ppGpp as there was no change in the levels of these cyclic nucleotides in the mutants when compared to wild type strains. On the contrary, in mungbean *Rhizobium* addition of cAMP/cGMP overcame the repression of uptake hydrogenase caused by glucose (Thimmaiah *et al.*, 1986).

Thus, it can be said that the regulation of hydrogenase via catabolite repression may prove to be complex and pool size of many metabolites may be involved.

Effect of combined N-sources : Since H_2 produced by nitrogenase system acts as an inducer of uptake hydrogenase expression, the factors regulating the expression of nitrogenase might indirectly regulate the expression of uptake hydrogenase. In addition, it is a well established fact that the presence of added nitrogen source in the growth medium is inhibitory to the expression of nitrogenase. Since the soil medium contains various types of combined N-sources and *Az. caulinodans* strain IRBG 46 express the nitrogenase and uptake hydrogenase activities under free-living conditions, an attempt has been made to study the effect of combined N-sources on the expression of uptake hydrogenase.

In the present study, addition of KNO_3 (10 mM) reduced the expression of the enzyme by 43%. Also the growth was reduced by 59% (see Table 13). The expression was not much affected in the presence of NH_4Cl, sodium glutamate and urea (all at 10 mM), although the growth was reduced by about 25%. On the other hand, NH_4NO_3 at 10 mM when replaced sodium glutamate (2.67 mM), inhibited the growth completely. However, when NH_4NO_3 was added to standard HUM at a concentration ranging from 2.5 mM to 10 mM, it allowed some growth to take place, but the expression of the enzyme took place only at 2.5 and 5 mM concentrations that too at a reduced level.

When KNO_3 at a concentration of 10 mM was added to standard HUM without replacing sodium glutamate, the expression of uptake hydrogenase was inhibited by nearly 50% and NH_4Cl at the same concentration did not show

any inhibitory effect on the expression (see Table 14). However, NH_4NO_3 even at a concentration of 2.5 mM showed 80% inhibition. This could be due to the synergistic effect of NH_4^+ and NO_3^- ions.

In different *Rhizobium* species, different responses to various nitrogen sources have been reported. In *R. japonicum* addition of NH_4Cl and KNO_3 did not effect the expression of hydrogenase (Maier *et al.*, 1979). In mungbean *Rhizobium*, all the N-sources tested (10 mM) were found to support the expression of hydrogenase (Thimmaiah *et al.*, 1986). Only NH_4Cl showed a stimulatory effect which was ascribed to secondary effects which act when there is no repression by carbon sources. However, Fukai *et al.* (1990) reported a stimulation in hydrogenase expression in free-living *B. japonicum*, when ammonium or nitrate was aded to the medium.

Localization of *hup* genes

Azorhizobium strain IRBG 46 used in the present investigation was found to contain a megaplasmid of approximately 280 kb size and another plasmid of smaller size. The presence of megaplasmid in *Azorhizobium* strain ORS 571, however, could never be demonstrated (Jarvis *et al.*, 1986).

In order to localize the presence of *hup* genes, it was thought worthwhile to hybridize both the genomic DNA and megaplasmid DNA with a 12.9 kb *hup* gene fragment from cosmid pHU52. Since both genomic as well as megaplasmid DNA were found to hybridize with the *hup* gene probe, there could be a possibility of the presence of multicopies of *hup* genes in *Az. caulinodans* strain IRBG 46. However, this observation can only be confirmed by curing the megaplasmid and then checking the presence of *hup* genes in the cured strain. In *R. japonicum*, the Hup determinants are chromosomal borne (Cantrell *et al.*, 1982), whereas in fast growing *R. leguminosarum* strain 128C53, H_2-recycling ability has been found to be plasmid encoded (Brewin *et al.*, 1980a).

As regards to the hybridization pattern of *hup* genes in IRBG 46 and its mutant B11, no observable difference could be detected. Nitrosoguanidine which is known to cause a point mutation was used for developing the mutant B11, the change of one or two bases might not have caused a major change

in the sequence. The difference in intensity of various bands could be due to the presence of some internal sites for restriction enzyme resulting in faint bands.

Thus, it can be concluded that *Azorhizobium caulinodans* strain IRBG 46 is a chemoautotroph which can utilize H_2 and CO_2 as sole source of energy and carbon, respectively. This strain is highly energy efficient both in free-living and symbiotic conditions, recycling all H_2 evolved by nitrogenase. On the other hand, the mutant B11 is unable to utilize any H_2. This study also demonstrates the presence of *hup* genes on the chromosome as well as on the megaplasmid. The study has further shown that free-living expression of uptake hydrogenase in IRBG 46 is regulated by many factors. Carbon sources (15 mM) like sucrose and fructose enhanced the expression, whereas malate completely repressed the enzyme activity. Other sources like glucose, gluconate and Na-succinate reduced the expression by 50%. Addition of cAMP failed to alleviate the repression brought about by malate. The expression of uptake hydrogenase was highly sensitive to pH changes but insensitive to high O_2 concentration. Among the combined N-sources tested, KNO_3 and NH_4NO_3 adversely affected the growth as well as the expression. Although the present study demonstrated the effect of various factors affecting the regulation, the mechanism by which the expression of uptake hydrogenase is regulated needs further investigation.

Chapter 6

SUMMARY AND CONCLUSIONS

Evolution of H_2 concomitant with the reduction of N_2 to NH_3, catalyzed by the enzyme nitrogenase, is a source of inefficiency in a legume-*Rhizobium* symbiotic system fixing atmospheric nitrogen. Some strains of rhizobia, however, possess an uptake hydrogenase (Hup) system recycling partly or whole of the H_2 evolved by the enzyme nitrogenase. The expression of uptake hydrogenase has been proposed to be under the control of many factors regulating its synthesis and activity. Some available reports show Hup system in *Azorhizbobium caulinodans* not to be repressed by high concentrations of organic carbon substrates. This is in contrast to all other Hup^+ rhizobia which show a repression of their Hup system in free-living cells. Therefore, during the present investigation, factors regulating the expression of uptake hydrogenase in *Az. caulinodans* have been studied. The significant findings of the present investigation are as summarised below :

(i) A H_2-uptake negative (Hup^-) mutant B11 of *Az. caulinodans* strain IRBG 46 was developed by nitrosoguanidine mutagenesis. This mutant was used to study the relationship of uptake hydrogenase with nitrogenase and RuBP carboxylase.

(ii) The *Az. caulinodans* strain IRBG 46 was assayed for *hup* expression under free-living conditions on low organic carbon containing HUM as well as medium containing high organic carbon (YEMA). The strain was found to exhibit very high specific activity in both the media tested though the specific activity was higher on HUM.

(iii) When tested for chemoautotrophic growth ability, IRBG 46 was found to grow very well, whereas B11 showed very less growth.

(iv) When cosmid pHU52 containing *hup* genes of *Bradyrhizobium japonicum* was transferred to Hup^- mutant B11, there was very little restoration of Hup activity in the transconjugant, B11 (pHU52).

(v) Addition of H_2 was not required for derepression of *hup* genes in IRBG 46 as this strain has a free-living N_2ase activity in the medium used for derepression of *hup* genes. H_2 produced via nitrogenase might be inducing the expression of uptake hydrogenase.

(vi) Studies on the H_2-recycling ability of *Az. caulinodans* strain IRBG 46 and its Hup^- mutant B11 have been carried out under *ex planta* as well as symbiotic conditions. Based on the relative efficiency (RE) estimate it was found that in Hup^- mutant B11 there was a significant loss of energy in H_2 evolution which was more or less equal under both the conditions, whereas strain IRBG 46 was able to recycle all the H_2 evolved via nitrogenase system.

(vii) The optimum pH for the expression of uptake hydrogenase was found to be 6.8. A slight decrease or increase in pH affected growth as well as expression of enzyme activity.

(viii) With the increase in O_2 concentration from 1% to 10% during incubation period, there was an increase in the expression of the enzyme activity and further increase in O_2 concentration did not affect the expression.

(ix) Complete removal of sodium gluconate (2.24 mM) as carbon source from the standard induction medium lowered expression of uptake hydrogenase by about 50% in free-living azorhizobial strain IRBG 46. Carbon sources (15 mM) like Na-citrate and malate did not allow the expression of uptake hydrogenase to take place. Glucose, gluconate and Na-succinate reduced the expression by 50% without any effect on growth. Sucrose and fructose increased the expression by about 40% and 35%, respectively. Mannitol and arabinose did not have much affect on the expression of the enzyme activity. Carbon sources like oxaloacetate and α-ketoglutarate completely inhibited the growth of the strain.

(x) With increasing concentration of glucose (5 - 25 mM) and Na-succinate (10 - 30 mM), the inhibition on the expression of hydrogenase also increased from 32% to 56% and 50% to 70% in case of glucose and succinate, respectively.

(xi) External addition of cAMP to the standard induction medium containing 15 mM malate stimulated the expression only to a very small extent (11%) suggesting thereby that cAMP is unable to alleviate repression brought about by malate.

(xii) Complete deletion of sodium glutamate (2.67 mM) from HUM decreased the growth as well as specific activity of the enzyme. Nitrogen-sources namely Na-glutamate, urea and NH_4Cl when added at a concentration of 10 mM did not effect the expression, whereas KNO_3 (10 mM) inhibited the expression by about 40%. NH_4NO_3 (10 mM) did not allow the growth to take place. Addition of lower concentrations (2.5 - 10 mM) of NH_4NO_3 to standard HUM were also inhibitory to a great extent with respect to growth as well as the expression of the enzyme activity.

(xiii) *Az. caulinodans* strain IRBG 46 revealed the presence of a megaplasmid of approximately 280 kb.

(xiv) Further experiments were performed to localize the *hup* genes present in this strain. For this a DNA : DNA dot blot hybridization of genomic DNA and southern hybridization of megaplasmid DNA was performed using a nick-translated 12.9 kb *hup* gene *Eco* RI fragment of pHU52 as probe. Both genomic as well as megaplasmid DNA gave a positive signal with the heterologous probe.

(xv) The Hup^- mutant B11 lacking H_2-oxidizing ability also showed low RuBP carboxylase activity, whereas the wild type Hup^+ strain IRBG 46 expressed both the activities at a high level.

It is concluded that *Az. caulinodans* strain IRBG 46 is highly energy efficient as compared to its Hup^- mutant B11 under both symbiotic as well as free-living conditions. Also, the study suggests the presence of multicopies of *hup* genes. The expression of uptake hydrogenase in free-living state is regulated by various factors such as carbon and nitrogen sources, pH and O_2 concentration. However, the understanding of regulatory mechanism needs further studies at molecular level.

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