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CONSTRUCTION OF A *RHIZOBIUM* sp. (*SESBANIA*) GENE BANK AND ISOLATION OF HYDROGEN UPTAKE GENES

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1992

CONSTRUCTION OF A *RHIZOBIUM* sp. (*SESBANIA*) GENE BANK AND ISOLATION OF HYDROGEN UPTAKE GENES

By

ANJALI MEHTA

A Thesis
submitted to 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

1992

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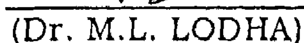

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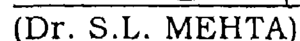

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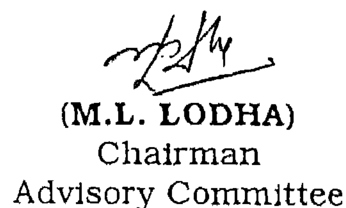
This is to certify that the thesis entitled, **Construction of a *Rhizobium* sp. (*Sesbania*) gene bank and isolation of hydrogen uptake genes** submitted in partial fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** of the Post-Graudate School, Indian Agricultural Research Institute, New Delhi, is a faithful record of the *bona fide* research work carried out by **Miss ANJALI MEHTA** under my guidance and supervision and no part of this thesis has been submitted for any other degree or diploma. The assistance and help received during the course of this investigation, has been duly acknowledged



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Dated : 9th September, 1992.

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge my heartfelt gratitude and deep appreciation for Dr. M.L. Lodha, Chairman of my Advisory Committee who has always furnished a congenial and stimulating work environment throughout the course of this study. My debts are due to him for his constant encouragement, thought provoking ideas and his ever sincere guidance and constructive criticism for the successful completion of this study.

My respectful acknowledgements are also due to Dr. S.L. Mehta, Head, Division of Biochemistry and other members of my Advisory Committee namely, Drs. N.D. Sharma, S.K. Kavimandan, P.B. Kirti and U.K. Sengupta for their valuable suggestions and cooperation during the course of my work.

I also owe my sincere thanks and gratefulness to Drs. S.P. Singh and K.R. Koundal, Biotechnology Centre for their invaluable guidance and indispensable assistance during the course of this study. But for their constant support, this study would have remained incomplete.

Sincere gratitude is extended to Dr. G.K. Barat, Professor, Division of Biochemistry, who has always been a source of strength and inspiration during the entire course of my study.

My thanks are also due to Dr. I.M. Santha, who has helped me in immeasurable ways.

My lab. colleagues, specially Kunni and Debasis need special mention, whose industrious help and moral support will forever be remembered by me.

My thanks are also due to my friends, Sandhya, Vibha, Sanghamitra and Umesh for their ever-lasting affection and tremendous help in immeasurable ways during the entire course of my work.

I gratefully acknowledge the help rendered to me by Shri Jai Singh during my lab. work.

Words cannot express my sentiments towards my parents who always helped to instil in me a strength of will throughout the course of my work.

Last, but not the least, the IARI Senior Research Fellowship awarded to me for my studies is gratefully acknowledged.

Anjali Mehta

Dated: 9 September, 1992

(ANJALI MEHTA)

CONTENTS

	CHAPTER	PAGE NO.
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	4
III.	MATERIALS AND METHODS	29
IV.	RESULTS	62
V.	DISCUSSION	75
VI.	SUMMARY AND CONCLUSION	85
	BIBLIOGRAPHY	i - xiii

LIST OF TABLES

TABLE No.	TITLE	PAGE No.
1.	Bacterial strains and plasmids used in this study	30
2.	Composition of Hoagland solution (Hoagland & Arnon, 1950)	34
3.	Composition of hydrogen-uptake medium (Maier *et al.*, 1978)	37
4.	Acetylene reduction, hydrogen evolution and relative efficiency in *Sesbania rostrata* nodules induced by *Sesbania* rhizobial isolates (30 day stage, Gibson tube experiment)	64
5.	Uptake hydrogenase activity of Hup$^+$ *Azorhizobium* strain IRBG-46 in different media under free-living conditions	65
6.	Antibiotic resistance and sensitivity profile of *Azorhizobium* strain IRBG-46	67
7.	Study on the expression of *hup* genes of plasmid cured and uncured *Azorhizobium* strain IRBG-46	69
8.	Uptake hydrogenase activity in trans-conjugants of *Cicer-Rhizobium* strain Rcd 301 under free-living conditions	74

LIST OF PLATES

PLATE No.	TITLE	BETWEEN PAGES
I.	Autoradiogram showing dot-blot hybridization of total DNA isolated from *Sesbania* rhizobial strains to nick-translated pHU52	66-67
II.	Megaplasmid profile of *Azorhizobium* strain IRBG-46 following *in situ* lysis on 0.4% agarose gel	68-69
III.	Electrophoretic analysis of small plasmid(s) of strain IRBG-46 on 0.7% agarose gel	68-69
IV.	Partial *Eco*RI digest of genomic DNA of strain IRBG-46 on 0.4% agarose gel	70-71
VA.	Electrophoretic analysis of fractions obtained after sucrose-density gradient centrifugation on 0.4% agarose gel	70-71
VB.	Vector and insert DNA on a 1% agarose gel	71-72
VI.	Restriction analysis of random clones from the gene bank	71-72
VII-XIV.	Autoradiograms showing colony hybridization of clones from gene bank using oligo-labelled 12.9 kb *Eco*RI fragment of pHU52 as probe	72-73
XV-XVI.	Restriction analysis of 13 clones	72-73
XVII.	Autoradiogram showing secondary screening of 5 positive clones	72-73
XVIII.	Transconjugants of *Cicer-Rhizobium* strain Rcd 301 containing recombinant plasmids, pSRH when grown on RMM-Tc	73-74

ABBREVIATIONS

ATP	-	Adenosine triphosphate
BSA	-	Bovine serum albumin
CIP	-	Calf intestine alkaline phosphatase
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribose nucleic acid
DTT	-	Dithiothreitol
EDTA	-	Ethylene diamine tetra acetic acid
HUM	-	Hydrogen uptake medium
hup	-	H_2-uptake gene
Hup^-	-	Hydrogen uptake negative
Hup^+	-	Hydrogen uptake positive
Hup^c	-	Hydrogen uptake constitutive
LB	-	Luria-Bertani
MSS	-	Mannitol salt solution
PEG	-	Polyethylene glycol
RE	-	Relative efficiency
RMM	-	Rhizobial minimal medium
SDS	-	Sodium dodecyl sulphate
SSC	-	Saline sodium citrate
TBE	-	Tris Borate EDTA
TCA	-	Tri-chloro acetic acid
TE	-	Tris EDTA
TY	-	Tryptone-yeast extract
TYM	-	Tryptone-yeast extract mannitol
YELA	-	Yeast extract lactate agar
YEMA	-	Yeast extract mannitol agar

INTRODUCTION

Nitrogen from the atmosphere can be fixed abiologically as well as biologically. Biological nitrogen fixation is much more important as it contributes to more than 70% of the input into the world's soil and water nitrogen. About 100 to 175 million tonnes of nitrogen are fixed annually by the process of biological N_2-fixation, and out of this, 50% is contributed by the legume-*Rhizobium* symbiosis.

Though nitrogenases from all known sources catalyze an ATP-dependent reduction of not only N_2 to NH_4^+ but also protons to H_2. This H_2 loss results in an inefficient use of the energy provided by the organism to the N_2-fixing process, as a majority of the nodulated legumes lose 40-60% of their nitrogenase electron flux as hydrogen (Schubert and Evans, 1976).

Many aerobic N_2-fixing bacteria, including some rhizobia are capable of synthesizing a hydrogenase system that oxidizes H_2 generated by the nitrogenase complex during the N_2 fixation process. Among these rhizobia, H_2-uptake hydrogenase positive (Hup^+) strains have been found in species of the three genera, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (Brewin, 1984; Stam *et al.*, 1984). Several potential advantages of the H_2-uptake (Hup) system have been demonstrated. These advantages include generation of energy for ATP synthesis, to provide reductant and to protect nitrogenase from O_2 inactivation. These advantages could help *Rhizobium*-legume symbiotic system fix more N_2 and produce more dry matter, and a consistent positive effect of the Hup system on overall plant productivity of legumes has been shown.

Assuming that H_2 recycling benefits legumes, it is desirable to transfer H_2-uptake (*hup*) genes to rhizobia which lack H_2-uptake capacity. Such transfers require the isolation of all *hup* genes from a strain with an efficient H_2-recycling system. Moreover, not much is known about the regulation of hydrogenase in *Rhizobium*. Maier *et al.* (1978) described the conditions required for the expression of hydrogenase in free-living *R. japonicum*. It has been suggested that high concentrations of carbon substrates and high O_2 partial pressures repress the hydrogenase synthesis/activity in Hup^+ rhizobia. But, recent reports on *Sesbania* rhizobia and azorhizobia have provided evidence showing that these rhizobia, unlike all others can be derepressed for Hup activity in the presence of high organic carbon under free-living conditions (de Vries *et al.*, 1984; Saini *et al.*, 1987).

A study of such naturally occurring rhizobial spp. insensitive to carbon substrate repression could be of potential agricultural significance, since these species as bacteroids could synthesize large amounts of hydrogenase. Such *Rhizobium* strains should also be useful in answering some basic questions concerning the regulatory aspects of hydrogenase synthesis and activity in *Rhizobium*.

Attempts to transfer *hup* genes from *R. japonicum* and *R. leguminosarum*, which are normally repressed by high concentrations of carbon substrates, to *Cicer*-rhizobia have been made. These *hup* gene transfers involving transfer of cosmid pHU52 containing Hup-determinants to Hup^- *Cicer*-rhizobia have been successful in conferring

a Hup$^+$ phenotype in the latter (Vasudev *et al.*, 1990; Kunnimalaiyaan *et al.*, 1992). However, no efforts have been made to transfer *hup* genes from Hup$^+$ azorhizobia to other Hup$^-$ rhizobia and to study the regulatory parameters governing the expression of these genes.

Thus, the present study was undertaken with the following objectives in view :

1. To construct a gene bank of *Sesbania*-Hup$^+$ *Azorhizobium* strain IRBG-46 and isolate H_2-uptake (*hup*) genes.

2. To transfer isolated *hup* genes into a Hup$^-$ *Cicer-Rhizobium* strain.

REVIEW OF LITERATURE

Nitrogenases from different sources exhibit ATP-dependent formation of both NH_4^+ and H_2. Many N_2-fixing microorganisms are able to recycle the nitrogenase mediated H_2 evolution using a membrane bound hydrogenase, thereby recovering some of the energy utilized in the formation of H_2 (Lambert *et al.*, 1985).

Improvement of legume productivity by increasing the N_2-fixing capability of *Rhizobium* species has become a major research objective. In order to realize 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 the uptake hydrogenase system and advantages conferred by H_2 oxidation in Hup^+ rhizobia in plants have been reviewed. In addition, progress in isolation, transfer and expression of *hup* genes both in free-living and symbiotic conditions in different species of rhizobia as well as the regulation of hydrogenase synthesis and activity have also been briefly reviewed.

2.1 Hydrogen evolution and recycling

The presence of a hydrogenase system was first reported by Phelps and Wilson in 1941 in nodules of pea formed by strain 311 of *Rhizobium leguminosarum*. This report was confirmed more than 25

years later by Dixon in 1967.

Hoch *et al.* (1957, 1960) discovered that N_2-fixing soybean nodules evolved H_2 and also concluded that the N_2 reduction and H_2 evolution reactions may be catalyzed by the same enzyme. Bulen *et al.* (1965) demonstrated H_2 evolution 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.

In some H_2-oxidizing bacteria H_2 is needed to induce hydrogenase, whereas in others, hydrogenase synthesis occurs without H_2 (Aragno and Schlegel, 1978; Friedrich *et al.*, 1984; Robson and Postgate, 1980; Bowien and Schlegel, 1981).

The magnitude of H_2 evolution in leguminous and non-leguminous nodules has been surveyed by Schubert and Evans (1976). They found only very few strains which evolved little or no H_2 which had been attributed to an uptake hydrogenase. For reasons not clear, only a minority of most species of *Rhizobium* express H_2 recycling capability. 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).

It is now well known that one of the main causes of inefficiency in the *Rhizobium*-legume symbiosis is the energy loss by H_2 evolution from the nitrogenase-catalyzed reaction in nodules. 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 systems (Ruiz-Argueso *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, fast-growing strains of cowpea rhizobia have also been found (Trinick, 1982). Isolates of urd bean (*Vigna mungo*) *Rhizobium* were tested for the presence of an Hup system using triphenyl tetrazolium chloride reduction as the screening procedure (Pahwa and Dogra, 1983). Hup^+, Hup^- strains and Hup^- mutants were compared on the basis of their effect on dry matter accumulation and N_2 fixation. Plants inoculated with Hup^+ strains produced higher N_2-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 role 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*.

2.2 Advantages of the Hup system

Several leguminous plant species form N_2-fixing symbioses. The bacteroids produce nitrogenase for the reduction of N_2 to ammonia. An inefficient property of the nitrogenase-catalyzed reaction is the

reduction of protons to H_2, which utilizes ATP (Eisbrenner and Evans, 1983; Maier, 1986).

The efficiency of the symbiosis is improved with uptake hydrogenase (Hup) 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 hydrogen 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.

Though Dixon (1972) has proposed several potential benefits of H_2 recycling to the N_2-fixing process, it was Hyndman *et al.* (1953) who had already shown that H_2 supported ATP synthesis by membrane particles from *Azotobacter vinelandii*.

Hydrogen recycling within a legume nodule leads to increases in N_2 fixation and growth is based primarily upon the premise that the supply of respiratory substrates to nodules may be limiting (Hardy and Havelka, 1975; Bethlenfalvay and Phillips, 1977). Since 25% or more of the ATP consumption in the N_2 fixation reaction is associated with H_2 evolution, recovery of a portion of the expended energy through an H_2 oxidation process should lead to increases in N_2 fixation and growth if the energy supply to nodules limits nitrogen fixation. In addition to

conservation of energy, Dixon (1972) has proposed that a functional H_2 oxidation system might be beneficial by :

a) removing O_2 from the nitrogenase environment thus decreasing the possibility of O_2 damage; and

b) removing H_2 as a product of N_2 fixation thereby preventing inhibition of nitrogenase by H_2.

More recently, Dixon and Blunden (1981) 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. 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.*, 1985). Earlier, several experiments to support Dixon's view of H_2-dependent synthesis of ATP have also been demonstrated with bacteroid preparations of *R. leguminosarum* and with *B. japonicum* bacteroids (Emerich *et al.*, 1979).

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). Uptake hydrogenase system in fast growing strains of *Rhizobium* sp. (*Sesbania*) was analyzed 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.

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, it has been shown by Vasudev *et al.* (1990) 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. Kunnimalaiyaan and Lodha (1992) reported that integration of the cosmid pHU52 into the *Cicer*-rhizobial genome significantly improved the RE of symbiotic N_2 fixation by imparting H_2-recycling capability to these Hup⁻ *Cicer*–rhizobial strains.

2.3 Purification of the enzyme hydrogenase

The hydrogenase from *R. japonicum* bacteroids was first reported to be a monomer with a molecular weight of about 65,000 (Arp and Burris, 1979). 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.*, 1986) all revealed that the enzyme contains two subunits with molecular weights of approximately 65,000 and 35,000. 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 by sucrose density gradient centrifugation to be 104,000 (Arp, 1985).

Antibodies raised against each of the two subunits did not cross-react with the other subunit, showing the 35 kDa subunit not to be a degradation product of the larger subunit (Harker *et al.*, 1984; Stults *et al.*, 1986). Zuber *et al.* (1986) have identified the structural genes for the components of *B. japonicum* uptake hydrogenase (Mw 60,000 and 30,000) by expressing these genes in *E. coli* and showing the products to cross-react with antibodies to the respective hydrogenase subunits.

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 (Leyva *et al.*, 1990), a dimeric ($\alpha\beta$) 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.*, 1986), *A. vinelandii* (Seefeldt and Arp, 1986) or *Rhodobacter capsulatus* (Seefeldt *et al.*, 1987) is being expected.

2:4 Regulation of hydrogenase synthesis and activity

The genetic and environmental factors influencing the expression of the hydrogen uptake (Hup) system of rhizobia have been analyzed. It was first shown by Maier *et al.* (1978) that in free-living cells, carbon substrates and oxygen prevent formation of the Hup system, whereas H_2 and CO_2 stimulate hydrogenase synthesis. 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. Schlegel *et al.* (1972) have also earlier shown that in some organisms, ample supply of organic carbon substrates and high O_2 concentration in the medium were also detrimental to hydrogenase expression.

Experiments done by Maier *et al.* (1978) also 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. This suggested that the inhibitory effect of oxygen on hydrogenase expression was probably due to repression of hydrogenase synthesis rather than

inactivation of preformed hydrogenase.

In an effort to answer some basic questions concerning the regulatory role of O_2 in *Rhizobium*, Merberg and Maier (1983) isolated mutant strains of *R. japonicum* that express H_2 oxidizing activity in the presence of high concentrations of O_2. These mutants were shown to have increased hydrogenase activity when harvested from soybean nodules. Further experiments also indicated that the mutants were also partially relieved of the normal repression of hydrogenase by carbon substrates. Since the mutant strains having higher hydrogenase activity in culture, also have higher activity in nodules, suggesting thereby that common elements might be involved in regulating the Hup system in free-living cells and bacteroids. These mutants selected for the ability to express hydrogenase in 10.0% partial pressure of O_2 were also less sensitive than the wild type to repression by carbon substrates such as arabinose, glycerol, gluconate and succinate.

Merberg *et al.* (1983) also revealed by 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. One of these peptides is presumed by them to be the hydrogen-activating enzyme; other peptides may be involved in regulation of hydrogenase. This observation is consistent with a hypothesis that the gene affected in the Hup^c mutant controls the synthesis of several enzymes. However, the concentrations of cyclic AMP and guanosine tetraphosphate were similar in strain SR and the Hup^c mutants during heterotrophic growth, 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 *et al.*, 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). They also showed that N_2-fixing cultures of *Rhizobium* ORS 571 contained considerable hydrogenase activity, and for the induction of hydrogenase in ammonia-assimilating cultures, addition of H_2 to the gas phase was 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 hydrogenase synthesis/activity. In this respect *Rhizobium* ORS 571 differs from *R. japonicum* in which the synthesis of hydrogenase is repressed by carbon substrates.

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) suggest nitrogen-fixation in nodules to be limited by the supply of carbohydrates, recent studies by Larue *et al.* (1984) suggest the condition of the bacteroids in the nodule to be oxygen-limited.

The conditions necessary for coordinate derepression of nitrogenase and O_2-dependent hydrogenase activities in free-living cultures of *R. japonicum* were studied by Graham *et al.* (1984). A positive correlation was observed 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 are 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 (LOKG), but nitrogenase activity was no greater than the wild-type in these Hup^c mutants. Therefore, the regulatory factor(s) in the Hup^c mutants causing increased hydrogenase, activity, do not correspondingly affect nitrogenase activity.

Expression of uptake hydrogenase activity in free-living cultures of mungbean *Rhizobium* strain S-24 has been studied by Thimmaiah *et al.* (1986). The results of their experiments also indicated that the expression of hydrogenase in the *Rhizobium* strain was greatly influenced by the quality and quantity of carbon source present in the H_2-uptake medium for its growth. Carbon sources like ribose, sodium citrate, glycerol and sodium succinate at 15 mM concentration were found to enhance the growth of the strain, but completely repressed the expression of the enzyme. The enhancement in the expression of the enzyme was maximum with sorbitol and fructose (~55%) followed by arabinose and sucrose (~22%). With increasing concentration of glucose from 5 to 25 mM, inhibition on the expression of the enzyme also increased. The repression effect (64%) brought about by 25 mM glucose was reversed by addition of both cAMP or cGMP. 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 of Lim and Shanmugam (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.

More recent studies by Saini *et al.* (1987) on the uptake hydrogenase system of *Rhizobium* sp. (*Sesbania*) have shown derepression of uptake hydrogenase in both poor as well as in rich carbon media in all isolated Hup^+ strains. Eight strains which were found to express *ex planta* hydrogenase on the H_2-uptake medium of Maier *et al.* (1978) also expressed uptake hydrogenase activity on media containing high carbon, viz nitrogenase induction medium (Dadarwal *et al.*, 1981) and YEMA medium. 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 also sufficiently high (30 - 70 μmol/h/g fresh nodules) as compared with that in pea nodules (0.2 - 0.8 μmol/h/g fresh nodules) and soybean nodules (3.7 - 6.7 μmol/h/g fresh nodules) (Ruiz-Argueso *et al.*, 1978; Hanus *et al.*, 1981). Thus there could be a possibility of these strains being less sensitive to high carbon repression.

Mechanisms by which carbon substrates repress Hup activity of cell suspensions were shown by van Berkum and Maier (1988) to be

changes in pH of the induction medium or limitations in the supply of O_2 to the cells caused by carbon substrate metabolism. In this study, expression of *ex planta* Hup activity in *Bradyrhizobium 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 activity 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).

More recent views indicate that certain genes which are regulated in response to environmental changes show altered expression that is dependent on DNA tertiary structure. A link has been demonstrated between some genes that are expressed anaerobically and gyrase activity

in different bacteria-like *E. coli* (Axley and Stadtman, 1988), *Salmonella typhimurium* (Yamamoto and Droffner, 1985), *Klebsiella pneumoniae* (Kranz and Haselkorn, 1986), in *B. japonicum* (Novak and Maier, 1987) and in *A. caulinodans* ORS 571 (Ratet *et al.*, 1988).

Synthesis of the uptake hydrogenase of *B. japonicum* is repressed by high levels of oxygen (Maier *et al.*, 1979; vanBerkum, 1987), and the synthesis of the enzyme has been shown to be prevented by inhibitors of DNA gyrase (Novak and Maier, 1987). But, mutants of *B. japonicum* SR have been described in which hydrogenase is expressed constitutively even under high-level oxygen conditions (Merberg and Maier, 1983; Merberg *et al.*, 1983).

It has been further shown that in the Hup^c mutants of *B. japonicum* SR, regulation of expression of hydrogenase is altered; the mutants synthesize hydrogenase constitutively in the presence of atmospheric levels of oxygen (Novak and Maier, 1989). In contrast to the wild type, the Hup^c mutants synthesized hydrogenase in the presence of high levels of DNA gyrase inhibitors-nalidixic acid, novobiocin, and coumermycin.

This alteration in regulation has been tied to alterations in gyrase activity or in the dependence of the cell on gyrase activity, both of which are, in turn, tied to the degree of supercoiling of the DNA. They suggest that in the mutants, an alteration probably occurred in a regulatory element, so that a normal environmental signal displayed an altered regulatory capacity.

Differential expression of uptake hydrogenase activity because of host influence has been recently demonstrated (van Berkum, 1990). Their 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 hydrogen oxidation capability only in symbiosis with specific host plants and were also not capable of having hydrogen 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 hydrogen uptake host-regulated (Hup-hr) phenotype has been suggested to describe strains belonging to this third class of hydrogen-oxidizing bradyrhizobia.

2:5 Localization of Hup determinants in Rhizobia

2:5:1 Hup determinants in slow-growing rhizobia

Although large plasmids have been detected in *R. japonicum*, 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 $49\text{-}290 \times 10^6$. Thus the Hup determinants whenever present seem to be chromosomally encoded.

2:5:2 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 al.*, 1985). Brewin *et al.* (1980b) reported that determinants for hydrogenase activity (*hup*) in a particular strain of *R. leguminosarum* 128C53 to be located on the non-transmissible plasmid pRL6J1 of molecular weight 19×10^7 and to be 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 and 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.

2:5:3 Hup determinants in Azorhizobia

Not much is known about the detailed genetic organization of *hup* determinants in azorhizobia. de Vries *et al.* (1988) have isolated four Tn5-induced Hup^- mutants of *A. caulinodans* ORS 571. The Tn5 insertions in these Hup^- strains have recently been found to map in the same region of the ORS 571 genome (upublished work of Hilgert *et al.* as cited by deVries *et al.*, 1988), which shares a high degree of DNA

homology with the *hup* locus of *R. leguminosarum*, described by Tichy *et al.* (1987).

2:5:4 Transfer of plasmid-encoded Hup determinants into Hup⁻ rhizobia

Since H_2 recycling capability is a desirable trait in a species of *Rhizobium* as far as legume productivity is concerned, it is desirable to transfer H_2-uptake (*hup*) genes to species of *Rhizobium* which lack this ability. Such gene transfer requires the isolation of all *hup* genes from a strain with an efficient H_2 recycling system (Lambert *et al.*, 1985). Since transfer of Hup activity can be facilitated if H_2-uptake determinants are located on plasmids, several experiments have been performed on these lines.

DeJong *et al.* (1982) reported effects of transfer of plasmid pIJ1008 (a recombinant of pVW5JI and pRL6JI) into several Hup⁻ *R. leguminosarum* strains. Since it carries *hup* determinants, its transfer produced strains with significantly better symbiotic properties. Ruiz-Argueso *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, but 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. melilotl* strain. These strains carrying both plasmids nodulated alfalfa but not pea. Reisolation and examination of the strains from alfalfa nodule tissue showed that pIJ1008 continued to be maintained but that the pea-nodulation ability was suppressed. In three separate plant growth experiments by Bekhi *et al.* (1985), alfalfa nodules induced by each of the *R. meliloti* strains carrying both *sym* plasmids were assayed for Hup activity. Though the activity was found to be 40-, 3.5- and 2 fold higher than the respective pIJ1008 free strains, this higher activity was not accompanied by an increase in plant biomass or nitrogen content of shoots.

Contrasting reports of the effect of transfer of *hup* genes on improvement of symbiotic efficiency has been provided (Sorenson and Wyndaele, 1986). Plasmid-encoded symbiotic determinants from the *R. leguminosarum* strain MA1 (817) with Hup activity and from the Hup^- strain MC1 (18a) were mobilized by recombination with the self-transmissible plasmid pVW5J1. The symbiotic determinants were transferred by conjugation from strain MA1 to strain MC1 and to a derivative of MC1 without the symbiotic plasmid, and vice versa, thus constructing four types of transconjugants. Strain MC1 was found to fix 60% more N_2 in pea root nodules determined as mg nitrogen per plant than strain MA1. The difference was not increased in the MC1 derivative that obtained hydrogenase activity. Plants inoculated with a derivative of strain MA1, however, where the symbiotic plasmid was replaced by that of strain MC1 had a higher percentage nitrogen content.

Conjugal transfer of plasmid pIJ1008 from *R. leguminosarum* to two Hup$^-$ *Cicer*-rhizobial strains G36-84 and BG4 has been reported by Vasudev *et al.* (1991). This was shown to confer Hup activity in the free-living state as well as under symbiotic conditions thereby improving the RE of N_2 fixation in the mentioned strains.

Transfer of a plasmid from Hup$^+$ Nod$^-$ *R. leguminosarum* strain Vm2 to Hup$^-$ Nod$^+$ strain Vm1 resulted into a Hup$^+$ Nod$^+$ transconjugant strain Vc4 (Garg *et al.*, 1990). The strain Vc4 showed Hup activity both *ex planta* and in nodules. Results obtained 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, and comparable to the parent Hup$^+$ field isolate Vp1. They also suggested that the improved symbiotic performance on transfer of pIJ1008 could not be solely attributed to Hup alone, since it carries other symbiotic traits as well. In contrast, in strains 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. Thus, 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) were transferred to two Hup$^-$ strains, viz. G36-84 and BG4 of *Cicer*-rhizobia by conjugation (Vasudev *et al.*, 1990). Transconjugants obtained after transfer of *Hup* complementing cosmid pHU52 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*. However, cosmids like pHU52 are beset with the problem of instability in *Rhizobium* (Hom *et al.*, 1985; Vasudev, 1989). Thus, the *hup* gene cosmid pHU52 was integrated into the chromosome of a Hup^- *Cicer-Rhizobium* strain G36-84 by using transposon Tn5 as an homologous sequence between them (Kunnimalaiyaan *et al.*, 1992). Functional characterization of the final recombinants has already demonstrated uptake hydrogenase activity in free-living cells and *hup* stability in nodules (Kunnimalaiyaan and Lodha, 1992).

2:6 Isolation of *hup* genes

A gene bank of *R. japonicum* 122 DES DNA was constructed by using the broad host range conjugative cosmid pLAFR1 (Cantrell *et al.*, 1983). A series of cosmids containing H_2-uptake (*hup*) genes was identified by transferring the gene bank into a H_2 uptake-negative (Hup^-) *R. japonicum* point mutant (pJ17nal) and screening tetracycline resistant colonies for the ability to grow chemolithotrophically and to reduce methylene blue in a colony assay method devised by Haugland *et al.* (1983). Though all the pJ17nal transconjugants were Hup^+, the frequency of Hup^+ transconjugants with another point mutant, PJ18 nal was approximately 10^{-3}. These results indicated the *hup* gene cosmid to contain one gene and a portion of another by their ability to complement

the Hup⁻ derivative of 122 DES PJ17 nal. Haugland *et al.* (1984) then characterized several of these cosmids with respect to organization of all the *hup* sequences within them as well as their stability and influence on Hup activity in bacteroids from soybean and alfalfa nodules. Transposon Tn5 insertions were made into one of the recombinant cosmids, pHU1 using the strategy of Ruvkin and Ausubel (1981) to demonstrate the *hup*-specific sequences spanning a region of about 15 kb.

Acquisition of pHU1 by Hup⁻ wild type *B. japonicum* and *R. meliloti* strains did not confer Hup activity in the free-living state. Lambert *et al.* (1985a) isolated an additional *hup* cosmid from the gene bank by complementation of one of the Tn5 generated Hup⁻ mutants. One of the new cosmids isolated, pHU52, was similar to pHU1 except that it possessed a 5.5 kb *Eco*RI fragment at the right hand end. Conjugal transfer of pHU52, in contrast to that of pHU1, conferred Hup activity in all Hup⁻ *B. japonicum* strains. Similarly conjugal transfer of pHU52 conferred Hup activity in Hup⁻ chickpea rhizobia in free-living as well as symbiotic condition (Vasudev *et al.* 1990).

The cosmid pHU52 also conferred Hup activity and autotrophic growth capability to Hup⁻ *R. meliloti* and *R. leguminosarum*. Conjugal transfer of pHU52 into these Hup⁻ rhizobia also conferred the ability to synthesize both the 60 and 30 kDa polypeptide components of the uptake hydrogenase (Harker *et al.*, 1985). These results suggested that all the determinants for hydrogenase subunit synthesis are present on pHU52.

Direct evidence for the presence of uptake hydrogenase structural genes (60 and 30 kDa) on the Hup-complementing cosmid pHU52 has been provided by Zuber *et al.* (1986). The strategy involved expressing these genes in *E. coli* and then showing immunological cross-reaction with antibodies to the respective hydrogenase subunits. The protein synthesized from the insert DNA of pHU52 in *E. coli* maxicells, was used to identify the clones that direct the synthesis of 60-and 30-kDa polypeptides in maxicells, to maximize their expression in *E. coli*, and then to show immunological cross-reaction. A transcriptional fusion vector, pMZ545 was constructed and used for this purpose. A 5.9 kb *Hin*dIII fragment and an adjacent 2.9 kb *Eco*RI fragment (located distal to the 5.9 kb *Hin*dIII fragment) directed the synthesis of 60 and 30 kDa proteins, respectively in *E. coli* maxicells.

To examine the contribution of the Hup phenotype of other rhizobia to plant N_2 fixation and growth has become essential. Thus, by using DNA from pHU1 as a hybridization probe, Nelson *et al.* (1985) found homology between *hup*-specific DNA of *B. japonicum* and genomic DNA from some Hup^+ *R. leguminosarum* strains but not from others. Thus they suggested the existence of two different types of *hup* sequences within Hup^+ strains of *R. leguminosarum*. But, later Leyva *et al.* (1987a) showed that all the strains of *R. leguminosarum* that induced significant hydrogenase activity in pea bacteroids contained DNA sequences homologous to *B. japonicum* 122 DES and that its organization is highly conserved within Hup^+ strains in this symbiotic species. In all instances the putative *hup* sequences were located on a plasmid that also contained

nif genes, the resulting *sym* plasmids ranging between 184-212 megadaltons.

The *hup* genes of *R. leguminosarum* strain 128C53 were also isolated by screening a gene library with *B. japonicum hup*-specific DNA probes (Leyva *et al.*, 1987b). Subsequently, the *hup* cluster from *R. leguminosarum* in recombinant cosmid pAL618 has been physically mapped by Tn5 insertions (Palacios *et al.*, 1988).

Further evidence was presented by Leyva *et al.* (1990) that pAL618 contains the entire *hup* DNA region of *R. leguminosarum* and that *hup* genes within this region are organized in six transcriptional units, designated *hup* I to *hup* VI. The large subunit of hydrogenase has been specifically localized to region *hup* I. To study *hup* gene organization, site directed transposon mutagenesis and complementation analysis were carried out. According to the Hup phenotype associated with the transposon insertions, *hup* genes were found to span a region of 15 kb in their pAL618 insert DNA. Complementation analysis revealed that Hup^- mutants fall into six distinct complementation groups that define six transcriptional units, designated regions *hup* I to *hup* VI. Region *hup* I was further subcloned by them and expressed in *E. coli* cells which produced a polypeptide of Ca. 65kDa that was cross-reactive with antiserum against the large subunit of *B. japonicum* hydrogenase. It was detected both in *E. coli* cells carrying the cloned *hup* I region and in pea bacteroids from strain UPM 791 indicating that region *hup* I codes for structural genes of *R. leguminosarum* hydrogenase.

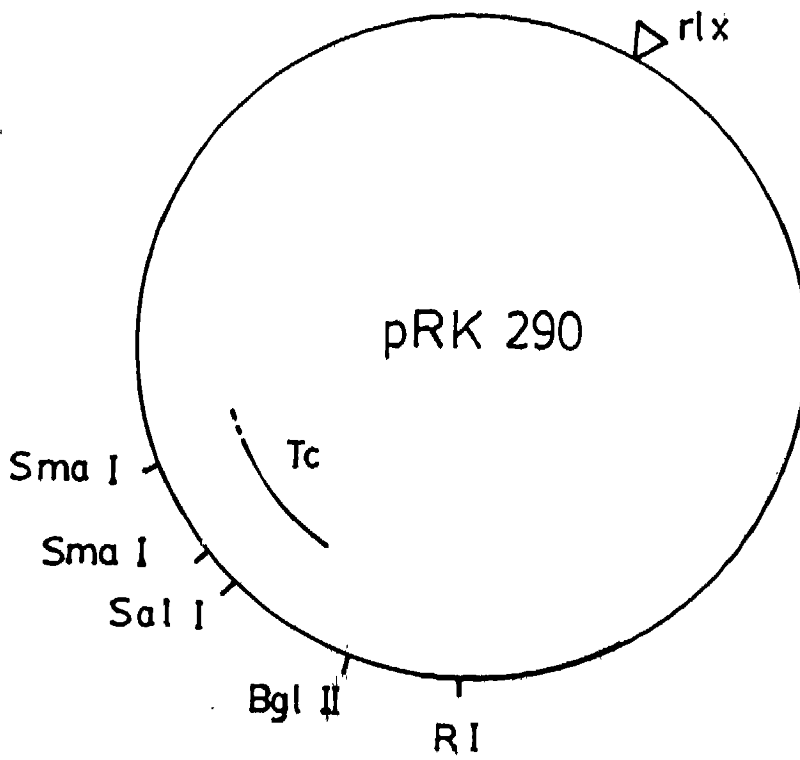

FIG. 1

2:7 pRK 290 - A broad host range DNA cloning system for gram-negative bacteria

The large size of native RK2 DNA (56 kb) is a serious drawback to its use as a plasmid cloning system in *E. coli*. In order to reduce the size and still retain overall broad host range transfer capability, a cloning system has been devised that separates RK2 transfer and replication functions onto separate plasmids (Ditta *et al.*,1980). The tetracycline-resistant plasmid component of this system pRK290, contains a functional RK2 replicon and can be mobilized at high frequency by using a helper plasmid, but is non-self transmissible. pRK290 contains single *Eco*RI and *Bgl*II sites, either of which can be used for insertion of foreign DNA without loss of essential functions (Fig. 1).

The kanamycin-resistant helper plasmid, pRK2013, consists of the RK2 transfer genes cloned onto a Col E1 replicon and its sole function is to trans-complement the vector for mobilization.

The plasmid pRK290 has been used for construction of gene banks of various bacterial spp. Ditta *et al.* (1980) constructed a gene bank of the DNA from a wild type strain of *R. meliloti* using pRK290 as a vector. Isolation of hydrogenase genes from *A. eutrophus* was made possible by construction of a gene bank of its DNA in pRK290 (Tait *et al.*, 1991).

MATERIALS AND METHODS

3:1 MATERIALS

3:1:1 Bacterial Strains

Pure cultures of *Escherichia coli* strains HB101, HB101 (pRK290), HB101 (pRK2073) and *Cicer-Rhizobium* strain Rcd301 (a Sm^R derivative of F75) were obtained from the Biotechnology Centre, Indian Agricultural Research Institute, New Delhi. Pure culture of *Rhizobium leguminosarum* strain B164 containing plasmid pIJ1008 was obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi. An *Azorhizobium* isolate of *Sesbania rostrata*, IRBG-46 was obtained from Dr. J.K. Ladha, International Rice Research Institute, Manila, Philippines and *E. coli* containing the cosmid pHU52 was supplied 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

L-arabinose, lysozyme, ethidium bromide, cesium chloride, sodium lauryl sulfate, RNase-A, Ficoll-400, salmon sperm DNA, pronase, agarose, calcium chloride, tetracycline and kanamycin were obtained from Sigma Chemicals Co., USA. Restriction enzymes *Eco*RI, *Hin*dIII and the Random Primed DNA labelling kit were obtained from United States Biochemical, USA. The nick-translation kit was obtained from Amersham, USA. Enzymes T4DNA ligase and alkaline phosphatase were from Promega, USA. Proteinase-K was obtained from Boehringer Manheim,

Table 1: Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source/Reference
E. coli		
HB101	*rec A, hsd R, hsd M, pro, leu,* Sm^R	Boyer *et al.* (1969)
HB101 (pRK2073)	Mob^+, Tra^+, Sm^R, Sp^R	Figurski and Helinski (1979)
Azorhizobium		
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)
Plasmids		
pHU52	pLAFR1::*hup*, Tc^R	Lambert, *et al.* (1985)
pRK290	Wide host range plasmid vector, Tc^R, *ori* (RK2)	Ditta *et al.* (1980)

Germany.

The Clean Genei kit was obtained from Bangalore Genei Pvt. Ltd., Bangalore. Bacto-agar, yeast extract and tryptone used were from Difco Laboratories, USA. Lambda DNA marker was supplied by CSIR Centre for Biochemicals, New Delhi. Sucrose, L-glutamic acid, inositol, pyridoxal-HCl, thiamine- HCl, Tris, EDTA were from Sisco Research Laboratories Pvt. Ltd., Bombay. Sodium gluconate was from Sarabhai M-chemicals, Baroda and nicotinic acid from BDH, England. Absolute ethanol was obtained from Bengal Chemicals Ltd., and ($\alpha^{32}P$) dCTP was supplied by BARC, Bombay.

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

3:2 METHODS

3:2:1 Preparation of growth media

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. Revival of lyophilized culture of IRBG-46 was done on yeast-extract mannitol (YEM) medium (Vincent, 1970) containing gL^{-1} mannitol, 10.0; K_2HPO_4, 0.5; NaCl, 0.1; $MgSO_4.7H_2O$, 0.2, and yeast-extract, 1.0 adjusted to pH 6.8. 1.5% agar was added to obtain the solid media. To obtain yeast-extract lactate (YEL) medium, mannitol was replaced by sodium-lactate (w/v). 1 ml of 2.5% aqueous solution of congo red per litre was

added to the YEMA medium to check the purity of the rhizobial culture. This strain was subsequently characterized and maintained on TYM medium (Khanuja 1991) containing bactotryptone, 5g; yeast extract, 0.5g; mannitol 10 g and $CaCl_2.2H_2O$, $1mML^{-1}$ Rhizobial strain Rcd301 was also maintained on TYM.

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 concentration of antibiotics (μg\ml) used in solid medium was as follows : tetracycline (Tc), 10; ampicillin (Ap), 50; spectinomycin (Sp.), 50; nalidixic acid (Nd), 25.

3:2:2 Sterilization

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

3:2:3 Incubation

For growth, cultures of *Rhizobium* were incubated at 30±0.5°C and *E. coli* cultures at 37±1°C using New Brunswick G-25 Refrigerated Incubator shaker and G-24 Environmental Incubator shaker, respectively.

3:3 Studies on nodulation and expression of *hup* genes of *Sesbania*-rhizobia under symbiotic conditions

Sesbania rostrata seeds were initially sterilized, for use in nodulation experiments as described by Adebayo *et al.* (1989). They

were intially treated with concentrated sulfuric acid for 30 min. This was followed by subsequently 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 12h. The sterilized seeds were then germinated in petri-plates on sterile Hoagland-Agar nitrogen-free nutrient medium (Hoagland and Arnon, 1950) (Table 2).

Meanwhile, slants of Hoagland-Agar were prepared in Gibson's tubes with a double layer of aluminium foil covering the rim of the tube. The tubes were filled with Hoagland nitrogen-free nutrient solution, and then the seedlings (~ 2 days old) were inserted into the tube with the help of a sterile forcep via a small hole made with the help of a sterile needle. Another hole was made opposite to the initial one through which the seedlings were inoculated with the rhizobial strains. These tubes were then incubated at 25±2°C under alternate light and dark periods of 16 and 8 hours respectively.

The plants were harvested 30 days after sowing. The roots with nodules were separated and were used for determination of nitrogenase (acetylene reduction) activity and hydrogen uptake (Hup) activity. Uninoculated plants were used as control. Four replications were used for each strain.

3:3:1 Estimation of H_2 evolved by nodules

Intact nodules were used for determining the rate of H_2 evolution in air. Nodulated roots were transferred to 25.5 ml vials which were

Table 2: Composition of Hoagland solution (Hoagland and Arnon, 1950).

Salt	Concentration	Qty. (ml/L)
	Solution A (macronutrient)	
K_2SO_4	0.500M	5
$MgSO_4.7H_2O$	1.000M	2
$Ca(H_2PO_4)_2$	0.050M	10
$CaSO_4$	0.010M	200
	Solution B (micronutrient) g/500 ml	
Boric acid	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	0.500%	1

The pH was adjusted to 6.5 using 0.1N 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 not used.

closed with serum stoppers and incubated at 25°C for 1h. H_2 evolved was estimated by the gas chromatographic method using an AIMIL-NUCON gas chromatograph equipped with a TCD and dual column (0.32x200cm) packed with molecular sieve 5A (80-100mesh). The results have been expressed as nmol H_2 evolved per g nodules fresh weight per h.

3:3:2 Assay of nitrogenase activity

The same nodules were assayed for nitrogenase activity by the acetylene reduction method (Hardy *et al.*, 1968). The reduction assay was performed in 25.5 ml capacity vials provided with serum stoppers. In each bottle 3.0 ml of air was replaced by 3.0 ml of C_2H_2 and incubated at 25°C for 1h. After incubation, gas samples (1 ml) were removed from each vial and analyzed for C_2H_4 using an AIMIL-NUCON gas chromatograph equipped with a FID and porapakT (60-80 mesh) dual columns (182 x 0.32 cm) maintained at 90°C with N_2 as carrier gas at a flow rate of 30ml/min. Both injector and detector were maintained at 110°C. Nitrogenase activity has been expressed as nmol C_2H_4 formed per g nodule fresh weight per h.

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

The expression of *hup* genes in nodules was revealed by measuring the relative efficiency (RE) of electron transfer to N_2 via nitrogenase as described by Schubert and Evans (1976) at 30 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. Only one strain having highest RE was used for subsequent studies.

3:4 Studies on expression of *hup* genes under free-living conditions

In order to study the strain *Azorhizobium* IRBG-46 for *ex planta* expression of *hup* genes in high organic carbon media, 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 3), yeast-extract mannitol (YEM) medium or yeast-extract lactate (YEL) medium as 1.5% agar slants. A 100 µl suspension of log phase cells were distributed 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 30°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 in sterile conditions in

Table 3 : 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	0.5g			
Sodium glutamate	0.5g			
Yeast extract	0.1g			
Distilled water	1.0L			
Agar	14.0g			
Part B				
Iron EDTA	28.0mg	14.000g		
Boric acid	3.0mg	1.500g		
$ZnSO_4.7H_2O$	2.0mg	1.000g		
$MnSO_4.7H_2O$	10.0mg	5.000g	In 250 ml H_2O	0.5ml
$CuSO_4.\ 5H_2O$	0.04mg	0.020g		
$CoCl_2.6H_2O$	0.025mg	0.013g		
KI	0.780mg	0.390g		
$Na_2MoO_4.2H_2O$	0.250mg	1.125g		
Part C				
$NaH_2PO_4.2H_2O$	150.000mg	2.500g	In 25 ml H_2O	1.5ml
Part D				
$CaCl_2.2H_2O$	150.000mg	0.375	In 25ml	10.0ml
$MgSO_4.7H_2O$	250.000mg	0.625	H_2O	
Part E				
Sucrose	0.5g	5.000g	In	2.5ml
L-arabinose	1.0g	3.571g	25 ml	7.0ml
Inostiol	100.0mg	0.500g	H_2O	5.0ml
Thiamine HCl	10.0mg	0.500g		1.0ml
Part F				
Nicotinic acid	1.0mg	0.010g	In 25 ml	
Pyridoxal HCl	1.0mg	0.010g	H_2O	2,5ml

Note : Part B was added to Part A, mixed thoroughly and the pH was adjusted to 6.8 using 0.1N 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), it was then added at the time of slant preparation. Seven ml of induction medium was added to each culture tube (16 x 150 mm).

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 30°C and gas samples removed after 3h and 5h of incubation, and analyzed for their H_2 content using an AIMIL-NUCON gas chromatograph as described in Section 3:3:1.

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 expressed as nmol of H_2 consumed per h per mg protein.

3:4:1 Soluble protein estimation

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 for 20 min at 12,000xg at 4°C, using a Sorvall RC5B refrigerated centrifuge. The cell pellet obtained was digested according to the method of Stickland (1951). The pellet was suspended in 3 ml of 0.75M NaOH and the protein in the sample precipitated by adding 0.6 ml of 25% TCA and 1.8 ml ethanol. After thorough mixing, the sample was centrifuged as described earlier. 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.

Since the *Azorhizobium* strain showed the expression of *hup*

genes in all the media used, it was selected for isolation of *hup* genes.

3:5 Selection of *hup* genes probe for *Azorhizobium* IRBG-46

In order to find out whether the *hup* genes of *Azorhizobium* IRBG-46 have homology with *hup* genes of *B. japonicum* 122DES cloned in pLAFR1 (pHU52), the following experiment was carried out. The rhizobial total genomic DNA was isolated, dot-blotted on nitrocellulose membrane filter and then hybridized with the labelled cosmid pHU52 as probe.

3:5:1 Isolation of genomic DNA

The genomic DNA of *Sesbania* rhizobial strains was isolated by the following method.

The cells of *Sesbania-Rhizobium* were grown in 10 ml TY medium. The cells after 24h of growth were collected by centrifugation at 2,000x g for 10 min. The cells were then resuspended in 5 ml of 50mM Tris containing 50mM EDTA, pH 8.0 and frozen at -20°C. 0.5 ml lysozyme (10mg/ml in 0.25M Tris, pH 8.0) was added to the frozen cell suspension and thawing was carried out with occasional mixing in a water bath kept at room temperature. When just thawed, the cells were placed on ice for 45 min. Later 1ml of proteinase K (1mg/ml in 0.5% SDS, 50mM Tris, 0.4M EDTA, pH 7.5) was added to the above and mixed well by inversion of the tubes. The tubes were then heated at 50°C for 60 min. 6 ml of buffered phenol was then added and mixed by inversion for 5 min to emulsify. The mix was then centrifuged at 5000 x g for

15 min to separate layers with DNA in the top aqueous layer. To the aqueous upper layer 0.1 volume 3M NaOAc was added and mixed gently by inversion. Addition of two volumes of ethanol with mixing precipitated the nucleic acids as a stringy glob. The precipitate was spooled out and dissolved in 5 ml of $T_{10}E_1$ [(10mM Tris, 1mM EDTA) (pH 8.0)]. After overnight dissolution at 4°C, an equal volume of chloroform was added to the tubes and mixed and centrifuged for 5 min to separate layers. The upper aqueous layer was transferred to a clean tube, and 0.1 volume 3M NaOAc followed by two volumes of ethanol were added. DNA precipitated as long threads was spooled out and dissolved completely in 0.1 ml $T_{10}E_1$ overnight at 4°C. Five µl of this DNA sample was loaded onto a 0.4% agarose gel. Electrophoresis was carried out at 60V for 8h and the DNA was viewed under the Fotodyne UV transilluminator to estimate the amount to be taken for the dot-blot experiment.

3:5:2 Dot-blot experiment

The total genomic DNA isolated as above was transferred to nitrocellulose membrane filter by using a dot-blot kit. Three pieces of Whatman 3MM filter paper and an NC filter were cut to the size of the apparatus. The prewetted NC filter 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 hooked to a vacuum source. Five µl of DNA samples were added to the wells. Vacuum was then turned-off and NC filter was removed. It was initially dried at room temperature and then soaked first in denaturing solution (NaOH, 0.5M, NaCl, 1.5M) for 5 min and then in a neutralizing solution (NaCl, 1.5M, Tris-Cl, 0.5M, pH 8.0) for 5 min. It was dried at room

temperature and baked between two fresh Whatman 3MM filter papers in a vacuum oven at 80°C for 2h.

The baked filter was then hybridized with the labelled probe as follows :

3:5:3 Isolation of pHU52

For probe making, first cosmid pHU52 was isolated. For this *E. coli* cells containing pHU52 were grown overnight in 5 ml LB medium with tetracycline. About 1.5-2 ml culture was poured into an eppendorf tube and centrifuged for 2 min in an eppendorf centrifuge. The supernatant was poured off and the pellet was dried as much as possible by inverting the tube on a filter paper. It was then resuspended in 100 µl of an ice cold Solution I [50mM glucose, 10mM EDTA, 25mM Tris-Cl (pH 8.0)], and 0.5 mg of lysozyme (25,550 U) was added just before use. The solution was kept on ice for 5 min and subsequently 200 µl of a freshly prepared Solution II (0.2N NaOH and 1% SDS) was added, and the contents were mixed thoroughly. The tubes were again kept on ice for 10 min. Then 150 µl of an ice cold Solution III (3M NaOAc, pH 4.8) was added, mixed well and kept on ice for 10 min. It was then centrifuged in an eppendorf centrifuge for 5 min. The supernatant was transferred to fresh tubes carefully and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuged for 2 min. The aqueous phase was transferred to fresh eppendorf tubes and an equal volume of chloroform/isoamyl alcohol (24:1) was added and centrifuged for 2 min at 8000 x g. The aqueous phase was then transferred to fresh eppendorf tubes and two volumes of ethanol (ice

cold) were added, mixed well and kept at -70°C for 30 min. It was then centrifuged at 8000 x g for 5 min, the supernatant was discarded and to the pellet, 100 µl of Solution IV [1M Tris-Cl (pH 8.0), 3M NaOAc] was added and tapped gently to dissolve the pellet. To this solution again two volumes of chilled ethanol were added, mixed well and kept at -70°C for 30 min. The tube was centrifuged again for 5 min. and the pellet was washed with 100 µl of 75% cold ethanol. It was then dried and dissolved in 20 µl TE buffer (10mM Tris; 0.1mM EDTA, pH 8.0). An aliquot of the DNA was then electrophoresed to determine its approximate concentration.

3:5:4 Preparation of probe by Nick-translation

For the preparation of probe, the pHU52 DNA isolated as above was nick translated using the Amersham nick-translation kit. The nick translation reaction was set up as follows :

DNA - 15 µl

Solution I - 20 µl

(dTTP, dGTP, dATP in equal concentration, in a concentrated nick translation buffer containing Tris-Cl (pH 8.0), $MgCl_2$ and 2-mercaptoethanol)

(α-^{32}P) dCTP (Sp. act., 2000 Ci/mmole)	-	5 µl
H_2O	-	50 µl
Enzyme solution	-	10 µl
Total volume	-	100 µl

The above mixture was incubated in a water bath at 15°C for 90 min. The reaction was stopped by adding 4 μl of 0.5M EDTA (pH 8.0). Salmon sperm DNA (10 μl of 10 mg/ml) was then added, the mixture was kept in boiling water for 5 min for denaturation and then cooled immediately on ice. The free nucleotides in the solution (both labelled and unlabelled) were removed by passing through a Sephadex G50 column. This denatured probe was then used for dot-blot hybridization.

3:5:5 Prehybridization

The baked NC filter was put in a dry polythene bag and the following prehybridization solution was added.

Prehybridization solution

20 x SSC	-	3 ml
20% SDS	-	200 μl
0.2M EDTA	-	80 μl
100xDenhardt's solution	-	200 μl
Water	-	15.1 ml
Denatured salmon sperm DNA (5 mg/ml)	-	100 μl

(100xDenhardt's solution contained 2% PVP, 2% BSA and 2% Ficoll 400).

The air bubbles were carefully removed and the bag was sealed and kept immersed for prehybridization in a water bath at 68°C for 2h.

3:5:6 Hybridization

After 2h of prehybridization, the excess prehybridization solution was discarded and only enough was left so as to wet the filter in the bag. Through a small cut in one corner of the bag, the denatured probe was added, the bag was sealed and incubated overnight at 68°C in a shaking water bath.

3:5:7 Washing of filters and Autoradiography

Following hybridization, the filter was carefully removed from the bag, immersed in 300 ml of 2xSSC and 0.1% SDS at room temperature and agitated gently for 10 min. It was then transferred to a fresh batch of the above mentioned wash solution and continued to be agitated gently. Subsequently, the filter was washed for one hour in 500 ml of a solution of 1xSSC and 0.1% SDS at 50°C. The filter was then dried, wrapped in a Saran wrap and exposed using Indu X-ray film for 48h at -70°C. The film was then developed using Indu developer for 3 min at room temperature in dark followed by washing in water for a minute and then fixed in Indu fixer for 5 min. Finally the film was washed under running tap water for 30 min and dried at room temperature.

3:6 Characterization of *Azorhizobium* IRBG-46

3:6:1 Antibiotic resistance profile

To determine the antibiotic resistance pattern of strain IRBG-46, the culture was grown on solid TY medium supplemented with the antibiotics mentioned in Table 6. The stock solution of tetracycline and rifampicin were prepared in 50% ethanol and methanol respectively.

Stocks of ampicillin, nalidixic acid and kanamycin were prepared in water.

3:6:2 Plasmid profile

Megaplasmid analysis of strain IRBG-46 was done according to the procedure of Plazinski *et al.* (1985). This is a double comb method which is normally used for megaplasmid isolation. A horizontal 0.65% agarose gel was made with a double-comb system and left for setting for 1h. The comb closest to the cathode was then removed and the wells were overfilled with a solution of 0.4% agarose - 1.5% SDS in the running buffer. After another hour, the second comb was removed, and the gel was placed into the electrophoresis tank covered with Ca.3mm of the running buffer. The electrophoresis tank containing the gel was then placed at 4°C, and the vacant wells were filled with 60 µl of sample. Preparation of sample was done as follows :

2ml culture pellet was washed with 300 µl of 0.1% Sarkosyl in $T_{50}E_{20}$ (pH 7.5). The pellet was then suspended in 60 µl lysis mixture consisting of lysozyme (1mg/ml), RNase A (1mg/ml), 0.1% bromophenol blue and 20% Ficoll 400. Samples were then immediately loaded into wells, and after 15 minutes, 8 µl of proteinase K was added (10 mg/ml) to each well. The wells were left as such for 10 min. Subsequently, the gel was run at 10mA for 2h and then at 45mA for 8h at 4°C. After electrophoresis, the gel was stained with ethidium bromide and photographed using an FCR-10 camera attached to a Fotodyne UV DNA transilluminator. The presence of small plasmid(s)

was analyzed by performing plasmid isolations using the alkaline lysis and the PEG procedures

3:6:3 Localization of *hup* genes in *Azorhizobium* IRBG-46

In order to determine whether the *hup* genes are present on the megaplasmid or on the chromosome of *Azorhizobium*

IRBG-46, a plasmid curing experiment was performed. Following the curing, the strain was checked for the expression of *hup* genes under free-living conditions on HUM.

For curing of the plasmid, a log phase culture of IRBG-46 was taken and inoculated into a series of culture tubes containing TY medium amended with different concentrations (10-200 µg/ml) of acridine orange as a curing agent. The cultures were grown at 30°C in an environmental incubation shaker.

The culture tube containing the highest concentration of acridine orange (150 µg/ml in this case), and displaying observable turbidity was used for plating dilutions and checking random individual colonies for loss of plasmid electrophoretically. Following electrophoretic determination of loss of plasmid, the strain was checked for expression of *hup* genes under free-living conditions on HUM as described in Section 3:4. The Hup activity of cured and uncured strains has been expressed as nmol of H_2 consumed per h per mg protein.

3:7 Construction of gene bank of *Azorhizobium* strain IRBG-46

3:7:1 Large scale genomic DNA isolation

Azorhizobium IRBG-46 was grown for 24h in 1L of TY medium. The culture broth was then centrifuged at 3800xg in a GS-3 rotor using a Sorvall RC5B refrigerated centrifuge. Each 250 ml culture pellet was then washed in 25 ml cold $T_{50}E_{20}$. The pellet was suspended in 21.5 ml $T_{50}E_{20}$ after washing, and 1.25 ml 20% Sarkosyl prepared in $T_{50}E_{20}$ was added. 2.5 ml pronase (5 mg/ml predigested 37°C/90 min.) was also added and incubation in plastic bottles was carried out at 55°C for 2h in a water bath. The mixture was then vortexed at maximum speed. Equal volume of buffered phenol was added and mixed by inversion for 5 min to emulsify. The emulsified mixture was transferred to sterile corex tubes and centrifuged at 5000xg for 15 min to separate layers. To the aqueous upper layer containing DNA, 0.1 volume of 3M NaOAc, (pH 4.8) was added and mixed gently by inversion. This was followed by addition of two volumes of ethanol with inversion to mix. The nucleic acids which precipitated as a stringy glob was 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 spun at 350,000 x g at 20°C in a VTi 65 rotor for 24h. The genomic DNA band was carefully removed with the help of a sterile 18 gauge needle into tubes carefully

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 1-butanol saturated with water. When all the pink colour disappeared, the lower aqueous phase containing DNA sample was dialyzed for 24h against several changes of TE buffer (pH 8.0) to remove the CsCl. The OD_{260} of the final solution of DNA was measured to calculate the concentration of DNA. This DNA was now stored in aliquots of 100 µl at -20°C.

3:7:2 Partial digestion of genomic DNA

Fragments of genomic DNA suitable for the construction of gene bank were prepared as follows :

Initially test digestions on aliquots of the batch of purified DNA with varying units of *Eco*RI restriction enzyme were carried out. This was done to determine the amount of enzyme required to reduce the modal size of the DNA to approximately 20 kb. After this pilot experiment in which all the conditions for partial digestion were established, large scale digestion of the genomic DNA was carried out to get maximum fragments in the range of 15-23 kb.

Test digestions included

DNA (15 µg)	-	15 µl
10 x *Eco*RI restriction enzyme buffer	-	30 µl
10 x BSA	-	30 µl
Water	-	225 µl
Total volume	-	300 µl

The DNA sample was distributed into five tubes. In the first tube 80 µl of DNA was added whereas the next four tubes were dispensed with 40 µl each of DNA. 1 µl of *Eco*RI enzyme (14U) was diluted to 10 µl in 1 x *Eco*RI restriction enzyme buffer. 3 µl of this diluted enzyme containing 4.2U was dispensed into the first tube containing 80 µl DNA (4.0 µg). It was thoroughly mixed and 40 µl of the mix was now dispensed from this tube into the second tube. A similar serial dilution was made in the next two tubes with the last tube left free of enzyme. Thus serial dilutions to get varying concentrations of enzyme such as 1U/µg, 0.5U/µg, 0.25U/µg and 0.125U/µg were made to check for maximum number of fragments in the range of 15-23 kb. The restriction enzyme digestion was carried out at 37°C for 1h. The samples were electrophoresed in 0.4% agarose gels to confirm the restriction digestions.

For large scale digestion, the concentration of 0.25U enzyme/µg DNA was taken to digest about 400 µg of the genomic DNA at 37°C for 1h. The restriction was terminated by addition of 10mM EDTA (pH 8.0). An aliquot of the digested DNA was electrophoresed in a 0.4% agarose gel to confirm the restriction digestion. Then the restricted DNA samples were extracted once with an equal volume of a 1:1 mixture of phenol:chloroform and again with a 24:1 mixture of chloroform:isoamyl alcohol. The aqueous phase was removed and precipitated overnight at -20°C with two volumes of ethanol and 0.1 volume of 3M NH_4OAc (pH 7.5). The DNA sample was pelleted at 11220 x g in a Sorvall RC5B refrigerated centrifuge for 20 min.

Finally the DNA pellet was dissolved in 250 µl of TE buffer and stored at -20°C.

3:7:3 Size fractionation of the restricted DNA

A 38 ml 10-40% linear sucrose density gradient was prepared in TE buffer using a gradient former in a Beckman SW28 polyallomer tube. The DNA sample (250 µl) was then heated to 68°C for 10 min, cooled slowly to 20°C and layered onto the gradient. The gradient was run at 90,000 x g in a SW28 rotor for 24h at 20°C. After centrifugation, the bottom of the tube was punctured using a 19 gauge sterile needle and 0.5 ml fractions were collected in sterile eppendorf tubes. Then 10 µl of every third fraction was mixed with 10 µl of water and 2 µl gel loading dye and analyzed on a 0.4% agarose gel using *λHind*III marker whose sucrose and salt concentration was adjusted to correspond that of the samples. After electrophoresis, the gradient containing DNA fragments in the 15-23 kb size range were pooled for precipitation. The pooled samples were diluted so as to reduce the sucrose concentration to less than 10%. Then the sample was precipitated with ethanol at -20°C for 4h and recovered by centrifugation at 4°C in a Sorvall RC 5B refrigerated centrifuge at 11,220 x g. The DNA pellet was dissolved in $T_{10}E_{0.1}$ and an aliquot was analyzed on a 0.4% agarose gel. This was stored at -20°C.

3:7:4 Isolation of Vector pRK290

A modified Birnboim and Doly (1979) procedure was followed for large scale isolation of plasmid pRK290. A freshly grown culture of *E. coli* HB101 (pRK290) was used for inoculation of 1L LB medium and

the overnight grown culture was used for plasmid isolation.

The cells were spun in a GS-3 rotor at 2661 x g at 4°C for 5 min. The pellets were resuspended in 16 ml Solution A (10mM EDTA; 25mM Tris, pH 8.0). Then 32 ml Solution B (0.2N NaOH; 1% SDS) was added, shaken to mix and left on ice for 5 min. After 5 min, 24 ml Solution C (3M NaOAc, pH 4.8) was added, mixed and left on ice for 1-2h. The sample was centrifuged later in GS-3 rotor at 6800xg at 4°C for 15 min. The supernatant was decanted carefully into clean bottles and 160 ml cold 100% ethanol was added, mixed and left at -20°C for 30 min. Following this, the DNA was centrifuged again at 7600xg at 4°C for 20 min. The supernatant was carefully drained off and the pellet resuspended in $T_{10}E_1$ so that the final volume was 7ml for 1L of original culture broth. For purification of DNA, 9.0 g of solid CsCl was added to the 7 ml DNA solution alongwith 1.5 ml ethidium bromide (5mg/ml) and 40 μl 30% Sarkosyl. The sample was centrifuged at 350,000xg in a Beckman VTi 65 rotor for 17h. The lower band of covalently closed circular (CCC) form of DNA was removed with a sterile 18 gauge needle. The ethidium bromide and CsCl were removed from the DNA sample as described earlier in Section 3:7:1.

3:7:5 Restriction of plasmid pRK 290

The plasmid pRK290 was subjected to complete restriction by restriction enzyme *Eco*RI to linearize it for subsequent ligation with the insert DNA. In order to linearize the plasmid, excess of *Eco*RI (5U/μg DNA) was used, and the restricted DNA was visualized on a 0.7% agarose

T-5498

gel. The unrestricted plasmid DNA showed three bands corresponding to linear, open circular (OC) and covalently closed circle (CCC) forms, whereas the restricted DNA showed only one band where all the forms had been linearized. The large scale restriction digestion reaction included :

DNA (10 μg)	-	20 μl
*Eco*RI (50U)	-	4 μl
10 x restriction enzyme buffer	-	15 μl
BSA (100 μg/ml)	-	15 μl
Water	-	96 μl
Total volume	-	150 μl

Restriction was carried out for 1h at 37°C.

3:7:6 Dephosphorylation of linearized plasmid DNA

Calf intestinal alkaline phosphatase (CIP) was used to remove 5'-phosphate groups from the linearized plasmid DNA according to Maniatis *et al.* (1989).

Once the restriction digestion of pRK290 was complete, the sample was extracted with phenol:chloroform (1:1, v/v) and precipitated with two volumes of ethanol for 4h at -20°C. The DNA was recovered by centrifugation at 12,000 x g for 15 min at 4°C in a Sorvall RC 5B refrigerated centrifuge. It was redissolved in 90mM Tris-Cl (pH 8.3). 10 μl of 10 x CIP dephosphorylation buffer and 0.05U CIP/μg DNA added and the DNA sample incubated at 37°C for 1h. 10xCIP dephosphorylation

buffer contained 500mM Tris-HCl, pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$ and 10mM spermidine.

At the end of the incubation period, the CIP was inactivated by heating to 65°C for 1h in the presence of 5mM EDTA (pH 8.0). The reaction mixture was then cooled to room temperature, and extracted once with phenol and once with phenol:chloroform (1:1, v/v). Then, 0.1 volume of 3M sodium acetate (pH 7.0) was added followed by addition of two volumes of ethanol. It was mixed well and stored at 0°C for 1h. The DNA was finally recovered by centrifugation at 12,000xg for 10 min at 4°C. The pellet was washed with 70% ethanol at 4°C and recentrifuged. The precipitated DNA was redissolved in sterile Milli Q water and stored at -20°C.

3:7:7 Ligation of restricted plasmid DNA with insert DNA

Initially a test ligation mixture was set up as follows : 0.1 μg of the vector DNA was transferred to a sterile microfuge tube. Since the molecular size of the insert and vector DNA was about the same, i.e., ~ 20 kb, an equal amount (0.1 μg) of insert DNA was added to the same tube. To this, water was added to make up the volume to 8.5 μl and the solution was warmed to 45°C for 5 min to melt any cohesive termini that may have reannealed. It was then chilled to 0°C. To this mixture was added 1 μl of 10xbacteriophage T4DNA ligase buffer containing ATP, and 0.1 weiss unit of bacteriophage T4 DNA ligase.

The 10xbacteriophage T4DNA ligase buffer contains : 300mM Tris HCl, (pH 7.8), 100mM $MgCl_2$, 100mM DTT and 10mM ATP.

The reaction mixture was incubated for 24h at 15°C. Two additional control reactions were also set up that contained : (1) the plasmid vector alone, and (2) the fragment of insert DNA. 1-2 μl of each of the ligation reaction mixture was used to transform competent *E. coli* HB101 cells. After checking for successful ligation reaction between insert and plasmid DNA, large scale ligation reactions were carried out involving 2 μg insert DNA, 2 μg linearized plasmid pRK290 DNA and 2 weiss units of bacteriophage T4DNA ligase.

3:7:8 Preparation of competent cells for transformation

E. coli HB101 cells were streaked on plain LB medium and grown overnight at 37°C. A single colony was picked up and inoculated into 10 ml LB broth and shaken overnight at 37°C in an environmental incubator shaker. One ml of this culture was used as inoculum for a 100 ml LB medium and was grown to OD_{550} = 0.5. Once the required OD was reached, the cells were centrifuged at 3800 x g in a Sorvall RC 5B refrigerated centrifuge at 4°C for 7 min in Oakridge polypropylene tubes. The cells were then resuspended in 50 ml cold 0.1M $CaCl_2$ and kept on ice for 20 min. The cells were again given a spin at 1247 x g for 3 min. The pellet was resuspended in 4 ml 0.1M $CaCl_2$. To this 1ml sterile glycerol was added. The cells were dispensed in sterile eppendorf tubes in 1 ml aliquots and frozen at -70°C.

3:7:9 Transformation

For each transformation reaction, not more than 50-100ng ligated DNA was used in a maximum volume of 10 μl. To this DNA sample, 100 μl of thawed competent cells were added and the tubes were

swirled gently several times in order to mix their contents. The tubes were then stored on ice for 30 min. Subsequently a heat shock was given to the cells by heating them to 42°C for exactly 2 min. This was followed by immediately chilling them on ice for 2 min. Then, 1 ml LB medium was added to the tube and the cells were transferred to a shaking incubator set at 37°C for 1h. The cells were agitated at 150 cycles/ min during this recovery period. Finally, the cells were concentrated by centrifuging them briefly in an eppendorf centrifuge and the pellet was suspended in 100 μl LB broth. This transformation mixture was plated on LB-Tc selective plates. A control transformation reaction with linearized, phosphatased pRK290 vector was also included to check for the extent of self ligation of vector.

3:7:10 Pooling of transformed colonies

The transformed colonies from the ligation reactions were pooled as follows : To each 90 mm plate, 5 ml of LB broth was added, the colonies gently scraped with the help of a sterile loop and a suspension of the cells made on the plate. This suspension in 2 ml aliquots was transferred to 3 ml storage vials and 176 μl dimethyl sulfoxide (DMSO) was added to it. It was mixed thoroughly by inverting the tubes and stored at -70°C.

After the gene bank was prepared, the colonies from different ligation mixtures were pooled in a final volume of 1 ml. This was centrifuged in order to remove the DMSO and again resuspended in 100 μl LB broth. Five spots of 20 μl each were then made with it on an LB-Tc plate and incubated overnight at 37°C. Three of the overnight grown

spots were separately suspended in 1 ml LB broth each alongwith 88 μl of DMSO to be stored at -70°C. Two spots were suspended in 1 ml LB broth each, in which one was stored at 4°C whereas the other was used immediately to determine the titre of the library.

3:7:11 Titre determination of the library

Serial dilutions of the 1 ml suspended culture were made. Dilutions of the order of 10^{-6}, 10^{-7} and 10^{-8} were plated in duplicate and incubated overnight at 37°C. The dilution corresponding to 10^{-7} gave 300 colonies and thus the titre was determined to be 3×10^{9}.

3:8 Screening of the gene bank for *hup* genes

Screening of *Azorhizobium* IRBG-46 gene bank was done to isolate the *hup* gene(s). The gene bank was poured at a dilution of 10^{-7} in order to obtain approximately 3000 colonies. From these plates, single colonies were picked up at random and spotted on oriented LB-Tc plates in duplicate with the help of sterile tooth picks. Each plate was spotted with 100 colonies. They were incubated overnight at 37°C and 2000 such clones were used for the initial screening by colony hybridization. The duplicate plates were stored at 4°C for picking up positive clones.

3:8:1 Colony hybridization

3:8:1:1 Tansfer of colonies to filters

Four pieces of Whatman 3MM paper were cut to an appropriate size and shape and fitted neatly onto the bottoms of 4 glass trays. These papers were saturated separately with the following solutions :

(a) 0.5N NaOH

(b) 1M Tris (pH 7.4)

(c) 2xSSC (pH 7.0)

(d) 95% ethanol

Using blunt-ended forceps, Whatman 541 filters were placed onto the spotted plates and all the air bubbles were removed slowly. The filters were then peeled off from the plates and placed colony side up sequentially on each of the above four solution impregnated 3MM papers for 5 min each. Following these treatments, the filters were air dried and kept in a dessicator for use in later hybridization experiments. The filters were oriented beforehand by marking them with pencil.

3:8:1:2 Preparation of probe

Cosmid pHU52 DNA was isolated as described earlier in Section 3:5:3 and restricted using *Eco*RI enzyme. The restricted sample was run on a 0.7% low melting point agarose gel. The band of interest i.e., 12.9 kb *Eco*RI fragment was cut with the help of a gel cutter. This was heated for 5 min at 65°C. To the 500 μl of sample was added 50 μl of 5M NaCl. After vortexing, the sample was given a brief spin in an eppendorf centrifuge. The sample was again heated for 5 min at 65°C. An equal volume of phenol was then added and was immediately vortexed. Following this the sample was again centrifuged in an eppendorf centrifuge from which the upper phase was eluted. It was phenolized once more after which two volumes of ether were added to the upper phase. The upper layer of ether was discarded and the tube was heated with its cap open at 65°C for 2 min. This enabled all the ether to evaporate, after which two volumes of ethanol were added. The DNA

sample was precipitated overnight at -20°C and recovered by centrifugation at 11220 x g in a Sorvall RC 5B refrigerated centrifuge.

3:8:1:3 Random primed DNA labelling

The eluted fragment after being checked on a 0.7% agarose gel was labelled using the USB Random Primed DNA labelling kit according to the methodology of Feinberg and Vogelstein (1983).

The labelling was performed according to the following procedure.

(a) The DNA fragment (12.9 kb) was denatured in a volume of 10 µl by heating for 10 min at 95°C and then chilling on ice.

(b) To an eppendorf tube on ice, the following were added :

5 µl Denatured DNA (50 ng)

3 µl - dATP, dGTP, dTTP mixture

(1:1:1 mixture of each dNTP)

2 µl - reaction mixture (random hexanucleotides in 10xreaction buffer)

5 µl - [α-^{32}P] dCTP, 50 µCi, 3000 Ci/mmole, aqueous solution.

4 µl - sterile Milli Q water

1 µl - Klenow enzyme (2 units)

(c) The reaction mixture was incubated for 1h at 37°C.

(d) The reaction was then terminated by heating to 65°C for 10 min.

3:8:1:4 Hybridization

The prehybridization and hybridization reactions were carried out as described earlier except that the probe used in this case was the 12.9 kb *Eco*RI fragment of pHU52 which was oligolabelled using the random primed oligolabelling kit, as described in Sections 3:8:1:2 and 3:8:1:3.

3:8:1:5 Restriction analysis of the recombinant plasmids

The positive clones were picked up from the duplicate plates and their plasmid DNA was isolated by the following procedure : 10 ml culture was grown overnight in LB-Tc broth at 37°C. The cells were centrifuged at 1,949 x g for 5 min. in SS34 rotor in a Sorvall RC 5B refrigerated centrifuge at 4°C. The pellet was resuspended in Solution A (25 mM Tris Cl; 10mM EDTA; pH 8.0) and vortexed. 100 µl of this mix was transferred to eppendorf tubes and 200 µl of Solution B (0.2N NaOH and 1% SDS) was added and mixed vigorously. It was kept on ice for 10-20 min with intermittent shaking. Later, 150 µl Solution C (7.5M NH_4OAc) was added, the tubes were shaken and kept on ice for 10-20 min. The mix was then centrifuged in an eppendorf centrifuge for 2.5 min and the supernatant was dispersed into fresh tubes having 1 ml cold ethanol. The tubes were again kept on ice for 20 min, and then centrifuged for 2.5 min. The ethanol was aspirated off twice. The pellet was redissolved in 100 µl water and let stand for 10 min at room temperature. Then, 50 µl Solution C was added, followed by addition of 300 µl cold ethanol. It was shaken well and kept on ice for 15 min. The plasmid DNA was recovered by centrifugation in an eppendorf centrifuge for 2.5 min. The supernatant was aspirated off and the pellet was air-

dried. It was then dissolved in 40 µl sterile Milli-Q water and used for restriction analysis and dot-blot hybridization for secondary screening of the positive clones.

The following restriction digestion reactions were set up for each positive clone obtained after primary screening.

DNA	-	10 µl
10 x restriction enzyme buffer	-	2 µl
Water	-	7 µl
*Eco*RI	-	1 µl (14U/µl)
Total volume	-	20 µl

The digestion reaction was carried out at 37°C for 3h; terminated by addition of bromophenol blue dye and loaded along with λ *Hin*dIII marker on a 0.7% agarose gel. The gel was photographed using a UV-Fotodyne transilluminator.

3:8:1:6 Secondary screening

Following restriction analysis, some of the recombinant plasmids were dot-blot hybridized with the 12.9 kb EcoRI fragment of pHU52 as described in Sections 3:5:5 and 3:5:6. The recombinant plasmids have been designated as pSRH plasmids.

3:9:1 Transfer of plasmids pSRH into *Cicer-Rhizobium* strain Rcd 301

The triparental mating system was used for transfer of recombinant plasmids pSRH to *Cicer-Rhizobium* strain Rcd 301. *Cicer-Rhizobium* strain Rcd 301 was grown on a TYM plate for 3 days to get a

log phase culture. Two to three loopfuls of this culture was mixed with one loopful of an overnight grown culture of *E. coli* HB101 containing the recombinant plasmids pSRH on a fresh TYM plate. 5 ml of overnight grown culture of *E. coli* HB101 (pRK 2073) was centrifuged at 3,000 x g and the cells were resuspended in 5 ml of mannitol salts solution (MSS). Then 0.1 ml of the supension was added as a helper plasmid and mixed in the minimum possible space to get a spot on the plate. This was subsequently dried at room temperature and incubated at 30°C for 24-36h. Later a loopful of cells from the grown spot were used for dilution plating on selective RMM-Tc plates. Since *E. coli* HB101 cannot grow on RMM, only the transconjugants carrying the recombinant plasmids pSRH were picked up for further studies of assaying the Hup activity under free-living conditions.

3:9:2 Studies on the expression of *hup* genes in free-living transconjugants

In order to study the expression of cloned *hup* genes in the transconjugants of *Cicer*-rhizobial strain Rcd 301, the activity of uptake hydrogenase was induced in them in the free-living conditions and then uptake of H_2 measured gas chromatographically as described earlier in Section 3:4. The only difference in the conditions maintained was that the time of induction was extended to six days in a H_2 gas atmosphere of 5%. Subsequently the assay of uptake hydrogenase was carried out in an atmosphere of 1% H_2 after 3h and 24h of incubation. The activity of uptake hydrogenase has been expressed as nmol H_2 consumed per h per mg protein.

RESULTS

Hydrogen may act as an energy source in N_2-fixing nodules and thus create less demand for potentially limiting respiratory substrates from the plant. In view of these observed or potential benefits of H_2 oxidation, transfer of H_2-uptake (*hup*) genes from an efficient Hup system to strains of several *Rhizobium* spp. that lack this phenotype might result in an improvement in their symbiotic performance. Comprehensive efforts to transfer *hup* genes to wild type Hup^- *Rhizobium* strains by genetic engineering techniques necessitates a basic understanding of the organization of Hup-determinants in the donor organism. In this excercise, it would be desirable if these determinants are closely linked.

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 *Sesbania*-azorhizobia are still at the preliminary stage. Some available reports show the presence of an Hup system in these rhizobia 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 in presence of high concentrations of organic carbon substrates or high (>4.0%) O_2 partial pressures. In view of this it was considered worthwhile to clone *hup* genes from an efficient Hup^+ *Sesbania Azorhizobium* strain and transfer these to Hup^- *Cicer-Rhizobium* in order to further analyze the expression of its *hup* genes when transferred to this slow-growing *Rhizobium*. The experimental findings of the present

investigation are reported under the following headings :

1. Studies on the H_2-recycling ability of *Azorhizobium* strain IRBG-46 and some isolates under symbiotic conditions.
2. Studies on the expression of *hup* genes of *Azorhizobium* strain IRBG-46 under free-living conditions.
3. Selection of *hup* gene probe for *Azorhizobium* IRBG-46.
4. Characterization of *Azorhizobium* IRBG-46 with respect to :
   - (a) antibiotic resistance profile,
   - (b) plasmid profile, and
   - (c) localization of *hup* genes.
5. Construction of gene bank of *Azorhizobium* strain IRBG-46.
6. Isolation of *hup* genes from the constructed gene bank.
7. Transfer of recombinant pSRH plasmids into *Cicer-Rhizobium* strain Rcd 301.
8. Analysis of expression of *hup* genes in the transconjugants under free-living conditions.

4:1 Studies on H_2-recycling ability of *Azorhizobium* strain IRBG-46 and some isolates under symbiotic conditions

The expression of *hup* genes in *Sesbania rostrata* root nodules formed by different rhizobial isolates was studied by measuring the relative efficiency (RE) of electron transfer to N_2 via nitrogenase as described by Schubert and Evans (1976). RE is an estimate of H_2-recycling ability.

The seeds of *Sesbania rostrata* inoculated with the *Sesbania*-rhizobial isolates, were grown in Gibson tubes as described in Section 3:3. Nodules were taken from 30-day-old plants and tested for H_2

evolution and acetylene reduction activity. The relative efficiency (RE) was then calculated, as presented in Table 4. The REs of the isolates S1 and S2 were found to be 0.61 and 0.59, respectively, whereas REs for the isolate S3 and strain IRBG-46 were 0.87 and 0.95, respectively (Table 4). Since strains showing RE greater than 0.8 are considered to be Hup^+, it is clear that only isolate S3 and strain IRBG-46 are having H_2-recycling abilities. Since the strain IRBG-46 showed high RE (0.95), it was selected for further studies.

Table - 4 : Acetylene reduction, hydrogen evolution and relative efficiency in *Sesbania rostrata* nodules induced by *Sesbania* rhizobial isolates (30 day stage, Gibson tube experiment).

Strain/ Isolate No.	Nodule fr. wt. (mg)	H_2 evolution ($nmol H_2 h^{-1} g$ fr.wt.nodule^{-1})	C_2H_2 reduction ($nmol C_2H_4 h^{-1} g$ fr.wt.nodule^{-1})	Relative efficiency *
S1	70	916	2347	0.61
S2	56	1271	3111	0.59
S3	58	1361	10647	0.87
IRBG-46	96	895	17436	0.95

Note : Each sample consisted of 4 plants.

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

4:2 Studies on the expression of *hup* genes of *Azorhizobium* strain IRBG-46 under free-living conditions

Azorhizobium strain IRBG-46 was assayed for *hup* gene

expression under free-living conditions as described in Section 3:4. The different media used for derepression of uptake hydrogenase in this strain were low organic carbon containing H_2-uptake medium (HUM) and media containing high organic carbon, viz. yeast extract-mannitol agar (YEMA) and yeast extract-lactate agar (YELA) media. For induction of uptake hydrogenase, the culture of the strain was grown on the slants of the different media in the presence of 10% H_2 for 5 days, and then assayed in the presence of 5% H_2 in air.

The results presented in Table 5 show the expression of uptake hydrogenase activity on both low as well as high organic carbon containing media. Although the specific activity of the enzyme was very high on all the media tested, the maximum specific activity was recorded on HUM. The specific activities obtained on YELA and YEMA media were more or less comparable, and they were 53% and 57% lower, respectively, as compared to specific activity obtained on HUM.

Table - 5 : Uptake hydrogenase activity of Hup$^+$ *Azorhizobium* strain IRBG-46 in different media under free-living conditions.

Medium	Protein (μg slant^{-1})	Uptake hydrogenase activity (nmol $H_2 h^{-1} mg$ protein^{-1})
HUM	285	5355 ± 1884
YELA	520	2504 ± 355
YEMA	265	2290 ± 815

Values are the mean ± SE of six replications.

4:3 Selection of *hup* gene probe for *Azorhizobium* IRBG-46

Since the genetic organization of *hup* genes of *Sesbania*-

Azorhizobium is not well known, it was considered necessary to perform preliminary experiments to determine the homology of its *hup* genes with an available probe of the *hup* genes of *B. japonicum* cloned in pLAFR1 (pHU52). For the above study, a DNA:DNA dot-blot hybridization experiment was performed in which the total genomic DNA's of strain IRBG-46 and some isolates (S1,S2 and S3) of *Sesbania-Rhizobium* were hybridized with nick translated pHU52 DNA. The results presented in Plate I clearly indicated strain IRBG-46 to have a good homology with the *hup* genes of *B. japonicum* as it gave a strong positive signal. Thus the *hup* genes of *B. japonicum* can be used as a heterologous probe in isolating the *hup* genes of *Azorhizobium* IRBG-46. However, at the same time isolate S3 which was found to be Hup$^+$ under symbiotic conditions (see Table 4), did not give any signal. This shows that the heterologous probe, nick translated pHU52, cannot be used for isolating *hup* genes from all *Sesbania* rhizobia. Similarly, the isolates S1 and S2, which have been shown to be Hup$^-$ under symbiotic conditions, also did not give any signal when hybridized with nick-translated pHU52.

4:4 Characterization of *Azorhizobium* IRBG-46

Since there are no available reports on the characterization of *Azorhizobium* IRBG-46, efforts were made to characterize the strain with respect to its antibiotic resistance profile, plasmid/megaplasmid profile and the localization of *hup* genes in it.

4:4:1 Antibiotic resistance profile

The growth of the strain IRBG-46 was tested on an agar medium with different concentrations (5,10,15,25 and 50 μg/ml) of

PLATE I

Autoradiogram showing dot-blot hydridization of total DNA isolated from *Sesbania* rhizobial strains to nick-translated pHU52

1 - *Sesbania-Rhizobium* isolate S1

2 - *Sesbania-Rhizobium* isolate S2

3 - *Sesbania-Rhizobium* isolate S3

4 - *Azorhizobium* strain IRBG-46

5 - Positive control (pHU52)

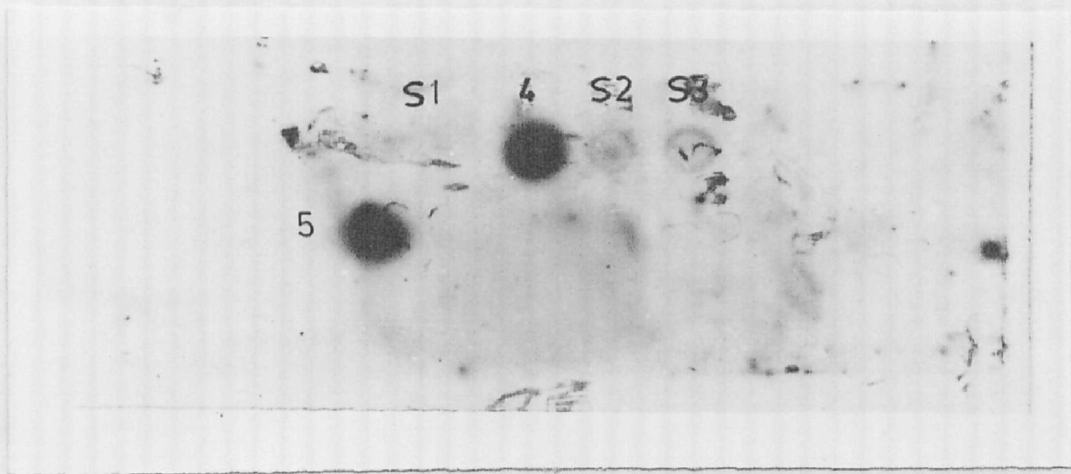

PLATE. I

antibiotics namely kanamycin (Km), ampicillin (Ap), nalidixic acid (Nd), tetracycline (Tc) and rifampicin (Rif). The growth of the strain on the respective plates was recorded after a period of two days.

The results presented in Table 6 show that the rhizobial strain was found to be resistant to Ap upto a concentration of 50 μg/ml and Nd upto 15 μg/ml. However, the strain was sensitive to Km, Tc and Rif.

Table - 6 : Antibiotic resistance and sensitivity profile of *Azorhizobium* strain IRBG-46

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

'+' indicates resistance to the antibiotic
'-' indicates sensitivity to the antibiotic.

4:4:2 Plasmid/megaplasmid profile

Megaplasmid analysis of cured and uncured strain of IRBG-46 was done according to the procedure of Plazinski *et al.* (1985). This is a double comb method which is used for megaplasmid isolation (Section 3:6:2). Plate II shows the megaplasmid composition of cured (lanes f-j) and uncured (lanes a-d) IRBG-46 obtained on a 0.4% agarose gel. In this study two megaplasmids of 350 kb and 280 kb sizes obtained from *R. leguminosarum* strain B164, have been used as size markers.

Since only the lower band comparable to a megaplasmid of approximately 280 kb size was obtained with reproducibility on performing several electrophoretic runs, it is suggested that the strain harbours only one megaplasmid. Plasmid curing experiments also demonstrated that only this band, corresponding to the presumptive megaplasmid disappeared upon curing (Plate II; lanes f-j). This conclusively demonstrates the presence of a single megaplasmid in this strain.

The presence of small plasmid(s) was analyzed by performing plasmid isolations using the alkaline lysis method and the PEG procedure. Plate III shows that only one small plasmid (approx. 55 kb) is present in this strain.

4:4:3 Localization of *hup* genes in *Azorhizobium* IRBG-46

Since a megaplasmid has been found with reproducibility in IRBG-46, it was necessary to determine the localization of *hup* genes in this strain, i.e., whether they are plasmid or chromosomal borne, in order to be able to isolate these *hup* genes in further studies. To localize the *hup* genes, a plasmid curing experiment was performed as described in Section 3:6:3, and the results of this experiment are presented above in Plate II. Following the curing, the strain was checked for the expression of *hup* genes under free-living conditions on HUM. The Hup activity of cured and uncured strains was found to be comparable (Table 7). This demonstrates presence of functional Hup determinants on the chromosome, though it does not eliminate the possibility of these determinants being present on the megaplasmid as well.

PLATE II

Megaplasmid profile of *Azorhizobium* strain IRBG-46 following *in situ* lysis on 0.4% agarose gel.

Lanes a-d, megaplasmid of IRBG-46

Lane e, megaplasmid markers

Lanes f-j, cured IRBG-46 (without megaplasmid)

Arrow indicates megaplasmid band.

PLATE III

Electrophoretic analysis of small plasmid(s) of strain IRBG-46 on a 0.7% agarose gel

Lanes a-b, Plasmid DNA isolated by alkaline lysis method

Lanes c-d, Plasmid DNA isolated by PEG procedure

Lane e, λ DNA marker (uncut)

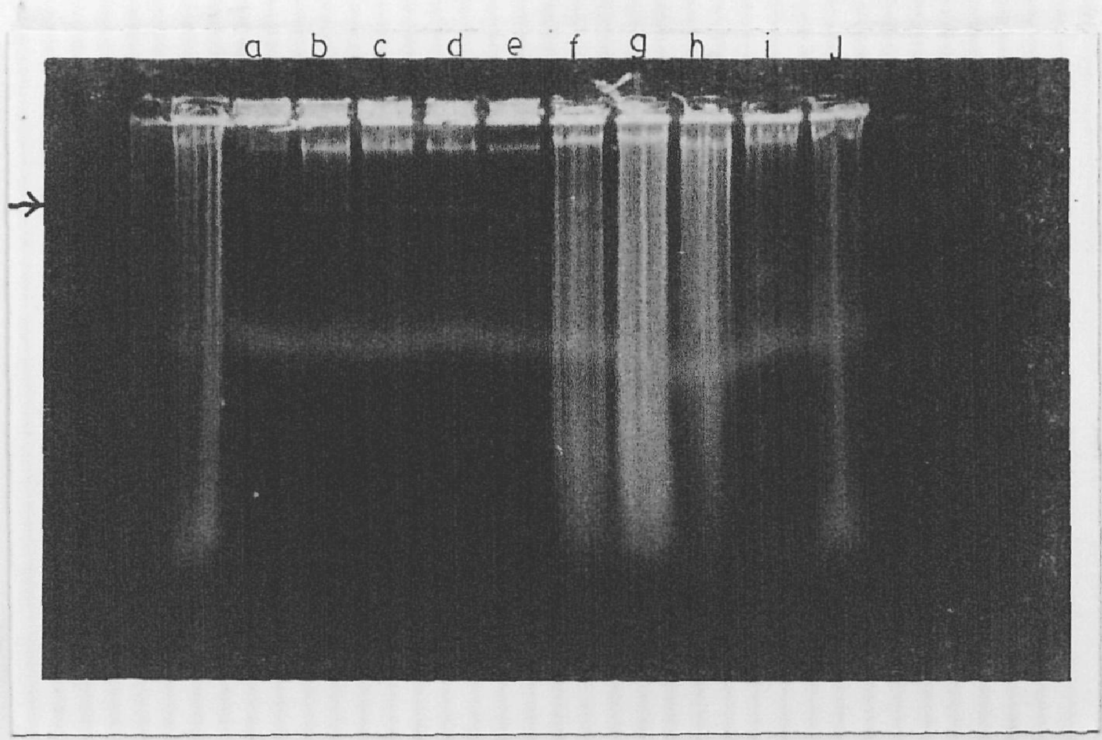

PLATE. II

PLATE. III

Table - 7 : Study on the expression of *hup* genes of plasmid cured and uncured *Azorhizobium* strain IRBG-46.

Strain	Protein (μg slant^{-1})	Uptake hydrogenase activity (nmol $H_2 h^{-1}$mg protein^{-1})
Uncured	217	3216 ± 862
Cured	133	3587 ± 621

Values are mean ± SE of six replications.

4:5 Construction of gene bank of *Azorhizobium* strain IRBG-46

Hup$^+$ *Azorhizobium* strain IRBG-46 was chosen for construction of a gene bank since it consists of an extremely efficient H_2-recycling system (see Table 5). Understanding the genetics of its Hup-system is possible only by isolation and characterization of the *hup* gene(s) from the gene bank.

The construction of the gene bank involved the use of plasmid pRK290 as a vector. Since this plasmid is a broad host range mobilizable vector, the recombinants can be introduced and maintained in *Rhizobium* spp.

4:5:1 Partial digestion of genomic DNA of *Azorhizobium* IRBG-46

Since *hup* genes in the fast-growing rhizobia are mostly found to span regions between 15 to 20 kb, a partial *Eco*RI digest of the genomic DNA of strain IRBG-46 was made to obtain maximum fragments in the size range of 15-23 kb. The conditions, i.e. the amount of enzyme and

the time of incubation required to obtain this partial digest were standardized by performing a pilot experiment as described in Section 3:7:2. An aliquot of the partially digested DNA was then electrophoresed on a 0.4% agarose gel in order to confirm the digestion (Plate IV).

Following confirmation of the restriction digestion, this partially digested genomic DNA was reprecipitated and dissolved in a volume of ~250 µl of $T_{10}E_1$ buffer. It was then run on a 10-40% linear sucrose density gradient as described in Section 3:7:3. The fractions obtained were collected and every third fraction was electrophoresed on a 0.4% agarose gel alongwith *Hin*dIII marker (Plate V A). Only those fractions containing fragments in the desired range (15-23 kb) have been pooled and reprecipitated as mentioned in Section 3:7:3. Plate VB (lane b) shows the pooled fractions electrophoresed on a 1% agarose gel.

4:5:2 Preparation of vector plasmid pRK290

The vector DNA which was isolated and purified as described in Section 3:7:4, was then run on 0.7% agarose gel to estimate its concentration. The vector DNA was then linearized with *Eco*RI and phosphatased with calf intestine alkaline phosphatase to prevent self ligation as described in Sections 3:7:5 and 3:7:6. The vector DNA is clearly shown as a single band of linearized plasmid DNA (Plate VB, lane a).

4:5:3 Ligation and transformation

It is clear from Plate VB that the concentration of the insert DNA is at least two times greater than the concentration of the vector DNA. Thus, for ligation the concentrations of vector and insert DNA were

PLATE IV

Partial *Eco*RI digest of genomic DNA of strain IRBG-46 on 0.4% agarose gel.

Lane a, uncut genomic DNA

Lane b, partial digest of genomic DNA

Lane c, λ *Hin*dIII marker

PLATE VA

Electrophoretic analysis of fractions obtained after sucrose-density gradient centrifugation on 0.4% agarose gel.

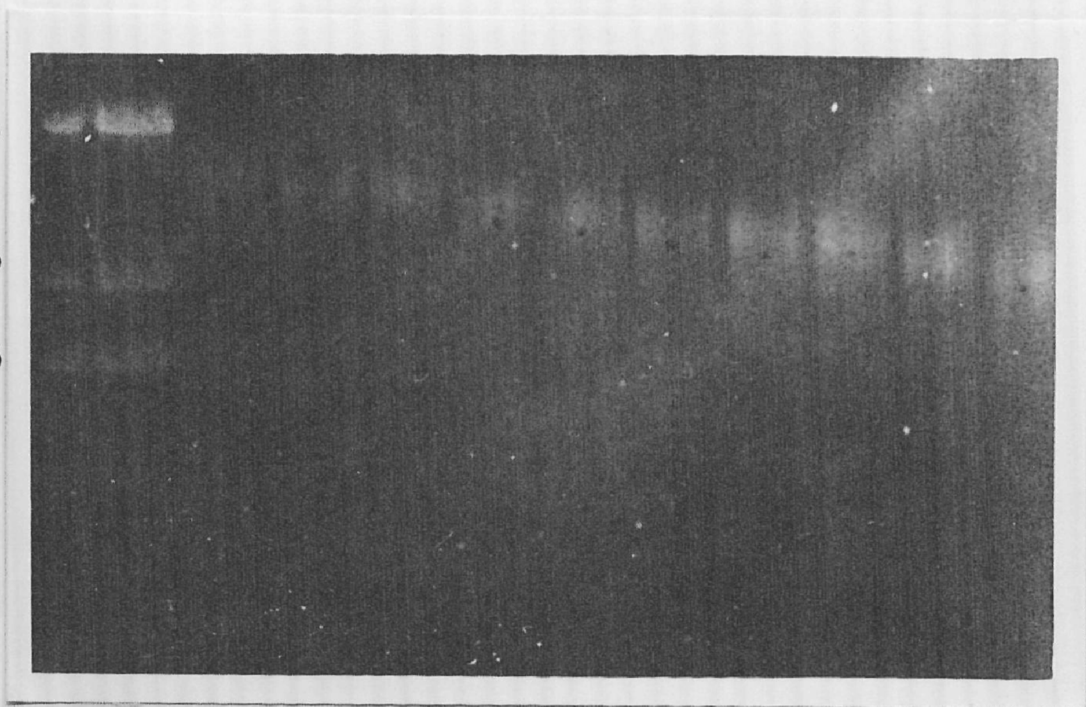

PLATE. VA

adjusted with sterile water so as to obtain an equimolar ratio of vector (~20 kb) and inserts (~15-23 kb) as they had approximately similar sizes. Following ligation as described in Section 3:7:7, the ligated DNA was transformed into *E. coli* HB101 and approximately 20,000 Tc-resistant colonies were obtained to constitute the gene bank. Since a number of small scale ligations were carried out, *Eco*RI restriction analysis of 11 randomly picked clones from a single ligation showed 5 of these (45%) possessing desired inserts (Plate VI).

4:6 Isolation of *hup* gene(s)

4:6:1 Primary screening of the gene bank for *hup* genes

In order to isolate the *hup* gene(s) of *Azorhizobium* strain IRBG-46 from the gene bank prepared, the bank was poured onto LB-Tc plates after determination of its titre. The screening of randomly picked 2,000 clones was done by colony hybridization using the 12.9 kb *Eco*RI fragment of pHU52 as a probe. Following hybridization, a total of 22 positive clones were obtained (Plates VII to XIV). However, for further studies, only 13 clones showing stronger signals were taken.

4:6:2 Restriction analysis of positive clones

The 13 positive clones obtained from the primary screening were subjected to restriction digestion with *Eco*RI enzyme as described earlier in Section 3:8:1:5. Out of these 13 clones, only 7 clones got restricted (Plates XV and XVI). In Plate XV, lanes b, h and i show 3 clones having the same restriction pattern with an insert size of approximately 22 kb and, lanes j and k show 2 clones having similar restriction pattern having an insert size of approximately 15 kb. Plate XVI, lanes b and c

PLATE VB

Vector and insert DNA on a 1% agarose gel.

Lane a, Linearized vector pRK290 DNA

Lane b, Pooled fractions of insert DNA (15-23 kb) following sucrose-density gradient centrifugation.

PLATE VI

Restriction analysis of random clones from the gene bank

Lane a,	λ *Hin*dIII marker
Lanes b-e & l,	*Eco*RI restriction pattern of random clones
Lanes f-k,	Clones not showing any insert

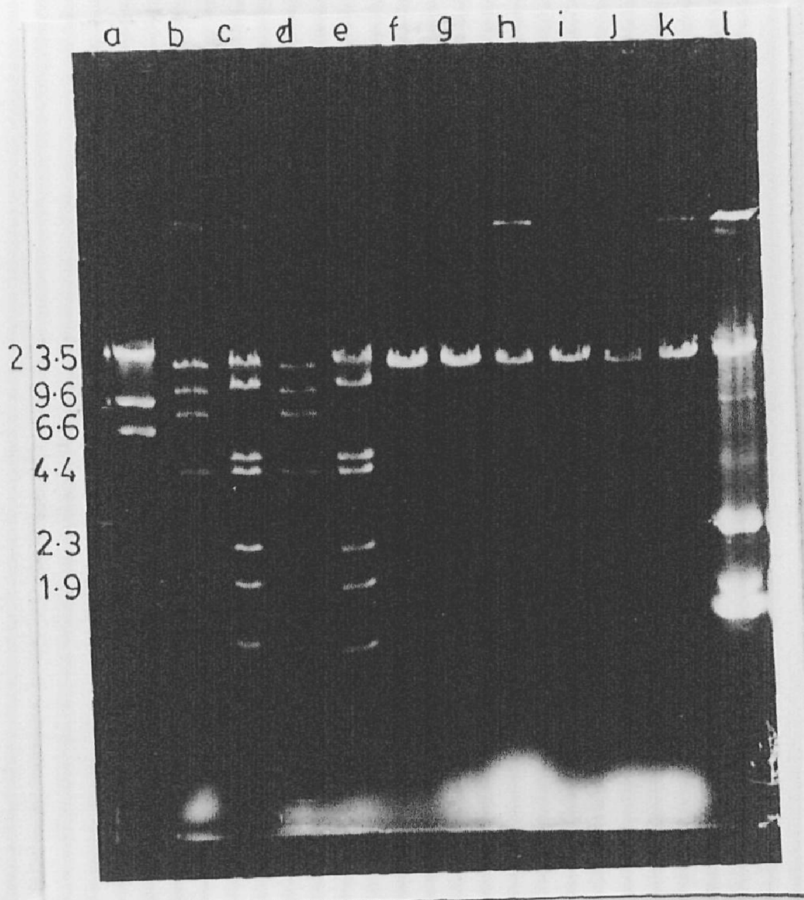

PLATE. VI

show 2 additional clones having different restriction patterns with insert sizes of approximately 20 kb and 19 kb, respectively.

Thus, for secondary screening by DNA:DNA dot-blot hybridization, only 5 clones represented by lanes b,h,i,j in Plate XV and lane c in Plate XVI, were taken, and the recombinant plasmids have been designated as pSRH1, pSRH2, PSRH3, pSRH4 and pSRH5, respectively.

4:6:3 Secondary screeening of the gene bank

The recombinant plasmids were isolated from the five clones and subjected to a dot-blot hybridization with 12.9 kb fragment of pHU52 DNA as a probe. Plate XVII shows the autoradiogram of the above hybridization. The positive signals obtained with all the 5 recombinant plasmids clearly demonstrates that they all have homology with the *hup* gene probe used. However, no signal was obtained when only vector (pRK290) DNA was taken for hybridization.

4:7 Transfer of recombinant pSRH plasmids into *Cicer-Rhizobium* strain Rcd 301

Since the recombinant pSRH plasmids, which are constructs of plasmid pRK290, are mobilizable but not self-transmissible (Mob^+, Tra^-) a helper plasmid pRK2073 having *tra* functions was used for their transfer to Hup^- *Cicer-Rhizobium* strain Rcd 301. The triparental mating system described in Section 3:9:1 was used for the conjugal transfer of the five recombinant pSRH plasmids. Plate XVIII shows the identification of the transconjugants on RMM-Tc plates.

PLATE VII-X

Autoradiograms showing colony hybridization of clones from gene bank using oligo-labelled 12.9 kb *EcoRI* fragment of pHU52

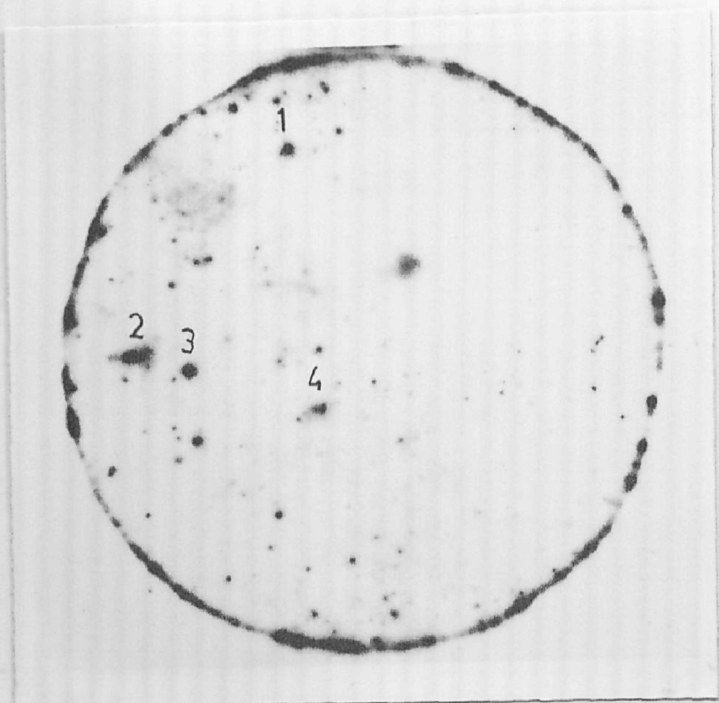

PLATE. VII

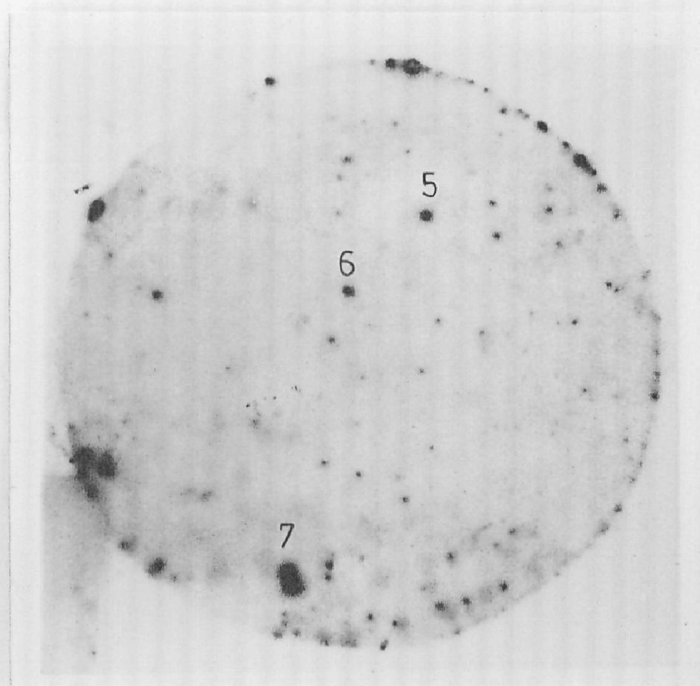

PLATE. VIII

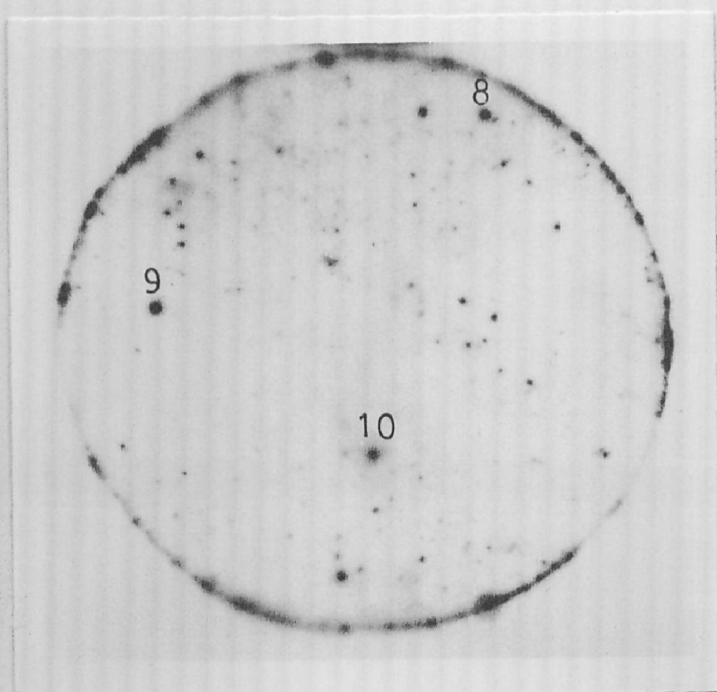

PLATE. IX

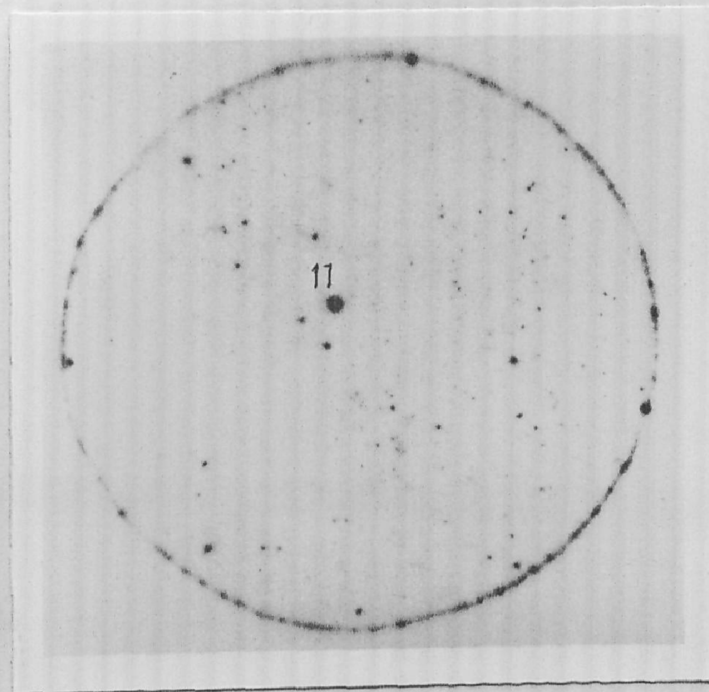

PLATE. X

PLATE XI-XIV

Autoradiograms showing colony hybridization of clones from gene bank using oligo-labelled 12.9 kb *Eco*RI fragment of pHU52

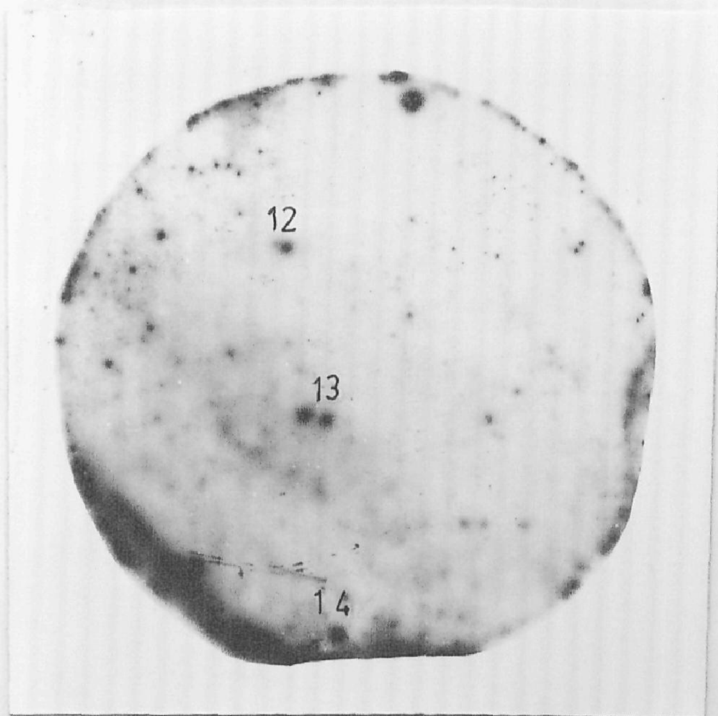

PLATE. XI

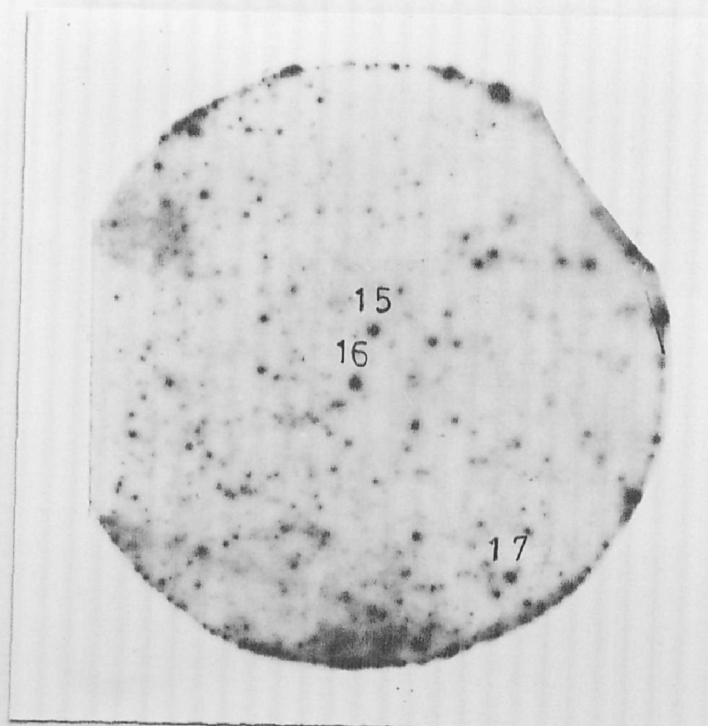

PLATE. XII

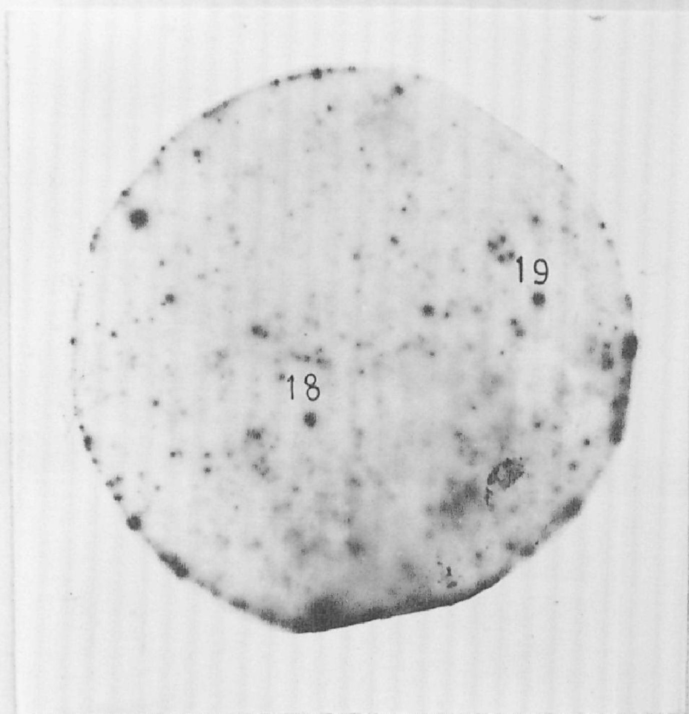

PLATE. XIII

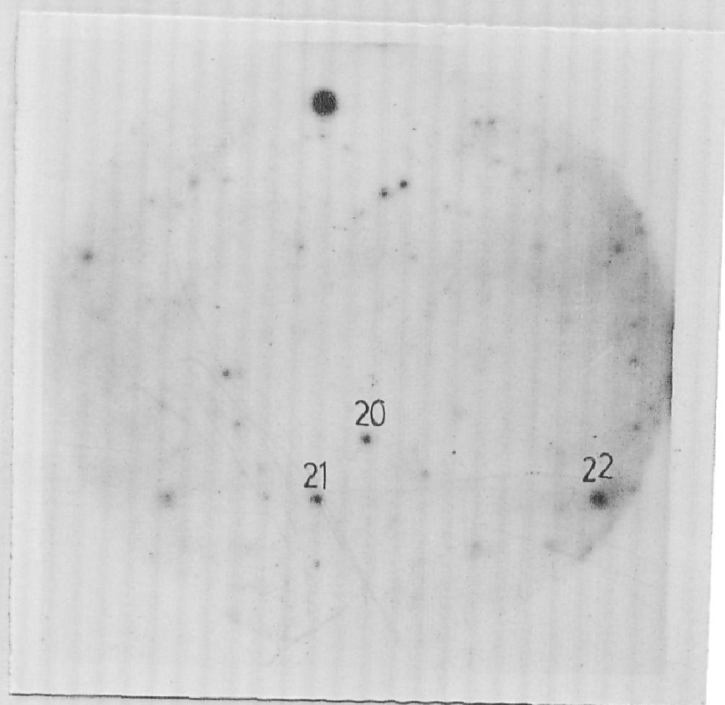

PLATE. XIV

Restriction analysis of 13 clones

PLATE-XV

Lane a, λ *Hin*dIII marker

Lanes b, h-k, *Eco*RI restriction pattern of 5 positive clones

Lane c-g & i, Clones not showing restriction

PLATE XVI

Lane a, λ *Hin*dIII marker

Lanes b & c, *Eco*RI restriction pattern of 2 positive clones

PLATE-XVII

utoradiogram showing secondary screening f 5 positive clones.

1-5, positive clones

6, negative control (pRK290)

C, positive control (pHU52)

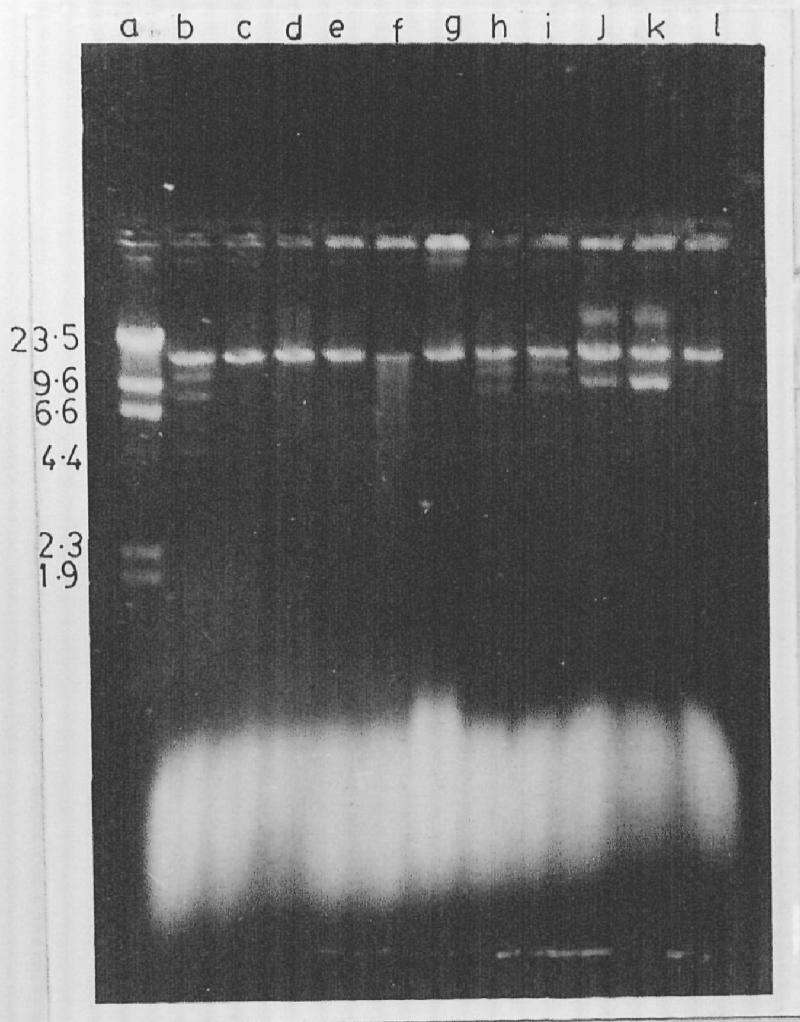

PLATE. XV

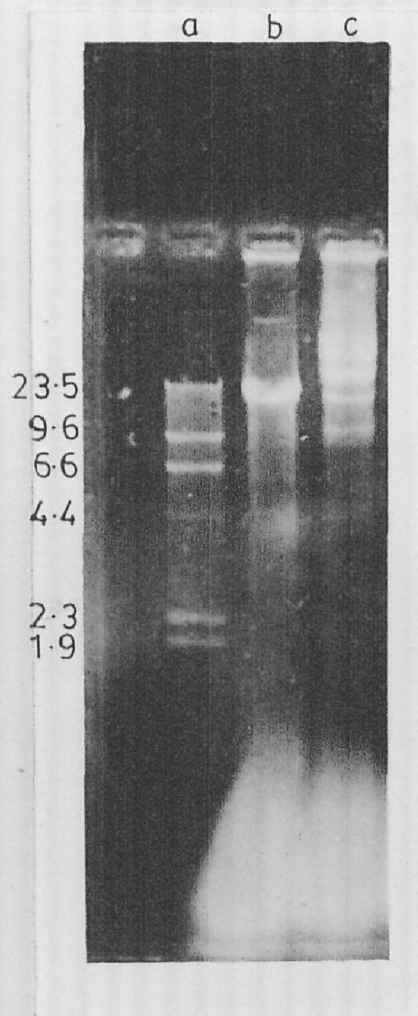

PLATE. XVI

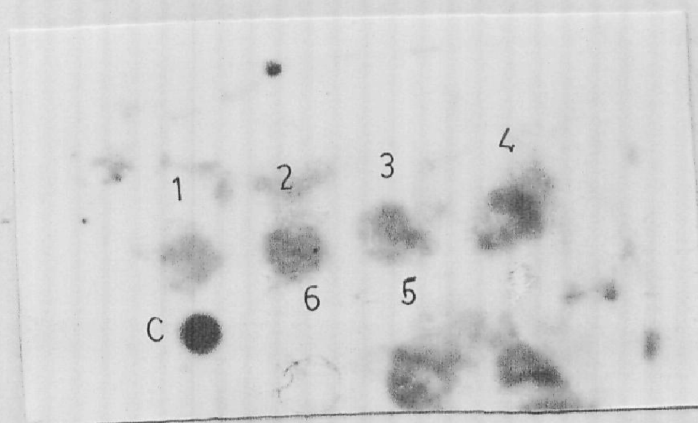

PLATE. XVII

The transconjugants obtained from the transfer of each of the recombinant plasmids were pooled separately and they have been designated as Rcd 301 (pSRH1), Rcd 301 (pSRH2), Rcd 301 (pSRH3), Rcd 301 (pSRH4) and Rcd 301 (pSRH5).

4:8 Analysis of expression of *hup* gene(s) in the transconjugants under free-living conditions

In order to find out whether the Hup-determinants of *Azorhizobium* IRBG-46 cloned in plasmid pRK290 are expressed in the genetic background of *Cicer-Rhizobium* strain Rcd 301, the *hup* genes were derepressed in the transconjugants as obtained above under free-living conditions and H_2-uptake activity determined in them gas-chromatographically as described in Sections 3:9:2.

The results presented in Table 8 show the expression of uptake hydrogenase activity in all the five transconjugants studied. The transconjugants Rcd 301 (pSRH1) and Rcd 301 (pSRH2) showed the highest activity of 392 and 340 nmole H_2 h^{-1} mg protein^{-1}, respectively. The transconjugant Rcd 301 (pSRH5) showed the lowest activity of 134 nmole H_2 h^{-1}mg protein^{-1}. However, no activity was detected in the recipient *Cicer-Rhizobium* strain Rcd 301.

PLATE-XVIII

Transconjugants of *Cicer-Rhizobium* strain Rcd 301 grown on RMM-Tc

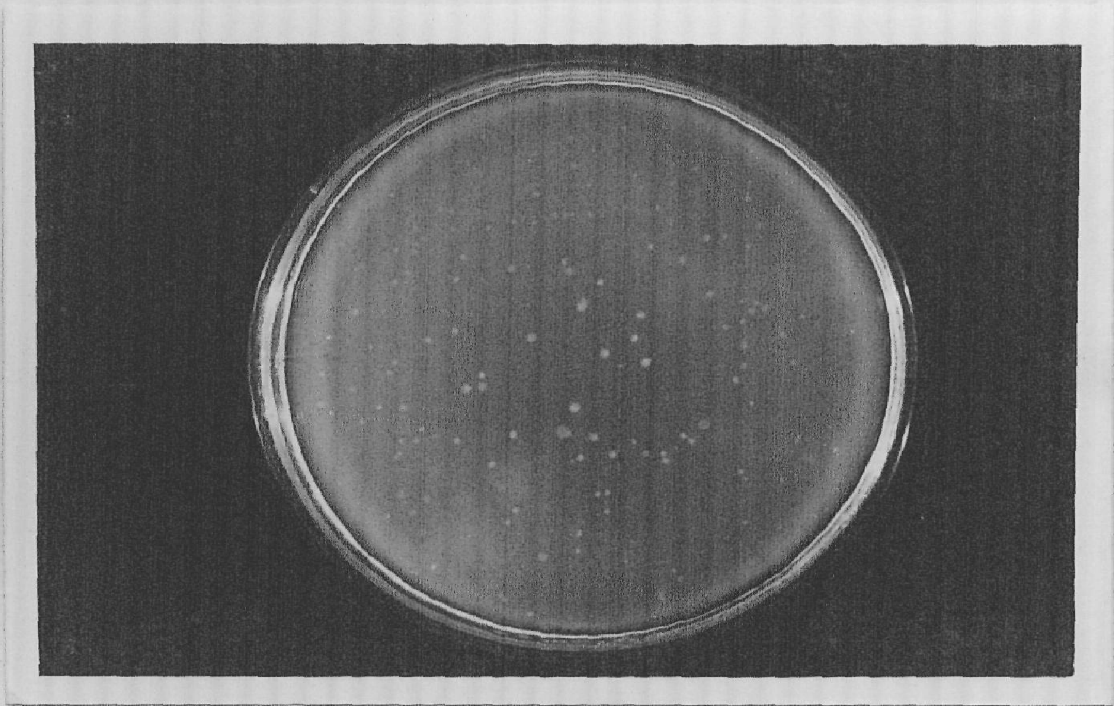

PLATE. XVIII

Table - 8 : Uptake hydrogenase activity in transconjugants of *Cicer-Rhizobium* strain Rcd 301 under free-living conditions.

Strain/Trans-conjugant	Protein (μg slant^{-1})	Uptake hydrogenase activity (nmolH_2h^{-1}mg protein^{-1})
Cicer-Rhizobium		
Rcd 301	-	nd
Transconjugants		
Rcd 301 (pSRH1)	154	392 ± 113
Rcd 301 (pSRH2)	156	340 ± 54
Rcd 301 (pSRH3)	141	219 ± 89
Rcd 301 (pSRH4)	231	184 ± 65
Rcd 301 (pSRH5)	156	134 ± 33

nd - not detected

Values are the mean ± SE of six replications.

DISCUSSION

All nitrogenase preparations obtained so far have been found to catalyze an ATP dependent H_2 evolution, even in the presence of N_2. Evidence has been presented indicating that this reaction also occurs *in vivo* in various N_2 fixing organisms. Schubert and Evans (1976) reported that 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 40-60% of the total energy flow through nitrogenase.

Oxidation of H_2 by nodule bacteroid H_2-uptake (Hup) system is known to protect nitrogenase from O_2 inactivation, generate ATP (Dixon, 1972), and provide reductant (Salminen and Nelson, 1984; Lodha and Naik, 1984). However, not even a single natural isolate of chickpea (*Cicer arietinum* L.) nodulating rhizobial strains with Hup^+ phenotype has been reported (Dadarwal and Sindhu, 1988; Vasudev *et al.*, 1990). Cosmid pHU52 containing *hup* genes of *Bradyrhizobium japonicum* has been transferred to *Cicer*-rhizobial strains and shown to express in this new genetic background. But the *hup* genes of *B. japonicum* cannot be derepressed in the presence of high organic carbon (Maier *et al.*, 1979). In contrast to this, *Sesbania*-rhizobia and stem nodulating azorhizobia are known to be derepressed in free-living *ex-planta* condition in the presence of high organic carbon substrates (de Vries *et al.*, 1984) Thus, the relevance of the differences in regulation of the Hup-system could be demonstrated by isolation and characterization of genetic determinants that are involved in the H_2 recycling process. Following identification of these determinants, they could be either used to complement Hup^-

mutant strains or transferred to other rhizobial spp. in order to study their complete organization.

Since not much is known about the H_2-uptake system of *Azorhizobium* which is derepressed even in the presence of organic carbon substrates, the present investigation was undertaken to clone *hup* genes of *Sesbania* stem nodulating *Azorhizobium* strain IRBG-46 and to transfer these to Hup$^-$ *Cicer-Rhizobium* strain Rcd 301.

Expression of *hup* genes of *Azorhizobium* strain IRBG-46

Before preparing the gene bank of *Azorhizobium* strain IRBG-46, it was necessary to see the expression of *hup* genes under symbiotic and *ex-planta* conditions, and to characterize the strain with respect to antibiotic resistance markers and plasmid profile.

The expression of *hup* genes under symbiotic condition was studied by calculating the relative efficiency (RE) of electron transfer to nitrogen through nitrogenase. According to Schubert and Evans (1976), if the RE value is more than 0.8, then it is considered that the bacterium possesses *hup* genes. Two out of the four strains of *Rhizobium* sp. (*Sesbania*) tested were found to be H_2-uptake positive as their RE was more than 0.8. Since the *Azorhizobium* strain IRBG-46 showed the highest RE of 0.95, it was selected for further studies.

For studying the effect of carbon substrates on the expression of Hup-activity, it is necessary that the activity is derepressed under *ex-planta* condition so that the growth conditions can be easily

manipulated. Carbon substrates have been reported to repress *ex-planta* Hup activity (Maier *et al.*, 1979; Simpson *et al.*, 1979) in *R. japonicum*. However, lack of carbon substrate repression of Hup activity in *B. japonicum* SR and in other strains of *B. japonicum* have been reported (van Berkum and Maier, 1988; vanBerkum, 1987). de Vries *et al.* (1984) provided the first report that in *Azorhizobium* ORS571, the presence of an organic carbon source does not repress or inhibit hydrogenase synthesis activity. More recently, Saini *et al.* (1987) also demonstrated Hup activity on high organic carbon media (YEMA and nitrogenase induction media) in all 8 Hup^+ strains of *Rhizobium* sp. (*Sesbania*) studied. In the present study also, when the *Azorhizobium* strain IRBG-46 was grown on high carbon media like YEMA slopes (mannitol, 10 g/L) and YELA slopes (lactate, 10g/L) for the derepression of Hup activity, high Hup activity (2290 and 2504 nmol H_2/h/mg protein) was observed. The activity on YEMA was 4 to 9 fold higher in this strain when compared to the activity reported for 8 Hup^+ strains of *Rhizobium* sp. (*Sesbania*) by Saini *et al.* (1987). When the low carbon medium (HUM slopes) was used (sucrose + arabinose + sodium gluconate, 2g/L), the Hup activity was 2.1 and 2.3 fold higher compared to the activity obtained with YELA and YEMA media, respectively (see Table 5). The derepression of Hup activity on high carbon media might be attributed to the fast depletion of carbon thereby reducing the O_2 concentration, since O_2 at high concentration is known to repress Hup activity (Merberg *et al.*, 1983). Another possibility could be that the *hup* genes in this strain constitute an hydrogenase operon which is less sensitive to high carbon repression. Study to unravel the functioning of

the Hup-system on these lines at the biochemical level has been made by Merberg *et al.* (1983), but this study has been performed on *R. japonicum*, in which the synthesis of Hup-system in free-living cells is repressed both by O_2 and carbon substrates. They have selected constitutive mutants which express hydrogenase in 10.0% partial pressure O_2 which were also simultaneously less sensitive than the wild type to repression by organic carbon substrates, and have suggested that a common regulatory element may be involved in the control of hydrogenase synthesis by O_2 and carbon. Thus, to experimentally prove the basis or cause for derepression in *Azorhizobium* strains, it would be essential to study the genetic loci of their *hup* genes with respect to the above parameters.

Once it was established that the *hup* genes of *Azorhizobium* strain IRBG-46 are expressed under both symbiotic and *ex-planta* conditions, the strain was characterized with respect to antibiotic resistance markers for the purpose of its maintenance in pure culture form. The strain was found resistant to ampicillin and nalidixic acid at concentrations of 50 μg/ml and 15 μg/ml, respectively and sensitive to tetracycline, rifampicin and kanamycin on TYM agar plates.

As regards the plasmid profile of the *Azorhizobium* strain used in the present investigation, it was found to contain one megaplasmid of approximately 280 kb size and one small plasmid of approximately 55 kb size. However, the presence of megaplasmid in *Azorhizobium* strain ORS 571 could never be demonstrated (Jarvis *et al.*, 1986).

Selection of *hup* gene probe for *Azorhizobium* IRBG-46

In order to be able to isolate the *hup* genes from the *Azorhizobium* strain, it was necessary to have an available *hup* gene probe which would show homology with *hup* genes to be isolated. Although, closer relatedness of an *Azorhizobium* ORS 571 to the bradyrhizobia than to the fast-growing rhizobia has been suggested (Jarvis *et al.*, 1986), studies of Tn5 insertions in the hydrogenase operon of the ORS 571 genome have shown that these insertions map in the same region of the genome which shares a high degree of DNA homology with the *hup* locus of *R. leguminosarum* described by Tichy *et al.* (1987). In the present investigation also it was found that *Azorhizobium* IRBG-46 DNA hybridizes with the *hup* genes of *B. japonicum* cloned in pLAFR1 (pHU52) (see Plate I). This thus shows the genetic conservation of some *hup* sequences in diverse species of rhizobia.

Localization of *hup* genes in *Azorhizobium* strain IRBG-46 and construction of gene bank

In order to localize the presence of *hup* genes, it was thought worthwhile to cure the megaplasmid using an appropriate curing agent like acridine orange at a concentration of 150 μg/ml. Electrophoretic determination of the loss of the megaplasmid was established by performing an *in situ* lysis of the cured strain. In cured strain, no band was visible corresponding to the megaplasmid band observed in the uncured strain. In order to further demonstrate whether *hup* genes are present on chromosomal DNA of strain IRBG-46, the expression of uptake hydrogenase activity was studied in the plasmid cured and

uncured strains by growing them on HUM slopes and measuring H_2-uptake gas-chromatographically. Since the Hup activities were found to be comparable in both the cured and the uncured strains (see Table 7), it is suggested that the *hup* genes are present on the chromosome of IRBG-46 and can be induced for expression of uptake hydrogenase activity in the free-living conditions. Further, it was decided to prepare a gene bank of *Azorhizobium* strain IRBG-46 in order to isolate the *hup* genes from this strain.

The preparation of a gene bank necessitates the use of a vector which can take up larger inserts, as the *hup* genes could span a region of 15-20 kb.A broad host range plasmid vector, pRK290 which can take up large inserts was chosen for the gene bank construction. Another advantage of cloning *Rhizobium* DNA in a vector such as pRK290, instead of in a ColE1 based plasmid or cosmid is that the clone bank can be used in genetic studies because the RK2 replicon will function in *Rhizobium* cells (Friedman *et al.*, 1982).

Plasmid pRK290 has unique *Eco*RI and *Bgl*II sites (see Fig.1), either of which can be utilized for the construction of the gene bank. In the present study, the unique *Eco*RI site of this plasmid has been utilized. A partial *Eco*RI digest of the strain IRBG-46 total DNA was made in order to get maximum fragments in the size range of 15-23 kb.

After checking the partial digest on a 0.4% agarose gel (see Plate IV), the partially digested DNA was subjected to a sucrose density gradient centrifugation to obtain fragments of the desired range. The

fragments following reprecipitation were used for ligation with the *Eco*RI restricted vector pRK290. Before ligation of the insert DNA with pRK290, the linearized vector was phosphatased using calf intestine alkaline phosphatase. This is a necessary step in order to reduce self ligations among the pRK290 DNA molecules so as to improve the efficiency of the gene bank. Following phosphatase treatment, the enzyme was removed to prevent its interference with the next step of ligation.

The insert DNA and the vector DNA were ligated in an equimolar ratio as the sizes of the two are approximately comparable. The recombinant plasmids were then transformed into *E. coli* HB101 in order to constitute the gene bank. The basis of selection of the transformants was the appearance of colonies on tetracycline (Tc, 10 μg/ml) supplemented LB plates. Plasmid pRK290 confers Tc resistance to the transformed HB101 cells which are otherwise sensitive to tetracycline. But, to determine whether the transformed colonies consist of recombinant pRK290 plasmids or self-ligated pRK 290 plasmids, an *Eco*RI restriction analysis of randomly picked clones is essential. In the present study an *Eco*RI restriction analysis of clones obtained from a single ligation showed 45% of them possessing inserts, (see Plate VI).

The scheme for the construction of gene bank of *Azorhizobium* strain is shown in Fig. 2.

Isolation of *hup* genes of *Azorhizobium* IRBG-46

In order to isolate the *hup* genes from the gene bank of

Fig. 2: A schematic representation of the cloning strategy used to construct the gene bank of *Azorhizobium* strain IRBG-46.

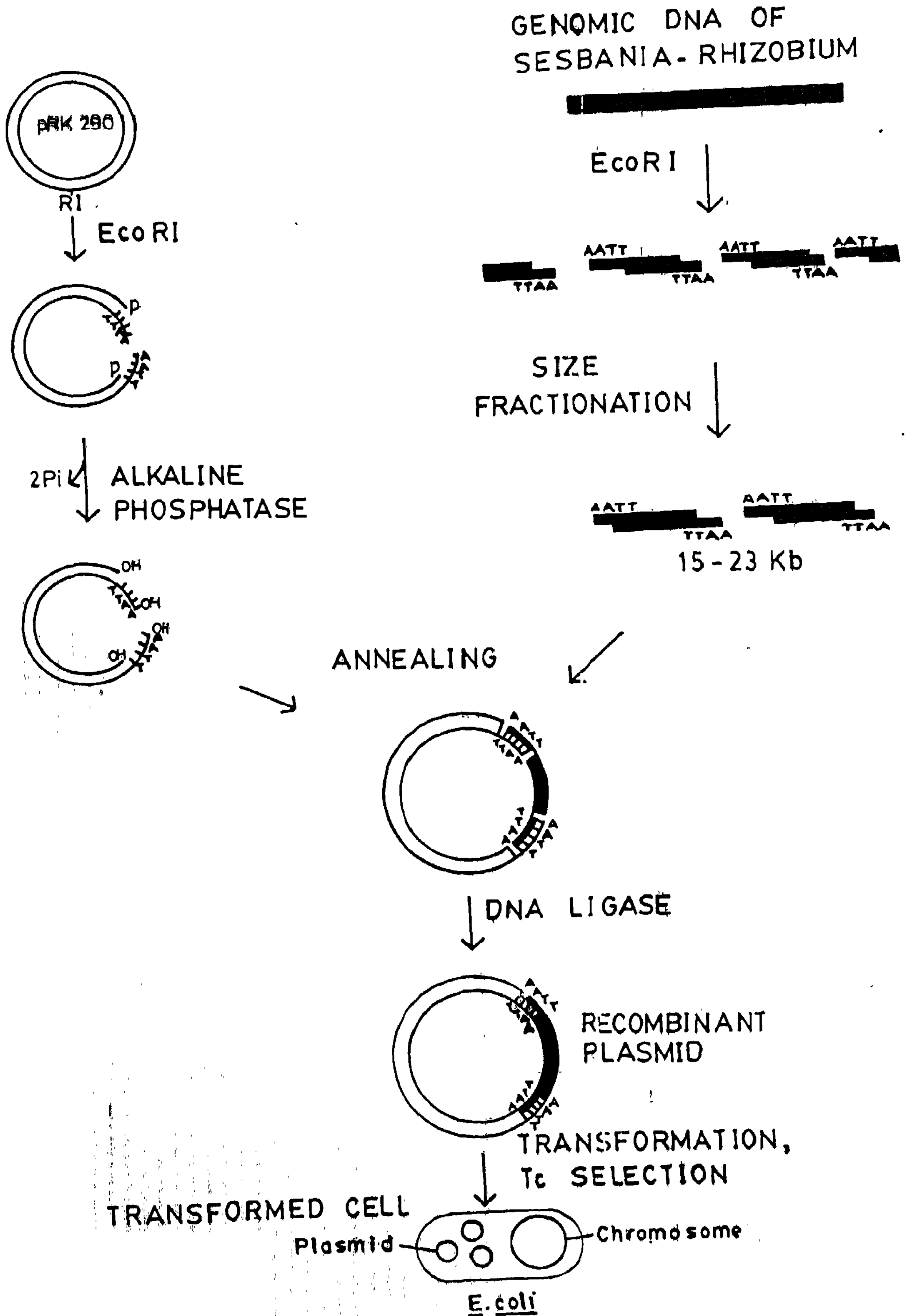

FIG. 2

Azorhizobium IRBG-46 constructed as above, a primary screening of 2,000 randomly picked clones was done by colony hybridization. The probe used was the *hup*-specific 12.9 kb *Eco*RI fragment of pHU52. A total of 22 positive signals (both weak and strong) were obtained following the primary screening. Earlier, the whole cosmid pHU52 was used as a probe to demonstrate homology between the genome of strain IRBG-46 and the *hup* genes of *B. japonicum*. Since, later only one *hup*-specific fragment has been used to initially screen the bank, it became necessary to follow it up with an *Eco*RI restriction analysis and secondary screening of the presumptive clones.

A total of 13 clones showing stronger signals were selected and subjected to a complete *Eco*RI restriction digestion (see Plates XV & XVI). Seven clones were found to harbour inserts of different sizes. Only 5 clones were chosen for a secondary screening by a DNA:DNA dot-blot hybridization. The recombinant plasmids, designated as pSRH1, pSRH2, pSRH3, pSRH4 and pSRH5 were taken for dot-blot hybridization with the 12.9 kb *Eco*RI fragment of pHU52. All the 5 recombinant plasmids showed positive signals and thus were showing homology with the probe used.

Since, only one *hup*-specific fragment has been used to probe for the *hup* genes desired to be isolated in the present study, it may be possible that all the *hup* genes are not present in the clones obtained. Thus, even though large DNA inserts have been taken for preparation of the gene bank in order to minimize the possibility of the *hup* genes getting truncated, it is essential to actually observe the Hup-activity

when these genes are transferred across genetic barriers to other Hup⁻ *Rhizobium* spp. In view of this, the expression of cloned *hup* genes was studied in *Cicer-Rhizobium* strain Rcd 301. Earlier report on the *hup* locus of *Azorhizobium* ORS 571 suggest that it spans regions between 9.0 and 16.6 kb (de Vries *et al.*, 1988).

Transfer of recombinant pSRH plasmids into *Cicer-Rhizobium* Rcd 301 and expression of *hup* genes

A functional Hup-system has not so far been detected in any natural strain of *Cicer-Rhizobium* (Vasudev *et al.*, 1990). Transfer of *B.japonicum hup* genes (pHU52) to *Cicer*-rhizobia has been successfully accomplished by Vasudev *et al.* (1990) and Kunnimalaiyaan *et al.* (1992). Since the above studies have shown the expression of the transferred *hup* genes in the genetic background of *Cicer-Rhizobium*, it was considered worthwhile to transfer the *hup* genes of *Azorhizobium* IRBG-46 to check for their expression in this *Rhizobium*. In view of this, the five pSRH plasmids were conjugally transferred to the *Cicer*-rhizobial strain Rcd 301, and the different transconjugants namely, Rcd 301 (pSRH1), Rcd 301 (pSRH2), Rcd 301 (pSRH3), Rcd 301 (pSRH4) and Rcd 301 (pSRH5) were tested for the Hup activity under free-living conditions on HUM slopes.

Though all the transconjugants showed Hup activity, the highest activity of 392 nmol H_2 h^{-1}mg protein^{-1} was observed in Rcd 301 (pSRH1) (see Table 8). Cosmid pHU52 containing *hup* genes of *B. japonicum* were conjugally transferred into Hup- *Cicer-Rhizobium* strains G36-84 and BG-4 (Vasudev *et al.*, 1990). The highest specific

activities obtained in G36-84 (pHU52) and BG-4 (pHU52) were 150 and 130 nmol H2h-1 mg proteing-1. The present study though demonstrated interspecies transfer of *Azorhizobium hup* genes and their expression in a Hup⁻ *Cicer-Rhizobium* strain, the activity obtained is very low when compared with the parent strain IRBG-46. Further studies are needed to demonstrate whether all the genes (structural and regulatory) have been isolated and cloned or whether the Hup activity in this strain is host-regulated in some way as demonstrated in soybean bradyrhizobia (van Berkum, 1990). For this, the physical and genetic analysis of the clones obtained should prove to be useful in elucidating the biochemical, genetic and regulatory factors involved in the complex H_2-uptake system of this *Azorhizobium*.

Thus, a gene bank of Hup^+ *Azorhizobium* strain IRBG-46 nodulating *Sesbania rostrata* has been prepared, and screened for recombinant plasmids, pSRH containing *hup* genes. These plasmids when transferred to Hup^- *Cicer-Rhizobium* Rcd 301 have conferred Hup activity in free-living conditions. However, it remains to be seen whether the Hup activity is expressed under symbiotic conditions. In addition, the gene bank can be further screened for getting clones with still higher Hup-activity, and studying the organization of *hup* genes. This would provide an answer as to why these genes are not repressed in the presence of high organic carbon substrates.

SUMMARY AND CONCLUSION

Nitrogenases from all known sources catalyzed an ATP-dependent reduction of not only N_2 to NH_4^+ but also protons to H_2. This H_2 loss results in an inefficient use of the energy provided for the N_2-fixing process. A minority of strains of *Rhizobium* possess the capability for synthesis of an H_2-recyling system that oxidizes the H_2 produced during N_2 fixation, thus recapturing some of the energy expended during H_2 evolution. Efforts leading to an understanding of H_2-uptake (*hup*) gene(s) organization in *Sesbania*-azorhizobia are still at the preliminary stage. Some available reports show the presence of an Hup-system in these rhizobia 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. In view of this, efforts have been made to isolate *hup* genes from the gene bank of Hup^+ *Sesbania Azorhizobium* strain IRBG-46, and to transfer these to Hup^- *Cicer-Rhizobium* strain Rcd 301 in an attempt to improve its energy efficiency. The salient findings of the present investigation are summarised below :

(i) Initially, studies on the H_2-recycling ability of *Azorhizobium* strain IRBG-46 and some isolates have been carried out under symbiotic conditions. Based on the calculations of relative efficiencies, strain IRBG-46 having a highest RE of 0.95 was selected for further work.

(ii) The *Azorhizobium* strain IRBG-46 was then assayed for *hup* expression under free-living conditions on low organic carbon containing H_2-uptake medium (HUM) as well as media containing high organic carbon, i.e. YEMA and YELA media. The strain was

found to exhibit high specific activity in all the media tested, though the specific activity was the highest on HUM.

(iii) In order to be able to isolate the *hup* genes from the strain IRBG-46, it was necessary to select a *hup* gene probe for it. For this, a DNA:DNA dot-blot hybridization experiment was performed in which the total DNA of the strain was hybridized with nick-translated pHU52 DNA as a *hup* gene probe. The strain IRBG-46 showed a strong positive signal with the pHU52 DNA.

(iv) Characterization of the *Azorhizobium* strain IRBG-46 revealed the presence of a megaplasmid and a small plasmid of approximate 280 kb and 55 kb sizes, respectively.

(v) Further experiments were performed to localize the *hup* genes present in this strain. Curing of megaplasmid and assaying the cured strain for Hup activity gas-chromatographically showed no loss of activity in this strain. Thus, it was concluded that the *hup* genes are present on the chromosome.

(vi) In order to isolate the *hup* genes from this *Azorhizobium* strain, a gene bank of the strain was constructed in *E. coli* HB101 using a broad host range mobilizable plasmid vector pRK290.

(vii) Initial screening of randomly picked 2,000 clones from this gene bank by colony hybridization using the oligo-labelled 12.9 kb *Eco*RI fragment of pHU52 as probe revealed 22 positive clones.

(viii) An *Eco*RI restriction analysis of 13 presumptive clones revealed the presence of inserts in 7 of them. The insert sizes ranged from approximately 15 to 22 kb. Five positive clones from these were picked up for a secondary screening of their recombinant plasmids by DNA:DNA dot-blot hybridization using the oligo-labelled 12.9 kb *Eco*RI fragment of pHU52. All showed a positive signal with the probe used.

(ix) The five recombinant plasmids containing Hup determinants of *Azorhizobium* strain IRBG-46 have been designated as pSRH1, pSRH2, pSRH3, pSRH4 and pSRH5. These pSRH plasmids were conjugally transferred into Hup^- *Cicer-Rhizobium* strain Rcd 301 in order to check the expression of *hup* genes in this new genetic background.

(x) Gas-chromatographic assay for Hup activity in the trans-conjugants Rcd 301 (pSRH1), Rcd 301 (pSRH2), Rcd 301 (pSRH3), Rcd 301 (pSRH4) and Rcd 301 (pSRH5) all showed expression of *hup* genes in this *Rhizobium* under free-living conditions on H_2-uptake medium. When compared with the parent strain, IRBG-46, this activity was low and varied from 134 to 392 nmol H_2 taken h^{-1} mg $protein^{-1}$.

In conclusion it can be said that recombinant plasmids containing *hup* genes of *Sesbania-Azorhizobium* strain IRBG-46 can be isolated from the gene bank prepared. Few clones isolated from the gene bank conferred H_2-uptake activity on the *Cicer-Rhizobium* strain Rcd 301. However, the bank can be used for isolating such more clones, which can be used for studying the genetic organization of *hup* genes.

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