PLASMID ANALYSIS IN AZOSPIRILLA

By
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CERTIFICATE - I

This is to certify that this dissertation entitled "Plasmid analysis in Azospirilla", submitted for the degree of Doctor of Philosophy in Microbiology of Haryana Agricultural University, is a bonafide research work carried out by Ms.Renu Kerpal (85-BS-49D) under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

(P. TAURO)
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CERTIFICATE - II

This is to certify that the dissertation entitled "Plasmid analysis in Azospirilla", submitted by Ms.Renu Kerpal (85-BS-49D) to the Haryana Agricultural University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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CHAPTER - I

INTRODUCTION

INTRODUCTION

Bacteria of the genus *Azospirillum* are free living diazotrophs that have been isolated from the rhizosphere and roots of forage and grain grasses. It is suggested that biological nitrogen fixation by *Azospirillum* species in association with the cereal roots may contribute significant amounts of nitrogen to plant growth. However, results from field experiments carried out since 1975 have led to the conclusion that *Azospirillum* spp. promote plant growth only under low fertility conditions and that the net beneficial effect observed by *Azospirillum* inoculation is the result of many other properties in addition to nitrogen fixation.

In recent years, a number of studies have been carried out to understand and improve the beneficial effects of *Azospirillum* application. To improve the process, however, a detailed knowledge of the bacterium is essential. So far little is known about the genetic organisation of this bacterium or molecular biology of its association with plants. Mutants impaired in nitrogenase activity by site directed transposon mutagenesis have been developed allowing a study of the *nif* genes in *Azospirillum* spp. A major difficulty in understanding the genetics of *Azospirillum* spp. is the lack of a suitable system for transposon mutagenesis like that applied to the genetic analysis of *Rhizobium* and *Pseudomonas* spp.

Azospirillum spp. contain plasmids of varying number and sizes. The number of such plasmids has been found to vary within a species. These plasmids in Azospirillum may confer resistance to various antibiotics and heavy metals. In addition, it is likely that they may also be involved in nitrogen fixation, hydrogen oxidation production of growth promoting substances etc., all properties associated with this bacterium. So far, however, none of these characterstics has been localized on any of the plasmids except in Azospirillum brasilense strain ATCC 29710 in which carbenicillin resistance has been shown to be associated with the plasmid. This study was aimed at a better understnading of the nature of plasmids in this bacterium in order to improve it with respect to its various beneficial activities.

CHAPTER - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Since the first report by Döbereiner and Day (1976) that azospirilla can fix atmospheric nitrogen in association with the roots of grasses, nitrogen fixation in tropical grass bacterial associations has now been well established (Pedresen et al., 1978; Nur et al., 1980; Döbereiner and Baldani, 1981; Haahtela et al., 1981; Hegazi et al., 1981 and Baldani et al., 1983). Amongst the various associative diazotrophs reported till date, Azopsirillum species (Table-1) have been of considerable importance and are thought to be major organisms responsible for nitrogen fixation in the roots of cereals and grasses (Döbereiner and Day, 1976). Further, among the bacteria that are able to fix nitrogen in free living conditions azospirilla display the maximum nitrogenase activity. As a result of this considerable interest has been displayed in understanding this organism and this is testified by the large number of papers and reviews published on this subject (Klingmüller, 1982, 1983, 1985; Okon, 1985). Other beneficial effects of Azospirillum are promotion of root hair development and root branching (Tien et al. 1979; Patriquin et al., 1983), increase in dry matter and mineral accumulation in plant parts (Kapulnik et al., 1983; Sarig et al., 1984) and improvement of water status of the plant (Okon, 1984).

The morphological and physiological characteristics of the genus Spirillum described by Döbereiner and Day (1976) agree with the description of Spirillum lipoferum (Beijerinck,

Table 1. List of *Azospirillum* spp.

S.No.	Species	Host	Reference
1.	*A.brasilense*	C-3 grasses(Wheat, *Digitaria*)	Tarrand *et al.*, 1978
2.	*A.lipoferum*	C-4 grasses(*Sorghum*)	Tarrand *et al.*, 1978
3.	*A.amazonense*	Cereals	Magalhaes *et al.*, 1983
4.	*A.halopraefrans*	Grasses	Döbereiner, 1987
5.	*Herbaspirillum seropedicae* (*Azospirillum seropedicae*)	*Sorghum*	Baldani *et al.*, 1984

1925) summarized in Bergey's Manual (Kreig and Döbereiner, 1984). Kreig (1977) observed that the DNA base composition of eleven S.lipoferum isolates was 69-71 moles % G + C, a value much higher than the average 38% found in the members of the genus Spirillum and he suggested the generic name Azospirillum Consequently, Tarrand et al. (1978) defined the Azospirillum genus and two species A.brasilense and A. lipoferum. Subsequently two more species viz. A.amazonense and A. halopraefrans were isolated (Magalhaes et al., 1983; Döbereiner, 1981).

Azospirillum species are aerobic, Gram negative bacteria, curved rods with a polar flagellum and contain globules of poly-β-hydroxybutyrate. The demonstration of high nitrogen fixing ability by the organism isolated from Digitaria roots when low concentration of energy sources and semisolid media were used (Döbereiner and Day, 1976), brought this organism into the forefront among free-living nitrogen fixing bacteria.

Azospirilla are capable of using a very wide variety of carbon and energy sources for growth on NH_3 or N_2. Azospirillum brasilense preferentially utilizes organic acids such as malate, succinate, gluconate ,lactate and pyruvate but also grows satisfactorily on sugars such as galactose, arabinose and fructose A.lipoferum utilizes glucose while A. amazonense can also use sucrose (Burris et al., 1978; Döbereiner, 1983; Del-Gallo et al., 1984). It has been shown by Del-Gallo et al (1984) that A. brasilense and A. lipoferum posess all the enzymes of the Embden-Meyerhoff-Parnas Pathway and of the TCA cycle. Both species poses

all the enzymes of the Entner-Doudoroff Pathway (Westby et al., 1983; Del-Gallo et al., 1984). These enzymes are however induced only by growth in the presence of gluconate. Oxidative hexose monophosphate pathway was not operative in these organisms. Similar results have been reported for A. brasilense Sp 7 by Westby et al. (1983).

A.brasilense Cd is reported to respond chemotactically to a wide variety of amino acids, sugars and organic acids (Barak et al., 1983). These observations were made using a chemotactic assay under conditions that prevented aerotaxis (Barak et al., 1983). Aerotaxis when measured by the capillary method was found to mask chemotaxis of Azospirillum to attractants (Barak et al., 1983).

When grown on N_2 at low pO_2, A.brasilense synthesizes poly-β-hydroxybutyrate (PHB) which comprises about 30% of the cell dry weight. In cells grown under high pO_2 and in the presence of NH_4Cl, PHB constitutes less than 1% of the cell weight (Burris et al., 1978). Since bacteria containing large amounts of PHB are found to be more resistant to high temperatures, drying or radiation and osmotic shock (Okon, 1985), the presence of this chemical in this organism may have a role in their ability to survive in soil.

The ability of Azospirillum to be chemotactically attracted to utilize carbon and energy sources present at low concentrations in the rhizosphere efficiently, as well as its ability to store carbon and energy as PHB makes this organism a good candidate for adaptation and colonization of the rhizosphere.

2.1 Nitrogen metabolism

Azospirilla adapt to the constantly changing nutritional conditions in the rhizosphere by their capacity for nitrogen fixation, assimilatory NO_3^- reduction, respiratory reduction of NO_3^- to NO_2^-, N_2O or N_2 and nitrate dependent N_2 fixation (Bothe et al., 1983; Döbereiner, 1983). In nitrogen free semi-solid medium, Azospirillum behaves as a typical micro-aerobic organism and forms a pellicle below the surface (Döbereiner and Day, 1976; Barak et al., 1982). Azospirillum is also capable of growth under fully aerobic conditions in the presence of combined nitrogen. Under steady state growth conditions in a chemostat, the protein content, succinate oxidase and superoxide dismutase activities of A.brasilense Cd cells increased sharply as compared to the respective activities detected in cell extracts under low dissolved oxygen tension (d.o.t.) (Nur et al., 1982). Under high d.o.t. Azospirilla tend to clump (Nur et al., 1981). A.brasilense Cd readily adapted in the chemostat to intermediate d.o.t. levels by producing red carotenoids (Nur et al., 1981; Nur et al., 1982). This observation has been confirmed by using mutants of A. brasilense Sp7 and Cd that do not produce carotenoids Hartmann et al., 1983 , suggested that carotenoids perhaps protect Azospirillum growth and nitrogen fixation under certain oxygen stress conditions.

Azospirillum grows well under very low d.o.t. In the presence of unlimited carbon supply, excess reductant is disposed off by synthesis of very large amounts of PHB (Okon, 1985). Under these conditions (d.o.t. 005 - 0.01) the cells produce less carotenoids, (Nur et al., 1982) and express nitrogenase activity

and grow in N-free medium(Burris et al., 1977; Burris et al., 1978). The nitrogenase in Azospirillum brasilense has been studied by Ludden et al.(1978). In order to obtain the nitrogenase components, the organism was cultured in a chemostat under oxygen controlled conditions (Okon et al., 1977; Ludden et al., 1978; Pedrosa and Yates, 1984). Properties of the nitrogenase complex are similar to those found in other nitrogen fixing bacteria (Ludden et al., 1978). The partially purified nitrogenase enzyme contained the normal Mo-Fe and Fe proteins as well as the activating factor of Fe protein (Ludden et al., 1978). Using antisera prepared against K. pneumoniae Mo-Fe and Fe proteins, cross reacting material was precipitated from nitrogen fixing cultures of A.brasilense Sp7 (Ludden et al., 1978). Molecular weights of the polypeptides after SDS gel electrophoresis were estimated to be 60,000 and 64,000 for the Mo-Fe protein and 33,000 and 36,000 for Fe protein (Nair et al., 1983), which agrees clearly with the values for these proteins reported for other organisms. These polypeptides are not detected in cells grown in ammonia or under aerobic conditions.

2.2 Hydrogen metabolism

In Azospirillum, like in other diazotrophs, hydrogen evolution is intrinsically associated with nitrogen fixation in an irreversible, energy dependent reaction catalysed by nitrogenase (Chan et al., 1980). In other aerobic nitrogen fixing bacteria such as Rhizobium and Azotobacter, the hydrogen evolved is recycled by an uptake hydrogenase (Smith et al., 1976; Bothe et al., 1977; Dixon, 1978). In Azospirillum brasilense Sp7, a high uptake hydrogenase activity has been

reported (Berlier and Lespinat, 1980; Chan et al., 1980; Kundu and Tauro, 1985; Gupta, 1987).

Chan et al. (1980) showed that hydrogen evolution in nitrogen fixing A.brasilense was presumably mediated by nitrogenase. However, hydrogen evolution was not detected in cultures grown in an inert atmosphere when nitrogenase mediated hydrogen production would be expected to be highest, suggesting the presence of an efficient uptake hydrogenase capable of oxidizing hydrogen at a rate equivalent to its rate of maximal production. This uptake hydrogenase was found to be stimulated by low levels of carbon sources (Pedrosa et al., 1980).

An enhancement of uptake hydrogenase in A.chroococcum under carbon limiting conditions has been reported (Maier et al., 1978; Walker and Yates, 1978). In Azotobacter hydrogen is used to reduce carbon dioxide to carbohydrate and lower concentration of C-source allows better derepression of hydrogenase (Walker and Yates, 1978). Pedrosa et al. (1982) reported that A.brasilense was capable of hydrogen dependent acetylene reduction by whole cells starved of carbon metabolites. Hydrogen did not support acetylene reduction when sufficient carbon was available, although, carbon substrate did not inhibit hydrogen dependent respiration. Mixed cultures of Hup^+ and Hup^- strains showed higher nitrogenase activity than that of pure Hup^- culture suggesting that Hup character is beneficial for nitrogenase (Wang et al., 1985). Tilak et al. (1986) reported that hydrogenase of Azospirillum spp. strain CC is located in the particulate fraction (cytoplasmic membrance bound) and it cross reacts with antibodies raised against the membrane bound hydrogenase of A. eutrophus.

Hydrogen oxidizing chemoautotrophic ability has also been reported in Azospirillum (Malik and Schlegel, 1981). Similar hydrogen dependent chemolithotrophy has been observed in Azospirillum isolated from wetland rice (Watanabe et al., 1982; Gowda and Watanabe, 1983). Gowda and Watanabe (1985a and b) confirmed stimulation of nitrogen fixation by hydrogen in the rhizosphere association with field grown, decapitated rice plants by $^{15}N_2$ incorporation. They found that addition of 10% hydrogen stimulated acetylene reduction associated with excised roots, decapitated and intact plants of rice grown in a continuously flooded field. Gupta (1987) has demonstrated using both varying growth conditions as well as mutants defective in hydrogenase of A.brasilense that loss of H_2ase leads to a decrease in growth and nitrogen fixation to the extent of about 30%. It is, thus, possible that the presence of an hydrogen dependent nitrogen fixation and hydrogen dependent carbon dioxide fixation (Gowda and Watanabe, 1983) could provide these bacteria an advantage of survival in nature, especially in carbon limited soil, endo and ectorhizospheres.

2.3 Genetics of Azospirillum

One of the main problems in the development of experimental programs in Azospirillum has been the unforeseen difficulty in obtaining stable mutations. Wood et al. (1982) concluded that the difficulty met in mutagenizing Azospirillum even after treatmen with powerful mutagens, is due to the peculiar structure of its genome. According to these investigators, the Azospirillum genome does not have a main chromosome, but is composed of a set

of different overlapping plasmids. The presence of multiple copies of each gene may explain the difficulty in obtaining mutations. These conclusions are, however, not confirmed by others (Plazinski et al., 1983; Del-Gallo et al., 1985), because both electron microscopy and gel electrophoresis have revealed the presence of plasmids in this organism and this hypothesis seems to be improbable because of its genome size which is only 1.8 times the genome of E.coli.

Among the classical techniques of mutagenesis previously described for E.coli (Miller, 1972) such as UV irradiation or treatment with ethyl methane sulfonate (EMS), N-methyl-N' Nitro-N-nitrosoguanidine (NTG) etc. only EMS and NTG were effective in raising mutation frequencies (Del-Gallo et al., 1985). The most likely explanation of the unusual behaviour of A.brasilense is that this bacterium, like Hemophilus influenzae, Proteus mirabilis etc. lacks SOS functions (Walker, 1984), as a result of which the DNA following treatment with NTG is not repaired and leads to a mutation.

Despite the difficulties isolation of mutants impaired in various functions has been reported. In A.brasilense Sp7 (Franche et al., 1981), A.brasilense 13t (Wood et al., 1982), and A.brasilense Sp6 (Polsinelli et al., 1980) using an enrichment procedure for auxotrophs the frequency of mutation is raised to $1x10^{-3}$ to $5x10^{-3}$ (Hartmann et al., 1983). Spontaneous antibiotic resistant mutants have been isolated without mutagenesis (Mishra and Roy, 1979). However, the spontaneous rate is low ($1x10^{-8}$).

Antimetabolite resistant mutants and those defective in nitrogen metabolism (Barberio et al.,1982; Hartmann, 1982; Hartmann et al., 1983) have also been isolated.

The suicide plasmid pJB4J1 (IncP, Gm, Mu,Tn5) used by Beringer et al. (1978) to introduce Tn5 in the chromosome of Rhizobium was found to be very useful in Azospirillum (Elmerich and Franche, 1982). Plasmids pSUP 2021 (Simon et al.,1983) and pGS9 (Singh and Klingmüller, 1986) which can not replicate outside the enteric bacteria were used for introduction of Tn5 in Azosprillum brasilense Sp7. Using suicide vector plasmids s.a. pBR 325 with Tn5, Tn5 Mob RP_4 (Simon et al., 1983) and Tn V (Tn5 Replicon pSC101, Furuichi et al., 1985), a variety of transposon and vector insertion mutants of A.brasilense (auxotrophic, chemotactic, motionless, IAA deficient and others) have been reported. These plasmids deliver transposon in A. brasilense at frequencies of 10^{-8} - 10^{-6} per receipient depending upon the nature of transposons and the recipient strains (Vanstockem et al., 1987; Borovok et al., 1988).

2.3.1 Genetics of nitrogen fixation

The genetics of nitrogen fixation in Klebsiella and few other bacteria has now been well demonstrated (Kennedy et al., 1981; Scott et al., 1982; Nguyen et al., 1983). In K.pneumoniae a cluster of 17 nif genes (nifregulon) is arranged in seven/ eight transcriptional units (Mac Neil et al., 1978; Merrick et al., 1980; Kennedy et al., 1981; Pühler and Klipp, 1981; Sibold, 1982). The structural proteins of nitrogenase are coded by the three genes nif H,nif D and nif K which are arranged in a single operon

(Kennedy et al., 1981). These three genes and the nif Y gene are transcribed in the order nif HDKY. This operon and part of nif E are carried on a 6.2 kb EcoR1 fragment which has been cloned in plasmid pSA30 (Cannon et al., 1979). These structural nif gene sequences are conserved between the diverse nitrogen fixing organisms (Nuti et al., 1979; Mazur et al., 1980; Ruvukun and Ausubel, 1980) including several strains of Azospirillum (Elmerich and Franche, 1982; Quiviger et al., 1982). The cloned nif HDK genes of K.pneumoniae have been used as radioactively labelled probes for Southern hybridization (Mazur et al., 1980; Hennecke, 1981; Ruvukun and Ausubel, 1981; Quiviger et al., 1982; Scott, et al., 1982) and on the basis of this it is established that the nif DNA sequences are highly conserved in different nitrogen fixing bacteria (Singh and Klingmüller, 1986). Two A.lipoferum strains and three A. brasilense strains were examined for structural nif gene sequences. The size of the homologous EcoR1 and Hind III fragments were different from one strain to another (Quiviger et al., 1982). With A.brasilense strain Sp7 the probe hybridized with a single 6.7 kb Hind III fragment and a 22 kb Bam H1 fragment. With A.lipoferum strain Br17, homology was found with two EcoR1 gragments of 16 and 1.8 kb and with a single 15 kb Hind III fragment (Quiviger et al., 1982). In the case of strains Sp7 and Br17, homology with a nif A probe was also detected (Nair et al., 1983).

2.3.2 Cloning of *nif* genes of *Azospirillum*

The structural genes for nitrogenase in *Azospirillum*, *nif HDK* are present on a 6.2 kb EcoR1 fragment and the three genes are transcribed as a single operon (Elmerich, 1984). Using homology with a *K.pneumoniae nif HDK* probe a 6.7 kb EcoR1 fragment designated as Abr1 was cloned from the total DNA of *A.brasilense* Sp7 (Quiviger *et al.*, 1982). Heteroduplex analysis performed with *nif HDK* cluster of *K.pneumoniae* established the approximate localization of the corresponding *Azospirillum nif H,D,K* genes in this fragment (Quiviger *et al.*, 1982). The Abr1 fragment carrying *nif* genes was subcloned in the broad host range vector pRK290 (Ditta *et al.*, 1980) and after construction of partial diploids in *Azospirillum*, complementation of a few Nif^- mutants was observed (Jara *et al.*, 1983). By heteroduplex analysis, the approximate localization of *nif H* was found in the 2.8 kb EcoR1-Pst 1 fragment of Abr1 (Perroud *et al.*, 1985) and by complementation analysis, it was established that the *nif HDK* genes are transcribed as a single operon.

Elmerich *et al.* (1988) cloned a 30 kb region from *Azospirillum* containing the structural *nif HDK* genes. The presence of *nif* genes in the 20 kb region located next to *nif HDK* was explored after subcloning various restriction fragments in the broad host range suicide plasmid pSUP2021. Over fifty Tn*5* induced mutations, randomly inserted in the 20 kb region were obtained and recombined in the host genome of Sp7. Two new *nif* loci were identified and located about 5 kb and 12 kb respectively downstream from *nif K*. Hybridization with heterologous

nif probes from *K.pneumoniae*, *Bradyrhizobium japonicum* and *Azorhizobium sesbaniae* was performed to characterize the new *nif* regions. These studies revealed that the region proximal to *nif K* contained *nif E* and the distal region contained genes homologus to *nif US* (Elmerich *et al.*, 1988). Hybridization with *fix* genes from *B. japonicum* and *A. sesbaniae* has revealed homology with *fix ABC* in the vicinity of *nif US*. The implications of this are at present not clear.

2.3.3 Characterization of *nif* mutants

Most mutants isolated by Bani *et al.* (1980) as poorly growing or unable to grow on nitrogen free medium had an Asm^- phenotype, while other mutants isolated by various mutagenic treatments fall into two categories viz. regulatory mutants and nitrogenase mutants (Elmerich, 1986). Pedrosa and Yates (1984) reported the isolation of Nif^- mutants with *nif A* and *ntrC* phenotypes. The *ntrC* mutants were complemented by plasmids pGE10 and pCK3 which carry *glnA ntrBC* and the *nifA* genes of *K. pneumoniae* respectively. Several other *Azospirillum* mutants derepressed for nitrogen fixation in the presence of high concentration of ammonium chloride (10mM) have been isolated (Pedrosa *et al.*, 1987) and used to determine the roles of *nif A* and *ntrC* genes in the regulation of nitrogenase synthesis and activity in *A. brasilense* Sp7. Mutant FP10 (*A.brasilense* $NifA^-$) was complemented by a recombinant plasmid (pEMS1, 48 kbp) from a gene bank contructed in the broad host range vector cosmid pVK102. These results suggest that the regulatory mechanisms in *Azospirillum* may be very similar to that of *K.pneumoniae*.

Two nitrogenase mutants defective in the structural genes

of Mo-Fe protein have been isolated (Jara et al., 1983; Pedrosa and Yates, 1984). One of them was characterized by complementation with pAB35 which contains the wild type nif HDK cluster, of K.pneumoniae and by lack of complementation by pAB36 which is depleted of nif D and a part of nifK but contains nif H (Jara et al., 1983). The mutant was also characterized biochemically by complementation of the crude extract with pure Mo-Fe protein of K.pneumoniae (Nair et al., 1983).

2.3.4 **Genetics of hydrogenase**

Studies on genetics of hydrogenase have developed only recently. The hup genes of R.japonicum that are involved in the synthesis Ni-hydrogenase have been characterized (Lambert et al., 1985). Cosmid pHU1 containing hup specific sequences from R.japonicum (Cantrell et al., 1983) complement with Hup^- mutants of A.chroococcum (Yates and Robson, 1985). The hup genes of R. japonicum are believed to be contiguous and comprise a 15.5 - 21.9 kb region divided into atleast three transcriptional units (Haugland et al., 1984). In A. eutrophus, the hox genes (hydrogen oxidation) are plasmid borne, encode both the membrane bound and a soluble NAD linked hydrogenase and contain the structural and regulatory genes in a 50 kb fragment (Cammack and Yates, 1986). The hup genes in R. leguminosarum and R.japonicum appeare to be plasmid borne while in A.chroococcum these appear to be located on the chromosome (Cammack and Yates, 1986). The genetics of hydrogenase system in azospirilla is relatively left unexplored except for a report on the isolation of mutant

strain Sp7029 (Gauthier and Elmerich, 1977) that was devoid of both nitrogenase and hydrogenase activities. However, subsequently both the activities were recovered. Contrary to this Gupta(1987) found that both the enzymes nitrogenase and hydrogenase are co-ordinately regulated but can be induced/derepressed indepdenent of each other though hydrogenase has a supporting role in the expression of N_2ase genes. The hup mutants decreased the N_2ase activity by 30% in A.brasilense Sp7.

2.4. **Phytohormone production**

In addition to nitrogen fixation it is believed that Azospirilla also help in plant growth by the production of growth stimulating factors. Appplication of Azorpirillum to cereals has been found to result in an increase in the number of lateral roots and root hairs (Tien et al., 1979; Umali Garcia et al., 1980; Kapulnik et al., 1981). This was most likely due to phytohormone production (Reynders and Vlassak, 1979; Tien et al., 1979; Barbieri et al., 1986). A.brasilense strain 13t, has been found to produce auxins (indole acetic acid and indole lactic acid), gibberellin and cytokinin like substances (Tien et al., 1979). Hartmann et al.,(1983) have examined 3 strains of A. brasilense including Sp7and Cd and three strains of A.lipoferum including Br 17 for indole acetic acid (IAA) and anthranillic acid production in malate medium. When the culture supernatants of all the strains were analysed, anthranillic acid was detected only in the strains of A. lipoferum whereas, IAA was found in both the

species. Addition of tryptophan stimulated the production of IAA in A.brasilense (Hartmann et al., 1983; Horemans and Vlassak, 1985) but the effect was small in the case of A.lipoferum (Hartmann et al., 1983). Mutants resistant to fluorotryptophan, which excreted higher amounts of IAA have been isolated from A.brasilense (Hartmann et al., 1983; Francesco et al., 1985). In these mutants the amount of IAA produced was much higher as compared to the wild type. However these overproducing mutants did not yield IAA in the absence of tryptophan.

2.5 Plasmids in Azospirillum

All Azospirillum strains examined so far have been found to contain plasmids of varying number. The size of these ranges from 4 Md to 300 Md (Franche and Elmerich, 1981; Heulin et al., 1982; Singh and Wenzel, 1982; Wood et al., 1982; Plazinsky et al. 1983; Vieille et al., 1987). These plasmids have been detected by electrophoretic migration in agarose gels of cell lysates obtained by various techniques such as those described by Meyers et al. (1976), Eckhardt (1978), Birnboim and Doly (1979), Casse et al. (1979) and Kado and Liu (1981). Several discrepencies between the number and the size of the plasmids within a given strain have been observed. For example, Singh and Wenzel (1982) found three plasmids of 5.4, 12 and 51 kb in A.lipoferum strain Br17 whereas Franche and Elmerich (1981) found a single large plasmid of about 200 Md in the same strain. Similarly in A. brasilense strain Sp7, Wood et al. (1982) reported the presence of seven plasmids, while in the same strain Michiels et al. (1985) reported the detection of only three plasmids and,

Vieille et al. (1987) found four plasmids. It is not clear whether this was due to instability of the plasmids, or due to technical artifacts or due to misnaming of the bacterial strain.

The taxonomic groups of Azospirillum can not be distinguished on the basis of their plasmid content as there is variation in the number even within the same species. Moreover, there is no indication that plasmids with the same apparent molecular weight belong to the same molecular species. The plasmids from various strains of Azospirillum have been purified by cesuium chloride density gradient centrifugation and their restriction endonuclease pattern has been studied (Franche and Elmerich, 1981; Singh and Wenzel, 1982). Hpa1 and Hind II were found to cleave plasmid pAL1 from A.Lipoferum whereas EcoR1 and Hind III had no site on the same molecule (Singh and Wenzel,1982).

So far, there is no evidence of any phenotype associated with the plasmids in Azospirillum except only one report by Singh and Wenzel (1982) who have found carbenicillin resistance to be associated with a plasmid in A.brasilense. Spontaneous loss (Franche and Elmerich, 1981), temperature curing (Heulin et al., 1982) and curing by acridine organe (Wood et al.,1982) of some plasmids has been reported but, no specific features were demonstrated as plasmid borne. Recently, homology was detected between total DNA from several Azospirillum strains and R. meliloti nodulation (nod and hsn) genes, which in Rhizobium are plasmid borne (Vieille et al., 1987). From A.brasilense Sp7, a 10 kb EcoR1 fragment sharing homology to R.meliloti hsn

region was cloned in pUC18 to yield pAB502 (Vieille et al. 1987). It was later found that the hsn homologous region was localised on the 90 Md plasmid, contained in strain Sp7 (Vieille et al., 1988). This was confirmed by mutagenesis of pAB502 with Tn5-Mob and recombination of the insertion into the plasmid p90.

2.6 Genetic transformation in Azospirillum

So far phages and plasmids of Azospirillum have not been used for the transfer of genetic material. Plasmid R68-45 (Haas and Holloway, 1978) can promote chromosome mobilisation in various Gram -negative bacteria including Rhizobium (Kondorosi et al.,1980)and A.brasilense strain Sp7 (Franche et al., 1981). Crosses were made with a series of multiple auxotrophs and the frequency of transfer was found to be above 10^{-6} per recipient. The ratio between recombinants and transcojugants which received R68-45 was always close to 10^{-5} and the plasmid was inherited by 90% of the recombinants. The marker transfer promoted by R68-45 appeared to be unpolarized and suggested the existance of multiple origins of transfer in Azospirillum (Elmerich and Franche, 1982), as has been previously reported in Pseudomonas aerugenosa (Haas and Holloway, 1978). Similar results were also obtained with A.brasilense strain Sp6 (Bazzicalupo and Gallori, 1983).

Recently vector pSUP5011 carrying Tn5-Mob has been used for mobilization of A.brasilense Sp245 plasmids in

plasmidless P.putida (Katzy et al., 1988). Kanamycin resistant transconjugants appeared from crossing with E.coli S17-1 (pSUP5011) with a frequency of 10^{-5} per recepient. With the help of plasmid RP_4 (Km^S derivative) kanamycin resistance was transferred from Azospirillum to P. putida AC340 (Matveev et al., 1988; Katzy et al., 1988). A number of Km^R Pseudomonas transconjugants have been obtained using 85 Md plasmid from Azospirillum brasilense but their stability in the new host has not been established.

CHAPTER - III

MATERIAL AND METHODS

MATERIAL AND METHODS

3.1 Bacterial cultures

Various bacterial cultures used in this study are listed in Table 2. Stock cultures of Azospirillum were maintained by regular transfers on malate medium (Pedrosa et al., 1980). E.coli cultures were maintained on nutrient agar and selective media slants.

The azospirilla were characterized by Gram staining, production of pigment by the cultures on malate medium, growth on various sugars, estimation of nitrogenase and hydrogenase activities and antibiotic resistance.

Chemicals used were from either Sigma Chemical Co., USA; Bethesda Research Laboratories, Neu Isenburg, W.Germany; Amersham International plc, Amersham, U.K.; Pharmacia LKB, Uppsala, Sweden; Hi-Media,Bombay; Sisco Research Laboratories, Bombay or CSIR Centre for Biochemicals New Delhi. Nitrogen and H_2 gases used in the N_2ase and H_2ase assay were from Indian Oxygen Limited, New Delhi.

3.2 Estimation of nitrogenase and hydrogenase

Nitrogenase and hydrogenase in Azospirillum were assayed by growing the cultures in Nfb medium (Pedrosa et al., 1980) with biotin (10 ug ml^{-1}) replaced by 200 ug ml^{-1} of yeast extract (Shukla and Kundu, 1986).

Table 2. Source of *Azospirillum* and *E.coli* strains used in the present study

Strain		Source
A.brasilense	Sp7	Prof. K.V.B.R. Tilak, IARI, New Delhi
	12S	Dr.B.S.Kundu, Department of Microbiology,H.A.U.Hisar
	B25	Prof.S.A.Dhalla, Bhavan's College, Bombay
	9	Dr.K.Chaudhary, Department of Microbiology,H.A.U.Hisar
A.lipoferum	A6	Dr.S.P.Wani, ICRISAT, Hyderabad
	708	Prof.R.H.Burris,Univ.Wisconsin,Modison, U.S.A.
A.amazonense	Y_1	Dr.B.S.Kundu,Department of Microbiology,H.A.U.Hisar
H.seropedicae	Z67	-do-
E.coli (pRK 290)		Dr.P.K.Sharma,Department of Microbiology,H.A.U.Hisar
E.coli C600 (RP4)		-do-
E.coli 1230 (R68-45)		Dr.S.Kumar, IARI, New Delhi

The composition of the medium was as follows:

Component	gl^{-1}
KH_2PO_4	1.200
Sod. malate	5.000
K_2HPO_4	0.800
$MgSO_4.7H_2O$	0.200
NaCl	0.200
Yeast extract	0.200
$CaCl_2$	0.200
$FeSO_4.7H_2O$	0.002
*Trace mineral soln	2.0 ml

*Trace mineral solution

$MnSO_4.H_2O$	1.75
H_3BO_3	1.40
$Na_2MoO_4.2H_2O$	1.00
$ZnSO_4$	0.12
$CuSO_4.5H_2O$	0.04

Agar was used at a concentration of 1.5% and 0.15% for solid and semi-solid medium, respectively. The pH of the medium was adjusted to 6.8 before autoclaving for A.brasilanse and A.lipoferum and 6.0 for A.amazonese, For preparing the inoculum, this medium was supplemented with 1.0 gl^{-1} of ammonium chloride (Pedrosa et al., 1980).

Inoculum was prepared by transferring a loopful of the culture from fresh (24 h old) malate agar plates into 50 ml of the malate medium containing ammonium chloride in 150 ml

Erlenmeyer flask. The culture was grown aerobically on a rotary shaker (250 rpm) at 30°C for 24h. The cells were then collected by centrifugation aseptically at 6,000 rpm for 10 min at 30°C in a Hittachi refrigerated centrifuge. The pellet was washed twice with sterilized N-free malate broth and resuspended in 5 ml of N-free malate broth. This was then used to inoculate 10ml of medium in a 50 ml flask and incubated at 30°C in a BOD incubator for 24 h under stationary conditions. From this, 0.1 ml was used to inoculate 5 ml of N-free semi-solid malate medium.

Nitrogenase activity was assayed as Acetylene Reduction Activity (ARA). Tubes (15 ml) containing 5 ml of the semisolid malate medium (Kundu and Tauro, 1987) were inoculated with 0.1 ml of the inoculum and after 24h of incubation, cotton plugs were replaced by presterilized serum stoppers (alcohol wahsed and UV irradiated) and 1.0 ml of the air was replaced with acetylene (C_2H_2) to create 10% C_2H_2 atmosphere in the assay tubes and incubated further for 24h. Gas samples (0.5 ml) were assayed for extent of acetylane reduction using a Nucon Dual Column Gas chromatograph equipped with Porapak Q column and Flame Ionization Detector (FID). Nitrogen was used as the carrier gas (flow rate: 30 ml min^{-1}) and H_2 as the fuel gas. Temperatures of oven, injector and detector were 150°, 110° and 110°C respectively. Standard ethylene was from EDT, Research, 65 IVY Crescent, London. Nitrogenase activity was calculated as n moles C_2H_4 produced h^{-1} mg^{-1} protein (Appendix-I).

The initial procedure used for estimation of hydrogenase was similar to that of nitrogenase except that 0.2 ml of the air was replaced by H_2 after 24hr of growth to create 2.0% H_2 atmosphere in the assay tubes. Gas samples (0.5ml) were assayed for initial and final levels of H_2 using a Perkin Elmer Sigma 3b Gas chromatograph having a Thermal Conductivity Detector (TCD) and moleculare seive 5A stainless steel column. Nitrogen served as the carrier gas with an inlet pressure of 180 KPa and a flow rate of 40 ml min^{-1}. The oven, injector and detector temperatures were 70°, 80° and 80°C respectively. The hydrogenase activity is expressed as n moles H_2 consumed h^{-1} mg^{-1} protein (Appendix-II).

3.2.1 **Bacterial cell protein determination**

Soluble proteins were extracted from the whole cells by adding 1.0 ml of 2N NaOH to the assay tubes containing 5 ml of the semi-solid medium and bacterial growth after estimting N_2ase and H_2ase activities. The tubes were kept in a boiling water bath for 10 min. The contents were then neutralized with 1.0 ml of 2 N HCl. The total soluble protein was then estimated in 1.0 ml of the neutralized extract using Folin Ciocalteau reagent (Lowry et al., 1951). Samples were centrifuged at 5000 rpm for 5 min to remove the agar particles before determining the colour intensi at 660 nm using a Bausch & Lomb Spectronic 20. Bovine serum albumen was used as the standard protein.

3.3 **Plasmid isolation and characterization**

3.3.1 **Small scale isolation**

For rapid isolation of small amounts of plasmid DNA, several methods were tested. These included the boiling method

(Holmes and Quigley, 1981); alkaline extraction method (Birnboim and Doly, 1979); Eckhardt's method (Eckhardt, 1978) and Simon's method (Simon, 1984). For analysis of the plasmids, cultures were grown in Luria broth (5 ml) for 24h, of the following composition;

Tryptone	10g
Yeast extract	5g
NaCl	10g
Distilled water	1L

3.3.1. **Boiling method** (Holmes and Quigley, 1981)

The culture was grown in 2.5 ml of Luria broth on a rotary shaker at 30°C. Cells were collected from 1.5 ml of the broth in Eppendorf tubes by centrifugation at 5000 rpm for 5 min using a Hettich Micro Rapid/K table top centrifuge at 25°C. The pellet was washed with 200 µl of TE-8 buffer (10mM TrisCl, 1mM EDTA, pH 8.0), and entrifugation and was then resuspended in 25 µl of S1 buffer of the following composition;

Sucrose	8%
EDTA	50mM
Tris	50mM
Lysozyme	2mg ml^{-1}

S1 buffer (except lysozyme) was sterilized at 10 lb in^{-2} pressure for 10 min and lysozyme was added just before use.

The suspension was vortexed and incubated on ice for 5 min and was followed by the addition of 25 µl of S2 buffer (S1 buffer + 10% Triton X 100). The contents were again vortexed

and incubated in a boiling water bath for 40-50 sec. The tubes were then quickly chilled by plunging in ice-cold water. When the tubes had cooled down, 250 µl of S3 buffer (500 mM NaCl, 10mM Tris, pH 8.0) were added and the contents were mixed thorughly by inverting the tubes several times. The contents were centrifuged at 12,000 rpm at 30°C for 20 min to separate the cell debris. The clear supernatant was transferred to another clean Eppendorf tube and the pellet was discarded. To the supernatant, an equal volume of phenol/chloroform mixture (1:1) was added and the contents were mixed gently by inverting the tubes followed by centrifugation for 5 min and the aqueous phase was transferred to another Eppendorf tube. The phenol/ chloroform step was repeated twice and finally, the aqueous phase was mixed with 500 µl of isopropanol, mixed thoroughly and kept at -20°C for 20 min. The contents were recentrifuged at 12,000 rpm for 20 min at 10°C to pellet the DNA. The DNA pellet was washed with 70% ethanol and dissolved in 50 µl of TE-8 buffer.

3.3.1.2 **Alkaline extraction procedure** (modified by Ish-Horowicz and Burke, 1981).

Bacteria were grown in 2.5 ml of Luria broth at 30°C overnight on a rotary shaker. One ml of the culture was transferred to Eppendorf tubes and centrifuged at 5000 rpm, for 5 min. The supernatant was drained off and traces were removed with the help of filter paper strips. The pellet was resuspended in 100 µl of solution I containing 5 mg ml^{-1} lysozyme.

Solution I

Glucose	50mM
Tris.Cl	20mM
EDTA	10mM
pH	8.0

Solution I was made in batches of 20 ml, autoclaved at 10 lb in^{-2} for 15 min and stored at 4°C. Powdered lysozyme was added just before use.

Cells were treated with lysozyme for 30 min in an ice bucket. This was followed by the addition of 200 µl of freshly made solution II (1% SDS in 0.2 N NaOH) which was prepared by dilution of a stock solution (20N NaOH containing 20% SDS). The contents of the tubes were mixed by gentle shaking on a vortex mixer and kept in ice for 5 min. To this, 150 µl of ice cold 3 M sodium acetate (pH 4.8) solution was added and the tubes were inverted several times to mix the contents and then kept in ice for 60 min. The contents were centrifuged at 12,000 rpm for 30 min at 4°C to settle the cell debris. The clear supernatant was transferred to a second tube and DNA was extracted twice with phenol/chloroform mixture (1:1) and finally with an equal volume of 95% cold ethanol. The pellet was finally suspended in 40 µl of buffer containing 5% Ficoll and 0.05% bromo-phenol blue.

3.3.1.3 **Preparation of phenol-chloroform mixture**

Phenol was distilled at 160°C and stored at -20°C in small aliquots. When needed, phenol was removed from the freezer, allowed to thaw and melted at 68°C. To this, 8-

hydroxyquinoline was added to a final concentration of 0.1%. This was then extracted several times with 1 M Tris (pH 8.0), till the pH of the aqueous phase was 7.6. This was followed by a final extraction with 0.1 M Tris containing 0.2% β-mercaptoethanol. The phenol so prepared was stored at 4°C.

Chloroform was mixed with isoamyl alcohol in the ratio 24: 1 v/v and phenol-chloroform mixture was prepared by mixing the above prepared phenol and chloroform in the ratio 1:1 v/v.

3.3.1.4 **Eckhardt's method** (modified by Rosenberg et al., 1981)

Cells were collected from 1.0 ml of an overnight grown culture as before by centrifugation at 5000 rpm for 5 min. The pellet was washed with 0.1% sarkosyl solution in TE-8 buffer, centrifuged and rinsed with the same buffer and was then resuspended in 20 µl of the lysozyme mixture of the following composition prepared in the electrophoretic buffer (Tris 89 mM, Boric acid 89 mM EDTA 2.5 mM, pH 8-8.2)

Lysozyme	7500 U ml^{-1}
RNase	0.3 U ml^{-1}
Bromophenol blue	0.05%
Ficoll	20%

After 5 min of incubation in the above solution, 30 µl of SDS mixture (0.2% SDS, 10% Ficoll in electrophoretic buffer) were added and mixed by gentle vortexing. Five µl of pronase solution (5 µg ml^{-1} in Tris-borate buffer) were added, mixed gently by tapping and kept for 5 min at room temperature. Hundered µl of 0.2% SDS and 5% Ficoll were then overlayed. The gel was loaded with the lysed cells and the wells sealed

with hot agarose to avoid any air bubbles. Electrophoretic buffer was then added in the chambers and electric current was passed initially for 30 min at 10mA followed by 40 mA for 4-6h.

3.1.5 **Simon's Method** (Simon, 1984)

Cell were harvested from 1.0 ml of an overnight grown culture as before and suspended in 20 µl of solution I.

Solution I (made in electrophoretic buffer)

Ficoll	7%
Sucrose	20%
RNase	1U ml^{-1}
Lysozyme	5 mg ml^{-1}

The solution was stored at 4°C. Lysozyme was added at the time of use.

The suspension was incubated for 10 min at room temperature Then 40 µl of solution II were added and incubated further for 20 min.

Solution II (in electrophoretic buffer)

SDS	1%
Sucrose	5%
Bromo phenol blue	0.05%

This solution was stored at 4°C.

The contents were mixed by shaking the tubes manually. After incubation, the gel was loaded with 50 ul of the lysate and 5mA current was passed for 30 min followed by 60 mA for 3-4h.

3.3.2 **Large scale isolation of plasmid DNA** (Godson & Vapnek, 1973)

Large scale isolation of plasmid DNA was done for restriction endonuclease digestion and transformation experiments.

The plasmid DNA for this purpose was isolated by the method of gentle lysis described by Godson and Vapnek (1973). The cultures were grown in 500 ml of Luria broth for 24h on a rotary shaker at 30°C. Cells were collected by centrifugation at 6,000 rpm for 5 min at 4°C in a Hittachi refrigerated centrifuge. The pellet was washed with 100 ml of an ice cold STE solution (0.1 M NaCl, 10mM Tris. Cl, ImM EDTA, pH 8.0) and resuspended in 10 ml of ice cold 10% sucrose made in 50 mM Tris.Cl (pH 8.0). The contents were transferred to a 50 ml centrifuge tube. To this, 2.0 ml of a freshly made lysozyme solution (10 mg ml^{-1} lysozyme in 0.25 M Tris. Cl pH 8.0) were added. The tube was shaken well and incubated for 10 min in ice. Then, 8.0 ml of 0.25 M EDTA were added and the contents were mixed by inverting the tubes several times and kept on ice for another 10 min. To this mixture 4.0 ml of 10% SDS were added. The contents were mixed quickly with a glass rod so as to disperse the SDS evenly throughout the bacterial suspension and gently so as not to shear the liberated bacterial DNA. Immediately after this, 6.0 ml of 5 M NaCl were added to give a final concentration of 1 M, mixed gently but thoroughly and placed in ice at least for 2h. This was followed by centrifugation at 20,000 rpm for 45 min at 4°C to remove high molecular weight DNA and bacterial debris. The pellet was discarded and the supernatant was saved. This was extracted twice with phenol/chloroform mixture and once with only chloroform. After each extraction, the aqueous layer was transferred to a clean tube. Following the final extraction, 2 volumes of 95% ethanol were added, the contents were mixed

thoroughly and kept at -20°C overnight. Next day the plasmid DNA was recovered by centrifugation at 20,000 rpm for 45 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol. The plasmid DNA was finally dissolved in 4 ml of TE-8 buffer (10mM Tris Cl, 1mM, EDTA, pH 8.0).

3.3.3 **Gel electrophoresis**

Tris-borate buffer of the following composition was used as the electrophoretic buffer;

Tris	89 mM
Boric acid	89 mM
EDTA	2.5 mM
pH	8 - 8.2

A 10X buffer was prepared and stored at room temperature. The buffer was diluted at the time of electrophoresis.

3.3.4: **Preparation of the Gel**

Agarose (0.7 - 0.8 g) was added to 100 ml of the electrophoretic buffer in a 250 ml flask. The slurry was heated in a boiling water bath until the agarose melted completely. The solution was kept at room temperature for cooling. In the mean time, the edges of a clean dry polystyrene plate were sealed with a cello type so as to form a mould of about 8 mm thickness. When the agarose solution cooled to 50°C, it was poured into the mould so as to form a gel 5-6 mm thick and immediately the comb was clamped, the teeth of which formed the sample wells. The distance between the plate and the teeth of the comb was kept 0.5 - 1.0 mm so that the wells were completely sealed at the bottom. After the gel was completely set (30-45 min at room

temerature) the comb and the tape were carefully removed. For vertical gel electophoresis the agarose was cast (1-3 mm thickness) in a vertical box. DNA samples, prepared by different methods were mixed with the loading buffer (5% glycerol, 7% Ficoll and 0.05% Bromo phenol blue) and loaded into the wells of the gel. The wells were then sealed and the gel was kept in an electrophoretic chamber filled with the buffer. The depth of the buffer was such that the gel was completely submerged. For vertical electrophoresis the upper and lower chambers were filled with the buffer.

3.3.5 Electrophoresis

The current was passed through the buffer for a defined period depending upon the method used. The loaded wells were kept on the side of the -ve electrode, so that the DNA would move towards the +ve electrode.

3 3 6 Staining and photography

For staining of the neucleic acids, ethidium bromide (0.5 $\mu g\ ml^{-1}$) was incorporated in the gel before pouring. Destaining of the excess dye was done by immersing the stained gel in 1mM $MgSO_4$ for 1 h at room temperature. Gel was then visualised using a UV transilluminator (Ultraviolet products Inc, U.S.A.) at 254 nm and photographed using a 400 ASA film.

3.4. Physical characterization of plasmids

3.4.1 Determination of molecular weights

Molecular weights of the plasmids were determined by comparing the mobility of the standard known molecular weight

plasmids RP_4 and R68-45 with the plasmids of Azospirillum brasilense B25. Vertical agarose gel electrophoresis was done by Simon's method. Electrophoresis was done initally at 5mA for 1 h followed by 60mA for 3-4h. The distance from the plasmid band to the sample well was measured (migration distance) and the relative mobility was calculated as:

$$\text{relative mobility} = \frac{\text{migration distance}}{\text{length of the gel}}$$

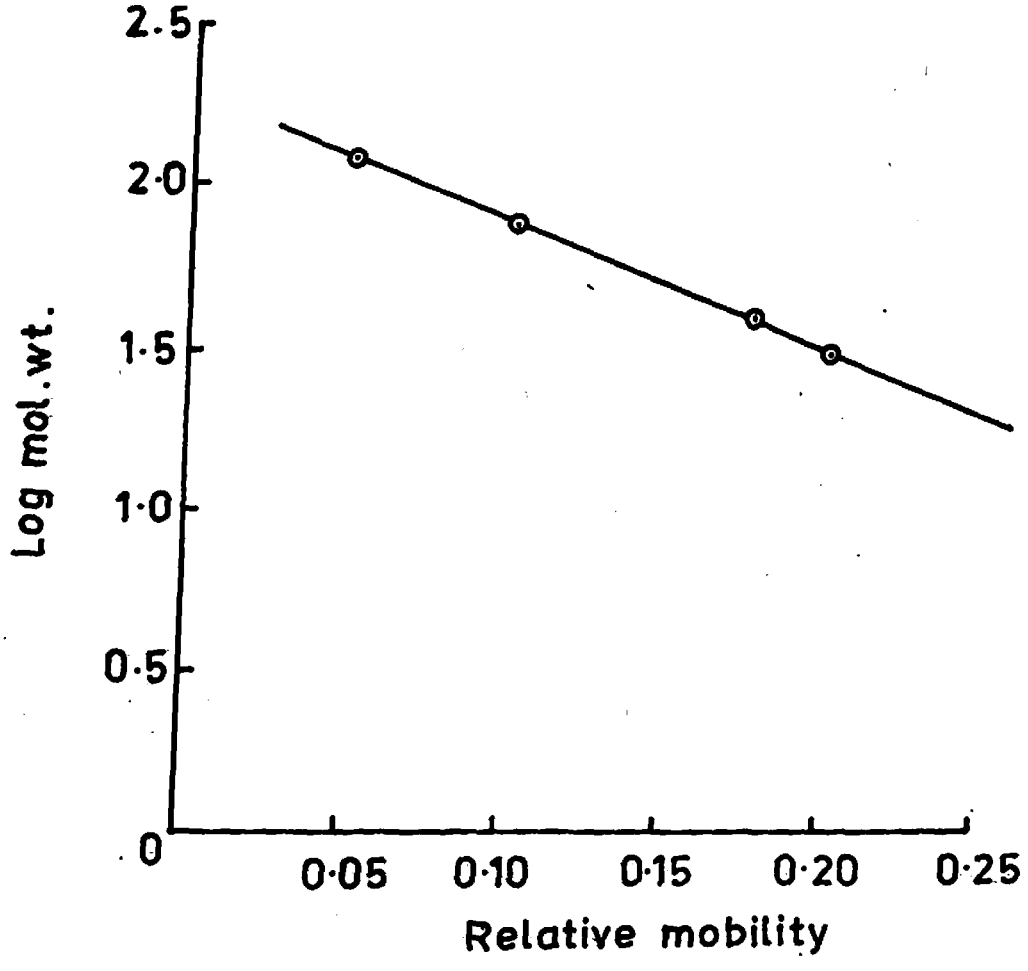
A standard curve was derived by plotting the log of molecular weight of the standards against their relative mobilities (Fig.1). The molecular weight of the unknown was determined by placing the value of the relative mobility of the unknown on the standard curve.

3.4.2 **Restriction analysis**

The cured strains (see 3.5.1) of A.brasilense B25 viz Ab1 and Ab6 carrying either of the plasmids pAb_1 and pAb_2 were grown in 500 ml of Luria broth on a rotary shaker for 24h. The plasmid DNA from both the strains was extracted by Godson and Vapnek's procedure described above. The purified plasmid DNA (250-300 ug) was finally suspended in 4.0 ml of distilled water From this, suspension, 18 µl was transferred to a clean sterile Eppendorf tube To this, 2 µl of 10 X digestion buffer was added (Appendix III). The contents were mixed by tapping the tube. Then 1 unit of each restriction enzyme (Eco R1, BamH1, Kpn1 and Sal 1) was added and the contents were again mixed (One unit of restriction endonuclease defined as the amount required to digest 1 µg of DNA to completion in 1 h in the recommended buffer and at the recommended temperature (Appendix-IV) in a 20 µl reaction mixture.

Fig.1 Molecular weight versus relative mobility of plasmid DNA

The plasmid DNA was isolated from A.brasilense B25, E.coli (R68-45) and E.coli (RP4) by Simon's method and subjected to electrophoresis. The approximate MW was calculated by comparing the relative mobility of pAb1 and pAb2 with R68-45 (39Md) and RP4 (36Md).


2.5
2·0
1·5
1·0
0·5
0
Log mol. wt.
0·05
0·10
0·15
0·20
0·25
Relative mobility

Incubation of DNA with the restriction enzymes was done at the appropriate temperature for 1h. The reaction was stopped by immersing the tubes in a water bath held at 70°C for 5 min. Then, 10 µl of the loading buffer without the tracking dye were added and the contents were mixed by tapping the tubes. The digested mixture was loaded in a 0.8% horizontal agarose gel. Electrophoresis was carried out in Tris-borate buffer at 20mA for 6-8 h.

The restriction enzymes were stored at -20°C. While carrying out the restriction enzyme digestion the reaction mixture was prepared to the point when all the reagents except the enzyme had been mixed. The enzymes were taken out of the freezer and immediately put in an ice bucket. They were used in the minimum possible time and placed back in the freezer after use. The gel was stained and photographed as described earlier.

3.5 Phenotypic characterization of the plasmids in A.brasilense Strain B25

This was based on curing, conjugation and transformation experiments.

3.5.1 Curing (Wood et al., 1982)

Plasmid curing was attempted using ethidium bromide, acridine organe, acriflavin at concentrations 2, 2.5, 3, 3.5, 4 and 5 µg ml^{-1}, SDS at concentrations 10, 25, 50, 75 and 100 µg ml^{-1} and a combination of ethidium bromide (2.5 µg ml^{-1}) and SDS (25 µg ml^{-1}). Stock solutions of the curing agents were prepared and stored at 4°C. Five ml nutrient broth in 15 ml tubes were prepared and suitable amounts of the curing agents

were added to give the required final concentrations. Tubes so prepared were inoculated with 0.1 ml of an overnight grown culture of A.brasilense B25 and incubated on a rotary shaker at 30° for 24h. Following incubation, the cultures were serially diluted and spread on nutrient agar plates so as to give around 50 colonies per plate. The plates were incubated at 30°C for 24-48 h which were then replicated on selective media plates containing various antibiotics viz. penicillin (500 $\mu g\ ml^{-1}$), ampicillin (500 $\mu g\ ml^{-1}$) amoxycillin (50 $\mu g\ ml^{-1}$), nalidixic acid (10 $\mu g\ ml^{-1}$), carbenicillin (100 $\mu g\ ml^{-1}$) erythromycin (10 $\mu g\ ml^{-1}$), streptomycin (10 $\mu g\ ml^{-1}$), chloramphenicol (10 $\mu g\ ml^{-1}$) and cephalosporidin (25 $\mu g\ ml^{-1}$) and heavy metals viz. Cadmium chlorid (200 $\mu g\ ml^{-1}$) and chromate (25 $\mu g\ ml^{-1}$). Growth on the plates was compared after incubation and clones missing on selective media plates were revived from the master plate and transfered on fresh nutrient agar plates for further testing. The clones which had lost one or the other marker were purified and maintaine on nutrient agar slants and kept at 4°C. Frequency of curing was calculated after five transfers as the percentage of total number.

To confirm that the strains were cured of the plasmids, gel electrophoresis of the cured variants was done according to the method of Simon (1984). Strains having lost the plasmids were selected and tested for the loss of other characteristics like nitrogenase, hydrogenase, carbon source utilization, polysaccharid utilization and growth hormone production.

3.5.1.2 **Antibiotic resistance pattern of the cured strains**

A.brasilense B 25 was tested for resistance against a

variety of antibiotics viz. penicillin, ampicillin, amoxycillin, carbenicillin, nalidixic acid, erythromycin, streptomycin, chloramphenicol and cephalospridin. Stock solutions of the antibiotics were prepared and sterilized by passing through presterilized sintered glass filter, Grade 5 (Corning). The solutions were kept in the refrigerator and suitable amounts were added to the medium to give the appropriate final concentration. The medium was cooled to 45-50°C before adding the antibiotics at the time of pouring. Suspensions of the cultures including the parent were streaked on these plates and incubated at 30°C for 48

3.5.1.3 **Heavy metal resistance pattern of the cured strains**

The cultures were tested for resistance against cadmium (100 μg ml^{-1}) and chromate (25 μg ml^{-1}). Stock solutions of these two compounds were prepared, sterilized by autoclaving and stored at 4°C. Suitable amounts from the stock solutions were added to the medium before pouring to give the required final concentrations. The cured strains along with the parent were streaked on these plates and incubated at 30°C for 48h.

3.5.1.4 **Ability to utilize different carbon sources**

A.brasilense B25 and the cured cultures were tested for growth on various carbon compounds viz. glucose, fructose, sucrose, lactose, maltose, mannitol, malate, galactose, arabinose, mannose, sorbitol, glycerol, xylose, xylene and toluene, which served as sole source of carbon and energy. The medium described by Pedrosa et al. (1980) was used in which malate was replaced by the desired carbon source (25 mM). The medium containing the carbon source was sterilized at 10 lbs in^{-2} for 10 min.

Various cultures along with the parent were tested for growth both in solid and liquid media having different carbon and energy sources and incubated at 30°C for 24-48 h or longer. The growth was estimated as + or - .

3.5.1.5 **Estimation of Indole Acetic Acid (IAA)**

Cultures were inoculated in malate broth supplemented with 1 g L^{-1} NH_4Cl and 100 µg ml^{-1} DL-tryptophan (Hartmann et al., 1983). Yeast extract and biotin were omitted to avoid any misinterpretation of phytohormone assay results. The tubes were incubated on a rotary shaker in dark for 6 days. After 6 days the cultures were centrifuged at 6,000 rpm for 10 min and the pellet was discarded. To 2.0 ml supernatant, an equal volume of Salkowski 's reagent was added.

Salkowski's reagent

$FeCl_3$	0.05 M
Perchloric acid	35%

The contents were mixed thoroughly and incubated at room temperature for 5 min for the development of pink colour, which was estimated colorimetrically at 500 nm using a Spectronic-21 spectrophotometer (Systronics). IAA (Hi-media) was used as the standard.

3.5.1.6 **Polysaccharide utilization**

Ability of the various strains to utilize polysaccharides such as pectin, starch and cellulose was also determined. The culture was grown in malate medium (+N) containing 0.25% pectin (Hubbel et al., 1978) and a loop-ful of cell suspension of each

strain was spotted on malate plates containing 0.25% pectin. The plates were incubated for 12 days at 30°C. Ability to utilize pectin was determined by flooding the plates with 2% aqueous solution of hexadecyltrimethyl ammonium bromide taking care not to dislodge the growth. After 20-30 min the plates were examined and the appearance of a clear zone around the colonies was considered as a proof of pectin utilizing ability of the strain.

For starch utilization, the cultures were directly spotted on plates containing 0.5% starch instead of malate. After 8 days of incubation at 30°C the plates were flooded with iodine solution. After 30 min. the plates were examined for the presence of a clear zone around the area of growth.

Cellulose utilization by Azospirillum strains was examined by directly spotting the cultures on plates containing 0.5% cellulose as the C-source. The plates were examined for growth after 10-12 days of incubation.

3.5.2. Conjugation

A.brasilense B25 was grown in nutrient broth at 30°C on a rotary shaker for 24h to give a titre of 2×10^8 to 3×10^8 cells ml^{-1}. The recepient (E.coli, pRK 290) was grown separately in nutrient broth and incubated overnight to give the same titre approximately.

Selective media plates containing a combination of tetracyclin (25 $\mu g\ ml^{-1}$) and either one of the other antibiotics viz. amoxycillin (50 $\mu g\ ml^{-1}$), streptomycin (10 $\mu g\ ml^{-1}$),

carbenicillin (100 μg ml^{-1}) or ampicillin (100 μg ml^{-1}) to which the recepient was sensitive, were prepared. Neither the donor nor the recepient could grow on these plates.

Conjugation was carried out by spot mating for this a drop of the recepient was first placed on a nutrient agar plate, and allowed to adsorb on the agar for 10-15 min. Then a drop of the donor was placed over the recepient spot and allowed to stand for another 15-20 min. Several spots were placed on 3-4 different plates and incubated at 30°C for 24-48 h in a BOD incubator. The thick growth from the spots was then scrapped and suspended in a small volume of sterile nutrient broth. The contents were mixed thoroughly on a vortex mixer. From this suspension 0.1 ml aliquots were spread on various selective media plates. The plates were then incubated at 37°C for 48 h and scored for exconjugants.

3.5.3. **Transformation**(Mandel and Higa, 1970)

3.5.3.1. **Preparation of cells**

The recepient (*E.coli*) was cultured in 250 ml of Luria broth and incubated on a rotary shaker for 24h. The cells were then chilled for 30-40 min on ice and transferred to sterilized cold centrifuge tubes and were harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The pellet was resuspended in 100 ml of cold 0.1 M $CaCl_2$ and incubated for 20 min on ice. The cells were collected again by centrifugation and resuspended in 5 ml of cold 0.1 M $CaCl_2$ and stored on ice for 24h in a refrigerator. Next day, the chilled cell suspension was vortexed and

sterile glycerol was added to a final concentration of 10%. Glycerol was added slowly and with gentle mixing to avoid heat release. The $CaCl_2$ treated cells were distributed in aliquots of 0.2 ml in sterile microfuge tubes for storage at -70°C in a Queue Cryostar (Japan). The tubes were removed from the freezer as and when required and each aliquot was sufficient for one transfomration.

3.5.3.2. **Transformation of E.coli with plasmid DNA**

Plasmid DNA for transformation was isolated by the method of Godson & Vapnok (1973). The required number of microfuge tubes containing $CaCl_2$ treated cells was removed from the freezer and thawed on ice. After thawing the cells were vortexed and 10 µl of the DNA solution was added to the tubes. The contents were mixed by tapping the tubes and reincubated on ice for 60 min. Following incubation, the cells were heat shocked for 2 min by transferring the tubes to a water bath at 37°C followed by incubation for 10 min at 25°C. Then 1.0 ml of sterile Luria broth was added to the microfuge tubes and incubated for 30 min at 37°C for phenotypic expression. The transformation mixture was then spread on selective media plates containing antibiotics and heavy metals. The plates were incubated at 37°C overnight and scored for transformants.

The transformants were maintained on selective medium containing the respective antibiotic/heavy metal.

CHAPTER - IV

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

4.1 Characterization of the strains

Before initiating studies on plasmid analysis in Azospirillum it was necessary to characterize the strains used in the present study. This was done by confirming their ability to i) fix N_2 and oxidize H_2 ii) to utilize various carbon sources iii) to produce pigmentation on nutritent and malate agar and antibiotic resistance.

For estimation of nitrogenase and hydrogenase, all the cultures were grown on nitrogen free semi-solid malate medium. It was found that all strains were positive for nitrogenase and hydrogenase (Table-3) confirming their identity as diazotrophs. The ARA and H_2ase levels, however, varied amongst these cultures. The ARA level was found to vary from 23 nm moles mg^{-1} protein h^{-1} to 106.4 n molas mg^{-1} protein h^{-1}, and the H_2ase level was found to vary from 13.5 n moles mg^{-1} protein h^{-1} to 291.5 n moles mg^{-1} protein h^{-1}. Further characterization was done on the basis of their ability to utilize malate, glucose, sucrose, fructose, citrate and glutarate (Table 4). It was found that malate and fructose were utilized by all the species. A. brasilense was not able to grow on glucose and A.amazonense was the only one to have the ability to utilize sucrose. Citrate and glutarate were utilized by A.lipoferum and H.seropedicae. A.brasilense and A.amozonense did not need biotin for growth. Azospirillum brasilense strains produce a pink pigmentation after 3-4 days

Table 3 . N_2ase and H_2ase in Azospirillum spp.

Species	Strain	ARA*	H_2ase*
	sp7	66.5	245.29
A.brasilense	12S	77.71	291.53
	B25	23.01	63.65
	9	75.36	69.66
A.lipoferum	A6	60.36	146.60
	708	94.46	316.84
A.amazonense	Y_1	106.40	13.50

*n moles mg^{-1} protein h^{-1}
Average of five replicates

Table 4. Characteristics of *Azospirillum* spp.

Characteristic	*Azospirillum* species			
	A.brasilense Sp7,B25,12S&9	*A.lipoferum* A6 & 708	*A.amazonense* Y_1	*H.seropedicae* Z67
Utilisation of				
Malate	+	+	+	+
Glucose	-	+	+	+
Sucrose	-	-	+	-
Fructose	+	+	+	+
Citrate	+	+	-	+
Glutarate	-	+	-	+
Requirements				
Biotin	-	+	-	-

of growth on nutrient as well as malate agar plates. All the cultures were long Gram-negative curved rods.

4.2.1. Standardisation of the method for plasmid analysis

In literature, the number of plasmids in some species and strains of Azospirillum has been detected and reported to contain 2-6 plasmids further, the same strain analysed in two different laboratories has been found to have varying number of plasmids for example in A.brasilense Sp7, presence of three, four and seven plasmids has been reported by different laboratories. Obviously, the method for investigation largely determined the efficiency of detection. In order to detect the correct number of plasmids in these strains several methods viz. boiling method (Homles and Quigley, 1981), alkaline hydrolysis (Birnboim & Doly, 1979), Eckhardt's method (Eckhardt, 1978); Simon's method (Simon, 1984) and the method of gentle lysis (Godson and Vapnek, 1973) were tried to determine which of the methods resulted in identifying the maximum and correct number of plasmids. The first four techniques were used for small scale preparation of plasmid DNA, two of which involved the isolation of DNA (boiling and alkaline hydrolysis methods) and then determining the number of plasmids by agarose gel electrophoresis The other two techniques (Eckhardt's and Simon's methods) involved gentle lysis of the cells directly in the gel wells or in the Eppendorf tubes. A.brasilense Sp7 and B25 were used in these stuides.

On gel electrophoresis the number of plasmids in A.brasilense Sp7, was found to vary from 0-2 when boiling and alkaline hydrolysis methods were used (Table 5) but with Eckhardt's and Simon's methods the number of plasmids obtained was 4 in the standard strain of A.brasilense Sp7. Although Simon's method and Eckardt's method gave equally good results but Simon's method was preferred since it is a simpler version of Eckhardt (1978), and was used for further studies. As regards Godson and Vapnek's method, this involves gentle lysis and minimum of shearing. It, therefore, worked very well with larger cell mass. This method also yielded 4 plasmids in strain Sp7 and was therefore, followed when plasmid DNA was required in larger amounts for transformation and restriction endonuclease digestion experiments.

4.2.2. **Detection of plasmid number in Azospirillum spp.**

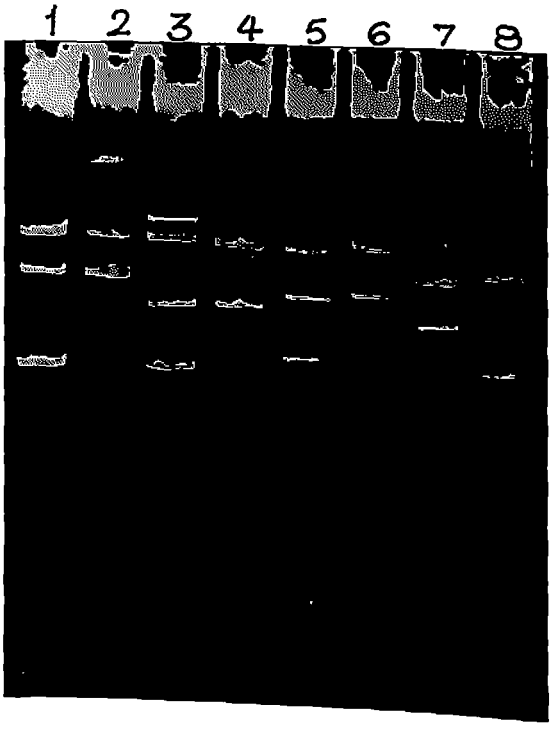
After standardizing the method for the detection of plasmids, the plasmid profile of the various strains belonging to different species (listed in Table 2) was examined. Gel analysis was repeated thrice so as to establish that the results are reproducible. Agarose gel electrophoresis by Simon's method revealed the presence of 2-4 plasmids (Fig.2) of various sizes in the various strains. The number of plasmids differed from one species to another for example, A.brasilense Sp7 had four plasmids, A.lipoferum three, A amazonense Y_1 had only two, while H.seropedicae Z67 had three. Interestingly, the number of plasmids varied from one strain to another within the same

Table 5. Plasmid pattern of A.bresilense as seen by different methods.

Method	No.of plasmids detected	
	Sp 7	B25
Boiling method	2	-
Alkaline hydrolysis	1-2	1
Eckhardt's method	4	2
Simon's method	4	2
Godson & Vapnek	4	2

Fig.2 Plasmid pattern of *Azospirillum* species

Lane 1. *A.lipoferum* A6
2. *A.brasilense* 12S
3. *A.brasilense* Sp7
4. *A.amazonense* Y_1
5. *H.seropedicae* Z67
6. *A.brasilense* 9
7. *A.brasilense* B25
8. *A.lipoferum* 708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species. A lipoferum strain 708 had 2 plasmids while strain A6 had 3 (lanes 8 and 1) similarly in A.brasilense the number varied from 2-4 (lanes 2,3,6 and 7), in different strains. The size of the plasmids also varied. As seen in Fig.2, the position of all the bands is not identical indicating that the molecular weight of the plasmids varies within the species. A.brasilense B25 (lene 7) having only two plasmids was selected for further studies. These are designated as pAb1 and pAb2 respectively

4.2 3. **Physical characterization of plasmids in A.brasilense B25**

Determination of molecular weights

The molecular weights of plasmids in strain B25 were determined by comparing their relative mobility with the standard known mol. wt. plasmid DNA molecules. The migration of plasmid DNA in agarose gels is inversly related to their molecular weights (Meyers et al., 1976). The larger the molecular weight the slower the rate of migration. Standard plasmid DNAs of known molecular weights viz. RP_4 (36 Md) and R68 - 45 (39 Md) carried in E.coli were also isolated and run along with A.brasilense B25. The cultures were lysed and loaded on the same gel and subjected to electrophoresis under identical conditions (Fig.3). In each case a single well defined band of DNA is observed for each plasmid species except for B25 which showed the presence of two distinct sharp bands The relative mobility of each band was calculated and plotted against log of molecular weight of the plasmid DNA (Fig.1).

Fig.3 Gel electrophoresis of DNA from A.brasilense B25 and E.coli

Lane 1,2 - A.brasilense B25;
3,4 - E.coli (R68-45);
5,6 - E.coli (RP4)


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From this, the molecular weights of the two plasmids viz. pAb_1 and pAb_2 in A.brasilense B25 were calculated (Table 6) which were determined to be about 90 and 120 Md, respectively. The chromosomal fragments, as is clear from the figure did not interfere with the detection of any of the plasmid bands.

4.2.4. **Restriction endonuclease digestion**

The two plasmids in A.brasilense B25 were further characterized by restriction endonuclease digestion since, restriction endonucleases cleave DNA molecules at specific sites (Maniatis et al., 1982). Four restriction endonucleases viz. Eco R1, Kpn1, Bam H1 and Sal 1 were used. To test the activity of the enzymes a standard plasmid pBR322 with known restriction sites was used and it was found that all the restriction enzymes were active (Fig.4). Plasmid pBR322 has only one site each for EcoR1, Bam H1 and Sal 1 but is not cleaved by Kpn1. The results are consistent with the published information (Maniatis et al., 1982).

The two plasmids pAb_1 and pAb_2 were purified separately from the cured strains Ab_1 and Ab_6. Before they were subjected to restriction endonuclease digestion, gel electrophoresis was carried out to detect any chromosomal contamination. The two plasmids were then incubated separately with the respective restriction enzymes as detailed in section 3.4.2, Appendix-III and Appendix-IV. On gel electrophoresis of the digestion mixtur it was found that only EcoR1 was able to cut the plasmid pAb_1 at 6 sites whereas the other three enzymes used did not cut any

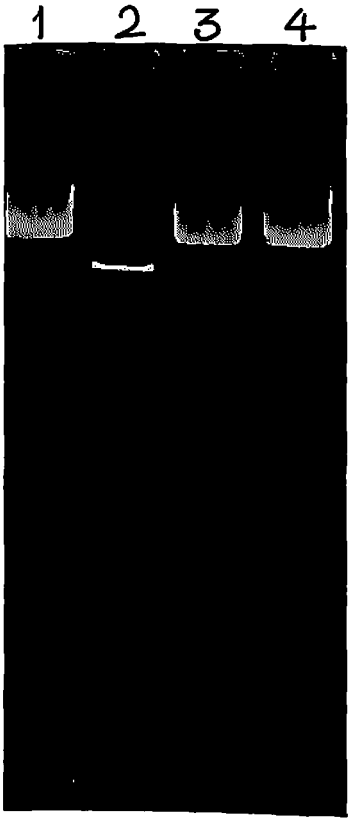
Table 6. Molecular weights of the plasmids from *A.brasilense* B25 and *E.coli*.

Culture	Plasmid	mol.wt.(Md)
E.coli	RP_4	36
E.coli	R68-45	39
A.brasilense B25	pAb_1	90
A.brasilense B25	pAb_2	120

Fig.4 RE digestion of the plasmid pBR322.

Plasmid pBR322 was purified and cleaved as described in Material and Methods and subjected to gel electrophoresis.

Lane 1. EcoR1;
2. Kpn1;
3. Bam H1;
4. Sal1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of the two plasmids at any sites (Fig.5) The enzyme and DNA concentration were varied but no difference in the results was observed. This suggests that either there is no site for these enzymes on these plasmid molecules or the DNA is modified to make it resistant to the restriction endonuclease attack (Yuan, 1981)

4.3. Phenotypic characterization of markers carried on the plasmids

Phenotypic characterization of the plasmids in A. brasilense B25 was done to undertstand their role in this organism. This was achieved in two ways i) by eliminating the plasmid and studying the phenotype of the cured strain and ii) by transfer of the plasmids to a recepient strain through conjugation or transformation and examining the change in the pheotypes of the recepient.

4.3.1. Curing

Ethidium bromide (EtBr), acridine orange (AO), acriflavin (Af) and sodium dodecyl sulphate (SDS) were used for the curing of plasmids. Before subjecting the culture to the curing treatments the minimum inhibitory concentration (MIC) of each agent for A.brasilense B25 was determined.

For this, nutrient broth tubes containing different concentrations of the curing agents were inoculated with same number of cells and incubated on a shaker at 30°C for 24 h. The MIC was found to be 2.5 μg ml^{-1} for EtBr, AO and Af, and50 μg ml^{-1} for SDS These concentrations were chosen for subsequent sutdies.

Fig.5 RE digestion of pAb1 and pAb2

The two plasmids were purified separately from the cured strains by Godson and Vapnek's method and subjected to RE digestion. Lanes 1-4 represent pAb1 while lanes 5-8 represent pAb2. Other details are:

Lanes	1,5	- EcoR1;
	2,6	- Kpn1;
	3,7	- Bam H1;
	4,8	- Sal1

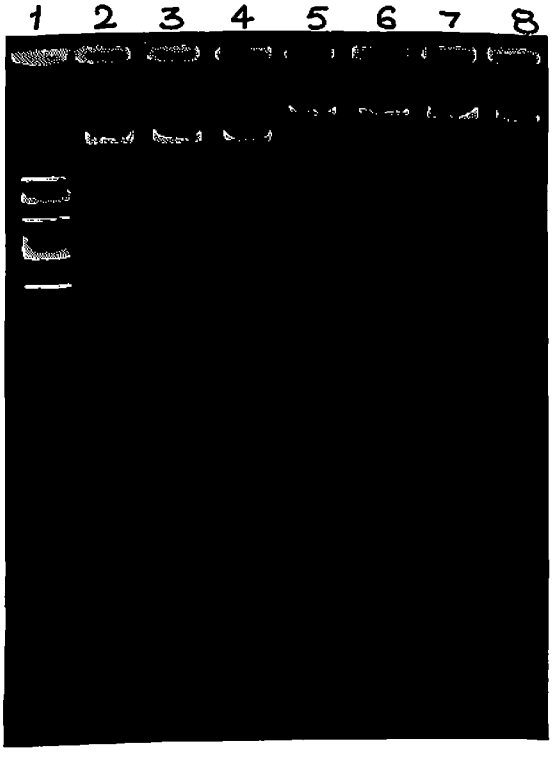
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Table-7 lists the different curing treatments and the number of clones screened for the loss of plasmids. None of the curing agents when used alone had any effect even on 5 serial transfers. Since individually these had no effect, a combination of the two chemicals, acridine orange and SDS was tested. When this was done few clones were obtained which were found to have lost either of the two plasmids. So as to enrich the medium with the cured strains (if at all they arose), spreading on selective media plates was done after 5 serial transfers.

4.3.2. Antibiotic resistance and plasmid analysis of the cured strains

To establish the identity of the cured strains, the mutants derived after the curing treatment were serially diluted and spread on nutrient agar plates (master plates) so as to give around 50 colonies per plate. The plates following incubation were replica plated on selective media plates and incubated for 24-48 h at 30°C. The clones not growing on selective media plates were picked up from the master plates and retested to confirm the loss of the respective marker. Table-8 shows the antibiotic resistance pattern of the various clones which had become sensitive to one or the other antibiotic. It is seen that all the clones are resistant to penicillin, ampicillin, cephalosporidine and nalidixic acid, however, some are sensitive to axomycillin, carbenicillin, streptomycin and erythromycin while others are sensitive to

Fig.3 Gel electrophoresis of DNA from A.brasilense B25 and E.coli

Lane 1,2 - A.brasilense B25;
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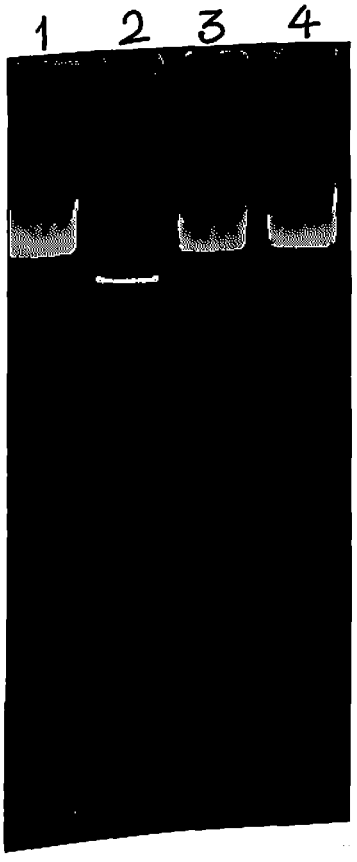
4. Sal1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	3,7	- Bam H1;
	4,8	- Sal1

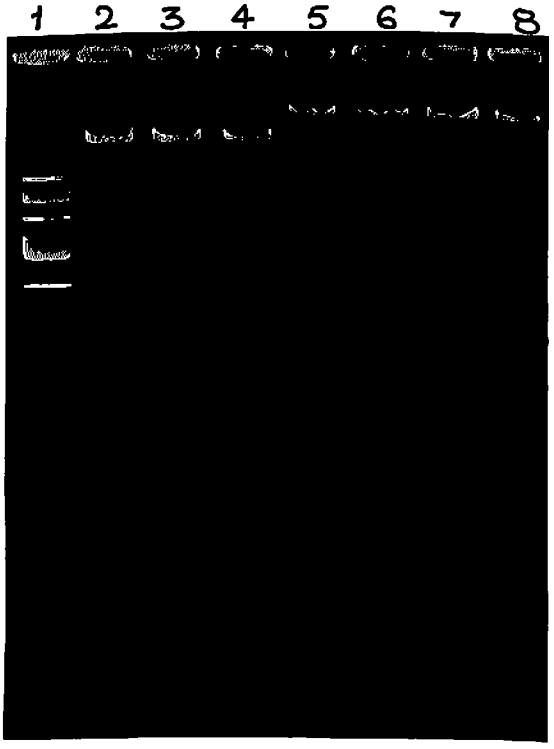
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Table 7. Frequency of plasmid curing in A.brasilense B25.

Curing agent	Conc. µg ml^{-1}	No.of* clones tested	No.of clones that lost plasmids	Frequency of curing (%)
Acridine orange	2.5	900	-	0
Acriflavin	2.5	700	-	0
Ethidium bromide	2.5	860	-	0
Sodium dodecyle sulfate	50	800	-	0
Acridine orange + SDS	2.5) + 25)	900	56	5.4
Control	0	500	0	0

*After five transfers.

Table 8. Antibiotic resistance pattern of the parent and the cured strains of *A.brasilense* B25.

Strain	Plasmid lost	P_{500}	A_{500}	Ax_{50}	Cb_{100}	CS_{25}	Nal_{10}	Cm_{10}	Em_{10}	Sm_{10}
B25	-	R	R	R	R	R	R	R	R	R
Ab1	pAb_1	R	R	R	R	R	R	S	R	R
Ab2	pAb_1	R	R	R	R	R	R	S	R	R
Ab3	pAb_1	R	R	R	R	R	R	S	R	R
AB4	-	R	S	R	R	S	R	R	R	R
Ab5	pAb_1	R	R	R	R	R	R	S	R	R
Ab6	pAb_2	R	R	S	S	R	R	R	S	S
Ab7	pAb_2	R	R	S	S	R	R	R	S	S
Ab8	pAb_1	R	R	R	R	R	R	S	R	R
Ab9	pAb_1	R	R	R	R	R	R	S	R	R
Ab10	pAb_1	R	R	R	R	R	R	S	R	R

chloramphenicol. This suggested loss of some genetic component.

To determine if the loss of antibiotic resistance was due to the loss of the plasmid/plasmids, DNA from representative clones was isolated and subjected to gel electrophoresis (Fig.6). It was found that loss of antibiotic resistance was associated with the loss of either of the two plasmids. In strains Ab1-3, Ab5 and Ab8-10, plasmid pAb_1 had been eliminated, while in strains Ab6 and Ab7, plasmid pAb_2 had been lost. Further, strains which had lost pAb_1 were sensitive to Cm and those which had lost pAb_2 were sensitive to Ax, Cb, Sm and Em.

4.3.3 Growth of cured strains in the presence of heavy metals

To verify if the loss of plasmids was related to their resistance to heavy metals, the ability of the cured strains to grow on medium containing either cadmium or chromium was also tested (Table-9). It was found that the strains which had lost pAb-1 had become sensitive to cadmium, whereas strains which had lost pAb_2 continued to be resistant to these heavy metals. This suggested that pAb_1 is also associated with determining resistance to cadmium

4.3.4 Determination of nif/hup character in cured strain

In Rhizobium the genes for nitrogenase and hydrogenase have been found to be carried on magaplasmids The nif HDK probe from K.pneumoniae hybridized with total DNA isolated from Azospirillum (Elmerich, 1984), but there is no evidence

Fig.6 **Gel electrophoresis of the plasmid DNA from cured strains of A.brasilense B25.**

Lanes 1,4 and 6 - strains without pAb2 (Ab6, Ab7 and Ab11)

Lanes 2,3,5,7 and 8 - Strains without pAb1 (Ab1, Ab2, Ab3, Ab5 and Ab8)

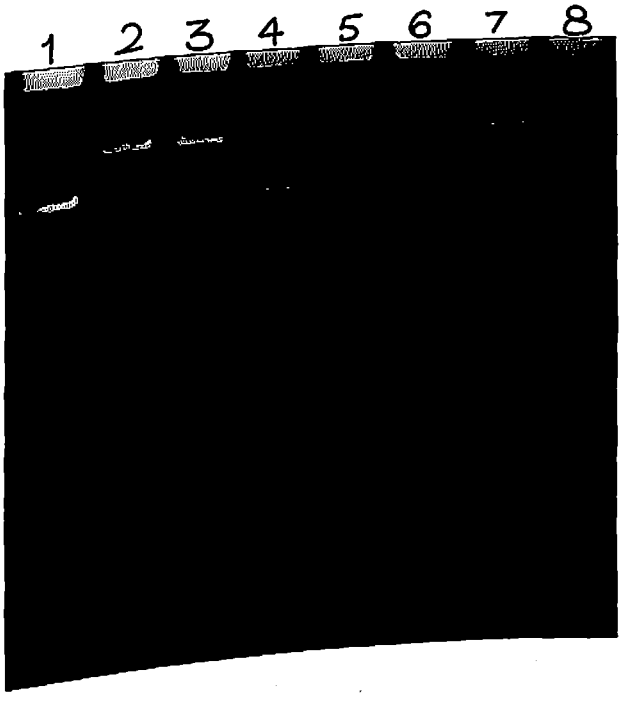
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Table 9. Growth of the parent and the cured strains of A.brasilense B25 on heavy metals.

Strain	Plasmid lost	Cd_{200}	Cr_{25}
B25	-	R	S
Ab1	pAb_1	S	S
Ab2	pAb_1	S	S
Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
Ab10	pAb_1	S	S

about the localization of these genes in Azospirillum. To verify if the loss of plasmid had resulted in the loss of either nitrogenase or hydrogenase activities the 10 strains listed in Table 8 were also tested for their ARA and hydrogenase activity by culturing in nitrogen free semisolid malate medium. Results in Table-10 show that these two activities in the cured strains are very similar to those of the parent. Loss of either plasmid had not affected these activities suggested that these plasmids are not involved either in nitrogen fixation or oxidation of hydrogen in A.brasilense strain B25.

4.3.5. **Production of indole acetic acid (IAA)**

Azospirillum spp have been found to promote root elongation in various grasses and cereals by their ability to produce growth promoting substances (Tien et al., 1979; Umali Garcia et al. 1980; Horemans and Vlassak 1985). To verify if growth promoting substances such as IAA production is associated with these plasmids, the cured strains were tested for any impairment in the production of IAA in malate medium supplemented with 100 $\mu g\ ml^{-1}$ tryptophan and 1 gl^{-1} NH_4Cl. After incubating the cultures for 6 days, IAA produced was estimated colorimetrically. It was found that there was no significant difference in the amounts of IAA produced by the parent or the two types of cured strains excepting in strain No Ab 4 and Ab 10 (Table 11) which produced lower amounts of IAA (10.66 and 4.58 $\mu g\ mg^{-1}$ protein) as compared to the parent (27.60 $\mu g\ mg^{-1}$ protein) The reduced levels of IAA may be due

Table 10. Levels of N_2ase and H_2ase in parent and cured strains of A.brasilense B25.

Strain	Plasmid lost	N_2ase*	H_2ase*
B25	-	26.91	65.20
Ab1	pAb_1	23.01	63.60
Ab2	pAb_1	26.56	58.08
Ab3	pAb_1	22.05	46.30
Ab4	-	24.18	50.50
Ab5	pAb1	23.67	62.80
Ab6	pAb_2	23.15	59.90
Ab7	pAb_2	26.12	58.85
Ab8	pAb_1	25.98	63.45
Ab9	pAb_1	24.03	63.60
Ab10	pAb_1	22.87	48.75

* n moles mg^{-1} protein h^{-1}

Average of five replicates

Table 10. Levels of N_2ase and H_2ase in parent and cured strains of A.brasilense B25.

Strain	Plasmid lost	N_2ase*	H_2ase*
B25	-	26.91	65.20
Ab1	pAb_1	23.01	63.60
Ab2	pAb_1	26.56	58.08
Ab3	pAb_1	22.05	46.30
Ab4	-	24.18	50.50
Ab5	pAb1	23.67	62.80
Ab6	pAb_2	23.15	59.90
Ab7	pAb_2	26.12	58.85
Ab8	pAb_1	25.98	63.45
Ab9	pAb_1	24.03	63.60
Ab10	pAb_1	22.87	48.75

* n moles mg^{-1} protein h^{-1}

Average of five replicates

Table 11 . Levels of IAA in parent and cured strain of A.brasilense B,25

Strain	Plasmid lost	IAA* $\mu g\ mg^{-1}$ prot
B25	-	27.60
Ab1	pAb_1	23.73
Ab2	pAb_1	23.78
Ab3	pAb_1	22.52
Ab4	-	10.66
Ab5	pAb_1	23.39
Ab6	pAb_2	23.75
Ab7	pAb_2	25.24
Ab8	pAb_1	21.51
Ab9	pAb_1	22.46
Ab10	pAb_1	04.58

* Average of three replicates.

Table 11 . Levels of IAA in parent and cured strain of A.brasilense B,25

Strain	Plasmid lost	IAA* $\mu g\ mg^{-1}$ prot
B25	-	27.60
Ab1	pAb_1	23.73
Ab2	pAb_1	23.78
Ab3	pAb_1	22.52
Ab4	-	10.66
Ab5	pAb_1	23.39
Ab6	pAb_2	23.75
Ab7	pAb_2	25.24
Ab8	pAb_1	21.51
Ab9	pAb_1	22.46
Ab10	pAb_1	04.58

* Average of three replicates.

Table 9. Growth of the parent and the cured strains of A.brasilense B25 on heavy metals.

Strain	Plasmid lost	Cd_{200}	Cr_{25}
B25	-	R	S
Ab1	pAb_1	S	S
Ab2	pAb_1	S	S
Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
Ab10	pAb_1	S	S

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Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
Ab10	pAb_1	S	S

Table 10. Levels of N_2ase and H_2ase in parent and cured strains of A.brasilense B25.

Strain	Plasmid lost	N_2ase*	H_2ase*
B25	-	26.91	65.20
Ab1	pAb_1	23.01	63.60
Ab2	pAb_1	26.56	58.08
Ab3	pAb_1	22.05	46.30
Ab4	-	24.18	50.50
Ab5	pAb1	23.67	62.80
Ab6	pAb_2	23.15	59.90
Ab7	pAb_2	26.12	58.85
Ab8	pAb_1	25.98	63.45
Ab9	pAb_1	24.03	63.60
Ab10	pAb_1	22.87	48.75

* n moles mg^{-1} protein h^{-1}

Average of five replicates

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Ab7	pAb_2	25.24
Ab8	pAb_1	21.51
Ab9	pAb_1	22.46
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Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
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* n moles mg^{-1} protein h^{-1}

Average of five replicates

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Strain	Plasmid lost	Cd_{200}	Cr_{25}
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Ab1	pAb_1	S	S
Ab2	pAb_1	S	S
Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
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Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
Ab10	pAb_1	S	S

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Average of five replicates

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B25	-	R	S
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Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
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Ab5	pAb_1	S	S
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Ab7	pAb_2	R	S
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* n moles mg^{-1} protein h^{-1}

Average of five replicates

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Ab8	pAb_1	21.51
Ab9	pAb_1	22.46
Ab10	pAb_1	04.58

* Average of three replicates.

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Strain	Plasmid lost	N_2ase*	H_2ase*
B25	-	26.91	65.20
Ab1	pAb_1	23.01	63.60
Ab2	pAb_1	26.56	58.08
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* n moles mg^{-1} protein h^{-1}

Average of five replicates

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* Average of three replicates.

Table 9. Growth of the parent and the cured strains of A.brasilense B25 on heavy metals.

Strain	Plasmid lost	Cd_{200}	Cr_{25}
B25	-	R	S
Ab1	pAb_1	S	S
Ab2	pAb_1	S	S
Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
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Ab4	-	24.18	50.50
Ab5	pAb1	23.67	62.80
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Ab8	pAb_1	25.98	63.45
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Ab10	pAb_1	22.87	48.75

* n moles mg^{-1} protein h^{-1}

Average of five replicates

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Strain	Plasmid lost	Cd_{200}	Cr_{25}
B25	-	R	S
Ab1	pAb_1	S	S
Ab2	pAb_1	S	S
Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
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Ab9	pAb_1	24.03	63.60
Ab10	pAb_1	22.87	48.75

* n moles mg^{-1} protein h^{-1}

Average of five replicates

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Strain	Plasmid lost	Cd_{200}	Cr_{25}
B25	-	R	S
Ab1	pAb_1	S	S
Ab2	pAb_1	S	S
Ab3	pAb_1	S	S
Ab4	-	R	S
Ab5	pAb_1	S	S
Ab6	pAb_2	R	S
Ab7	pAb_2	R	S
Ab8	pAb_1	S	S
Ab9	pAb_1	S	S
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Ab5	pAb1	23.67	62.80
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Ab7	pAb_2	26.12	58.85
Ab8	pAb_1	25.98	63.45
Ab9	pAb_1	24.03	63.60
Ab10	pAb_1	22.87	48.75

* n moles mg^{-1} protein h^{-1}

Average of five replicates

Table 11 . Levels of IAA in parent and cured strain of A.brasilense B,25

Strain	Plasmid lost	IAA* $\mu g\ mg^{-1}$ prot
B25	-	27.60
Ab1	pAb_1	23.73
Ab2	pAb_1	23.78
Ab3	pAb_1	22.52
Ab4	-	10.66
Ab5	pAb_1	23.39
Ab6	pAb_2	23.75
Ab7	pAb_2	25.24
Ab8	pAb_1	21.51
Ab9	pAb_1	22.46
Ab10	pAb_1	04.58

* Average of three replicates.

to random mutation in the genes coding for IAA biosynthesis.

4.3.6 Utilization of various carbon sources and polysaccharides by the cured strains

To verify if the plasmids control any other essential function such as different carbon sources and polysaccharides utilization the growth of the parent and the cured variants was tested on various carbon cources (arabinose, sucrose, fructose, glucose, malate, lactose, galactose, mannose, maltose, mannitol, sorbitol, glycerol, xylose, ribose, salicin, xylene and toluene) and polysaccharides (cellulose, starch and pectin). All these were supplemented singly in minimal medium and served as the sole source of carbon and energy. It was found that all the cured variants were similar to the parent (Table-12). Similarly there was no difference in utilization of polysaccharides between the parent and the cured strains (Table 13) This suggests that plasmids in Azospirillum are associated with some other function and not with any of those tested above.

4.3.7. Frequency of reversion

To establish that the loss of antibiotic markers is due to the elimination of the plasmid and not as a result of a mutation the cured strains which had lost pAb1 or pAb2 were tested for reversion. The results in Table 14 show that none of the mutants reverted when 10^7 cells were spread on selective media plates. This allows us to conclude that the loss of antibiotic and cadmium resistance is due to the loss of plasmids.

Table 12. Carbon utilisation by parent and cured strains of *A.brasilense* B25.

Strain	Ara	Suc	Fru	Glu	Mal	Lac	Gal	Man	MaL	Mol	Sor	Gly	Xyl	Rib	Sal	Xyl	Tol
B25	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab1	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab2	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab3	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab4	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab5	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab6	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab7	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab8	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab9	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
Ab10	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+

Table 13. Utilization of polysaccharides by the parent & the cured strains of A.brasilense B25.

Strain	Plasmid lost	Cellulose	Starch	Pectin
B25	-	-	+	-
Ab1	pAb_1	-	+	-
Ab2	pAb_1	-	+	-
Ab3	pAb_1	-	+	-
Ab4	-	-	+	-
Ab5	pAb_1	-	+	-
Ab6	pAb_2	-	+	-
Ab7	pAb_2	-	+	-
Ab8	pAb_1	-	+	-
Ab9	pAb_1	-	+	-
Ab10	pAb_1	-	+	-

Table 14. Frequency of reversion of cured strains of *A.brasilense* to the wild type.

Strain	Total count x 10^7	Growth on malate medium	No. of revertants on selective medium
Ab1	146	Confluent	-
Ab2	192	"	-
Ab3	98	"	-
Ab5	118	"	-
Ab6	104	"	-
Ab7	208	"	-
Ab8	192	"	-
Ab9	173	"	-
Ab10	168	"	-
Parent	205	"	Confluent

4.4 Transformation experiments

To further establish that the two plasmids are involved in antibiotic and heavy metal resistance an attempt was made to transfer these plasmids to E coli by conjugation and transformation and then examining the phenotype of the transconjugants and transformants. For this the pRK290 strain of E.coli was used as the recepient which is restriction endonuclease negative and does not have the ability to restrict the entry of foreign DNA. A.brasilense B25 was used as the donor. Before proceeding for the conjugation and transformation experiments the genotype of the donor and the recepient was established to facilitate selection of the transconjugants and transformants. Growth of the donor and the recepient was tested in the presence of Ap, Cb, Ax, Cm, Em, Sm, Tc and Cd (Table-15) The donor strain A brasilense B25 was resistant to Ap, Cb, Ax, Cm, Em, Sm and Cd, and sensitive to Tc the recepient E.coli was sensitive to these antibiotics and resistant to tetracyclin. Thus for the selection of exconjugants and transformants each plate contained only one antibiotic, in the selective medium along with tetracycline since this medium allowed only the excojugants or the transformants to grow.

4.4.1. Conjugation tests

Conjugation was carried out by the spot mating technique as described in section 3 5.2. The nutrient agar plates were first spotted with the cultures and then transferred to

Table 15 . Genotype of A.brasilense B25 and E.coli (pRK290).

Ab.	Conc. $\mu g\ ml^{-1}$	A.brasi B25	E.coli (pRK290)
Amp	100	R	S
Cb	100	R	S
Ax	50	R	S
Cm	10	R	S
Em	10	R	S
Sm	10	R	S
Tc	25	S	R
Cd	100	R	S

selective media plates after suspending them in fresh sterile nutrient broth. This was done to avoid killing of both the donor and the recepient. Following incubation of the plates at 37°C for 24-48h, they were scored for the presence of exconjugants. Growth occured only on the plates containing chloramphenicol and cadmium (Table 16). No clones were found on any other plate suggesting that only one of the two plasmids could be transferred through conjugation.

To verify whether the acquisition of characters by E.coli was due to the acquisition of plasmid by conjugation with A.brasilense gel electrophoresis of the exconjugants was done. As seen in Fig.7, all the exconjugants have gained a plasmid pAb1 of the parent strain. In lane 2, only one band corresponding to the host can be seen at the bottom of the figure, whereas in all the exconjugants two bands are seen which confirms the earlier observation that chloramphenicol and cadmium resistance is coded by the 90Md plasmid of pAb_1 of A.brasilense B25.

Since the phenotype of pAb_2 could not be established by transfer of the plasmids to E.coli by conjugation, an attempt was made to see if this could be done by transformation using plasmid DNA of A.brasilense B25.

4.4.2 Transformation

For this, plasmid DNA was isolated by the method described in section 3.3.2. The purity of DNA was verified by gel electrophoresis and transformation was carried out by incubating this plasmid DNA with $CaCl_2$ treated E.coli cells.

Table 16. Drug resistance pattern of the donor B25 and exconjugants.

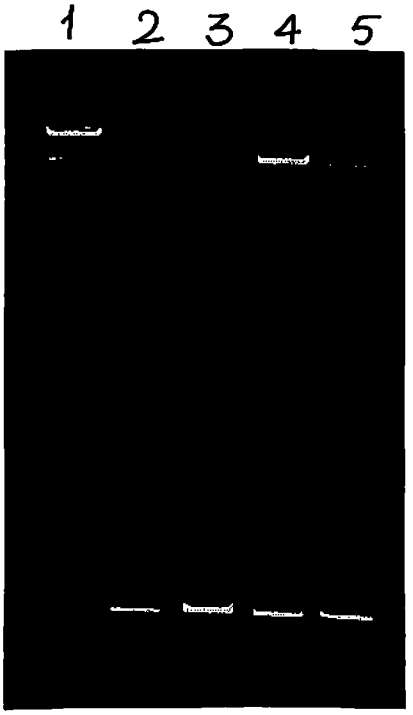
Culture	Ax	Cb	Cm	Em	Sm	Cd	Tc
B25	R	R	R	R	R	R	S
E.coli	S	S	S	S	S	S	R
ex 1	S	S	R	S	S	R	R
ex 2	S	S	R	S	S	R	R
ex 3	S	S	R	S	S	R	R
ex 4	S	S	R	S	S	R	R
ex 5	S	S	R	S	S	R	R

Fig.7 **Gel electrophoresis of plasmid DNA from exconjugants of *E.coli***

Lane 1. *A.brasilense* B25

2. *E.coli* (pKR290)

3-5. exconjugants ex_1, ex_2 and ex_3


1 2 3 4 5

The transformation mixture was then spread on selective media plates containing either Ap (500 µg ml^{-1}), Cb (50 µg ml^{-1}), Ax (50 µg ml^{-1}), Sm(10 µg ml^{-1}), Em (10 µg ml^{-1}), Cm (10 µg ml^{-1}) or Cd (200 µg ml^{-1}). Tetracyclin 25 (µg ml^{-1}) was added to all the plates to allow only E.coli to grow. The plates were incubated at 37°C for 48 h. After incubation, growth was seen on all plates except the one containing ampicillin (Table 17). The number of transformants on all the plates was determined and the frequency of transfer of the drug resistance marker was calculated for all the antibiotics tested. The results in Table-17 show that transformation occured at a frequency varying from 1.7×10^{-5} to 5.68×10^{-5}. Chloramphenicol and cadmium markers were transferred at a much higher frequency than the other drug markers tested.

Around 20 transformants were randomly selected from each plate and counter tested for resistance to all the other antibiotics and heavy metals used in the experiment. On this basis it was found that these 20 could be divided into three groups TpZ1, TpZ2 and TpZx (Table-18). It was also seen that resistance to chloramphenicol and cadmium is co-transferred in TpZ1 while the other 4 markers were co-transferred together in TpZ2. The third group of transformants, TpZx had gained resistance to all the markers tested except Ap which means that both the plasmids had been transferred in this group.

4.3. Gel electrophoresis of the transformants

To further verify that the transformants had acquired the new phenotypes by virute of their having gained the plasmids

Table 17. Frequency of drug resistance trait in *E.coli* transformants using total plasmid DNA preparation of *A.brasilense* B25.

Culture	Growth in the presence of Tc (25 µg/ml) and							No.of trans-formants	No.of cells plated	Frequency of trans-formation
	Ap500	Ax50	Cb100	Cm10	Sm10	Em10	Cd200			
E.coli (control)	-	-	-	-	-	-	-	-	$6x10^6$	0
	-	+	-	-	-	-	-	124	$6x10^6$	$2.06x10^{-5}$
	-	-	+	-	-	-	-	108	$6x10^6$	$1.88x10^{-5}$
E.coli transfor-mants	-	-	-	+	-	-	-	341	$6x10^6$	$5.68x10^{-5}$
	-	-	-	-	+	-	-	102	$6x10^6$	$1.70x10^{-5}$
	-	-	-	-	-	+	-	141	$6x10^6$	$2.35x10^{-5}$
	-	-	-	-	-	-	+	327	$6x10^6$	$5.45x10^{-5}$

Table 18. Drug resistance pattern of transformants of *E.coli*

Group	Ax	Cb	Sm	Em	Cm	Cd	Tc
A.brasilense B25	+	+	+	+	+	+	-
E.coli	-	-	-	-	-	-	+
TpZ_1	-	-	-	-	+	+	+
TpZ_2	+	+	+	+	-	-	+
TpZx	+	+	+	+	+	+	+

gel electrophoresis of the transformants was done. As seen in Fig.8, that in lane 1, two plasmids can be seen, in lane 2 only one plasmid can be seen, whereas in all the other lanes, two or three plasmids can be seen, one corresponding to the upper band (120 Md) of lane 1 and the other corresponding to the lower band (90 Md) of lane 1 and third lower most band corresponding to the host, E.coli. This confirms that the transformants had acquired the new phenotypes by acquiring the plasmids and that the phenotype of pAb1 is resistance to chloramphenicol and cadmium and of pAb2 is resistance to carbenicillin, amoxycillin, streptomycin and erythromycin.

4.4.4. N_2ase and H_2ase activities in the transformants

To see if the plasmids had also transferred any other characteristic one representative, of TpZ_1 and TpZ_2 was selected and further tested for both ARA and H_2ase activities. As seen in Table -19, only the donor strain B25 displayed both the ARA and H_2ase activities confirming that the plasmids in A.brasilense B25 do not code for either nitrogenase or Hydrogenas

4.4.5. IAA production and carbon source utilization by the transformants

Earlier (section 4.3.5) it was found that strains cured of either of the plasmids had not lost the ability to produce IAA. In conjugation experiment, however, no exconjugants with pAb_2 were detected. Since, in transformation this was possible clones with pAb_1 and pAb_2 were tested for IAA production and carbon source utilization as before.

Fig.8 Gel electrophoresis of plasmid DNA from transformants of *E.coli*

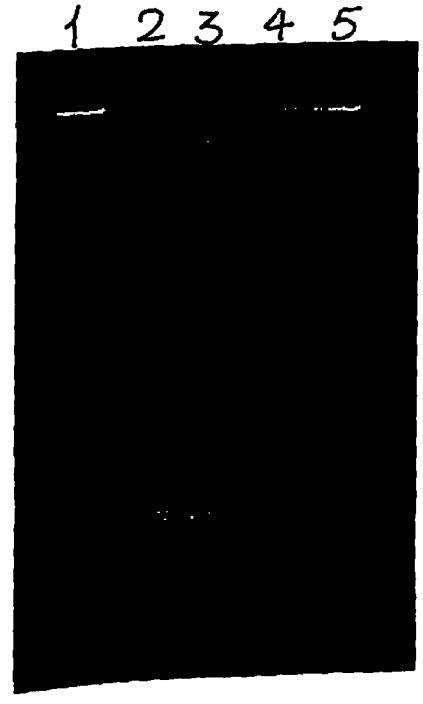
Lane 1. *A.brasilense* B25;

2. *E.coli* (pRK290);

3,7 & 9. Tp Z1;

4,6,8,10 & 11. TpZ2;

5. TpZx


1 2 3 4 5

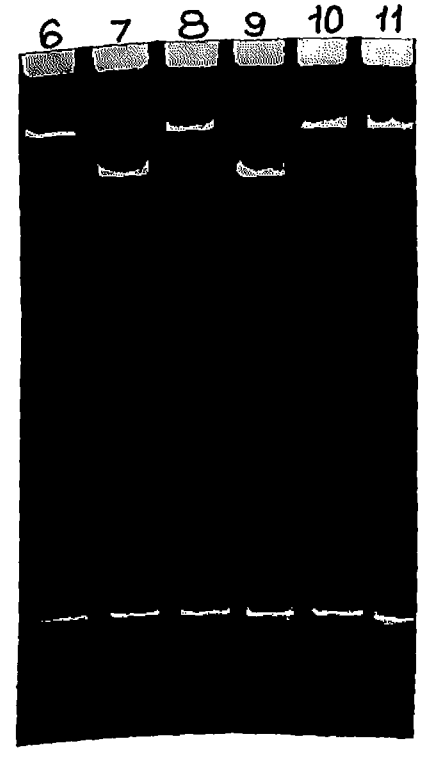

6 7 8 9 10 11

Table 19. Levels of N_2ase and H_2ase in *A. brasilense* B25 and the transformants.

Culture	Plasmid gained	*N_2ase	*H_2ase
A.brasilense B25	-	26.28	64.34
E.coli(pRK290)	-	-	-
E.coli(TpZ_1)	pAb_1	-	-
E.coli(TpZ_2)	pAb_2	-	-

* n moles mg^{-1} protein h^{-1}

Average of five replicates

Table 20. Level of IAA production in *A. brasilense* B25 and the transformants.

Culture	Plasmid gained	IAA* $\mu g\ mg^{-1}$ prot.
A.brasilense B25	-	27.6
E.coli (pRK290)	-	20.58
E.coli(TpZ_1)	pAb_1	22.36
E.coli (TpZ_2)	pAb_2	20.02

* Average of three replicates

Table 21. C-utilisation by A.brasilense and the transformants.

Culture/C-source	Suc	Fru	Glu	Lac	Gal	Ara	Man	Cel	Amy	Mal	Xyl	Sor	Gly	Xyl	Tol
A.brasilense B25	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+
E. coli (pRK290)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E.coli TpZ_1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E.coli TpZ_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Concentration of the carbon source used = 25mM.

Results in Table-20 show that there is no change in the level of IAA produced following a gain of the plasmid By *E.coli*. When the transformants were tested for growth on various carbon sources (Table 21) there was no change in their ability to utilize any of the carbon sources tested. The results obtained by curing and conjugation are further confirmed by these results that plasmids pAb_1 and pAb_2 do not code for any of these functions in *A.brasilense* B25.

CHAPTER - V

DISCUSSION

DISCUSSI

The discovery in the seventies that some bacteria associate with the roots of tropical grasses and fix substantial amounts of nitrogen (Döbereiner and Day, 1976) suggested that this could be a potential area for development and use in cereal crop production. Today, a number of bacteria are known to associate with the roots of cereal plants but among these species of Azospirillum have drawn the most interest. These bacteria fix substantial amounts of nitrogen and also associate with cereal roots, but the molecular basis of the complex process of bacterium-host association and other beneficial effects of Azospirillum inoculation, have not been explored. One reason for this is the lack of information regarding the genetic organization in this organism. Like many other diazotrophs, Azospirilla are also endowed with plasmids. Their number and size, however, has not been consistently reported. Detailed genetic studies in Azospirillum can help to uncover some plasmid determinants which may have a role in its various activities and the complicated process of its association with the plant roots. This may also allow improvement of these bacteria for better plant association and improved crop yields.

Until now, the genetic techniques applicable to Azospirillum have been few. As a consequence, there are only a few studies in this area related to this organism. Although, plasmids have been reported in Azospirillum, their number within one strain has been

reported differently (Franche and Elmerich, 1981; Singh and Wenzel, 1982; Vieille et al.,1987). This difference may be due to the methods adopted. From our studies on standardizing the technique to obtain reproducible results, we found that the method of Simon (1984) was best suited and gave reproducible results. Using this method, the plasmid number in different Azospirillum species and strains was examined (Fig.2) and as expected the number of plasmids varied amongst the various strains and within a species. This makes it difficult to distinguish the genus Azospirillum on the basis of their plasmid content. Recently, detailed serological studies using ELISA technique carried out by Wani et al. (1988), have shown that all the Azospirillum species are serologically distinct and this corelates to a certain extent with the plasmids. Strain A. brasilense B25 was found serologically similar to A.brasilense strain Sp7; yet, the number of plasmids in these two strains examined under identical conditions is different. This discrepency at this moment can not be sorted out.

A.brasilense strain B25 was selected for further plasmid studies because of its high N_2ase and H_2ase activities as well as satisfactory growth in minimal medium and it contained the least number of plasmids among the different A.brasilense strains. The molecular weights of the two plasmids in this strain were determined by comparing their relative mobilities with the standard ones (RP_4 and R68-45) in agarose gels. The approximate molecular weights were found to be 90 and 120 Md for pAb1 and pAb_2 respectively. Restriction endonuclease digestion showed

that EcoR1 has six sites on pAb1 while pAb2 could not be cleaved by any enzyme. The reason for this may be that either the DNA is modified or the degree of supercoiling is such that the sensitive sites are inaccessible to the enzyme (Maniatis et al. 1982). Similar results have been reported by Singh and Wenzel (1982) who could not detect any site in the plasmid pAL1 (6.2 kb) from A.lipoferum strains 29708 and 29709 using EcoR1 and Hind III.

To understand the genetic determinants of plasmids in this organism two approaches were used:
i) to eliminate the plasmids and ii) to transfer the plasmids to E.coli cells through a) conjugation and b) transformation and then examining the change in the phenotype of the cured and the transofrmed cultures.

Among the various curing agents tested to eliminate the plasmids for A.brasilense B25, none yielded any results. However, curing was possible only when SDS and acridine orange were combined. Detection was done only after five transfers and the frequency was determined at the end of this period. Hence, the frequency dtermined does not represent the actual frequency of curing and this may be much higher or lower. Further, despite this, only strains with the loss of one plasmid were derived and the reason for not eliminating both is not known. However, our primary interest was to understand the genetic elements on these plasmids and hence, the clones carrying either of the two plasmids were studied further.

When properties such as nitrogenase, hydrogenase, IAA

production, carbon source and polysaccharide utilization by the cured or transformed strains were compared with the wild type, there was no significant difference which suggests that in this strain the genes coding for these activities are not plasmid borne. Elmerich et al. (1988) have detected hybridization between nif probes from Klebsiella pneumoniae, Bradyrhizobium japonicum and Azorhizobium sesbaniae and total DNA from Azospirillu This study supports their findings regarding the presence of nif genes on chromosome.

These two plasmids, however, determine resistance to various antibiotics. The loss of pAb1 makes the strain sensitive to chloramphenicol as well as cadmium while loss of pAb2 makes it sensitive to carbenicillin, amoxycillin, streptomycin and erythromycin. Azospirillum strains are highly resistant to ampicillin and penicillin but no change in these was noticed in the cured strains. It is, therefore, concluded that in this strain penicillin and ampicillin resistance is not plasmid borne. This is consistent with the report by Singh and Wenzel (1982).

To further establish the genotype of the two plasmids conjugation and transformation were tried in E.coli, keeping in mind the ability of Azospirillum genes to express in other hosts(Franche and Elmerich, 1981; Fani et al., 1985, 1986). It was possible to transfer only plasmid pAb1 through conjugation and all the exconjugants obtained were resistant to chloramphenicol and cadmium. The reason for not being able to transfer pAb2 are i) this may be a non-conjugative plasmid and ii) the conditions

for conjugation need to be further standardized. By deriving exconjugants for pAb1 the genetic determinants have been further established.

Since plasmid pAb2 could not be transferred through conjugation transformation of E.coli with purified plasmid DNA was performed. When this was done transformants with either or both the plasmids were detected. The frequency of transformation with pAb1 was about 5 times that of pAb2 which is probably because of its smaller size. Double transformants were also obtained but their number was negligible as compared to the single transformants These double transformants were of no interest in this study since the objective was to detect the transfer of either of the plasmids and to identify their determinants.

It allows us to conclude that the two plasmids in A.brasilens B25 determine antibiotic and heavy metal resistance. It appears that only the smaller plasmid is of the conjugative type. Unfortunately, the essential determinants such as nitrogen fixation and hydrogen oxidation etc. are not carried by these plasmids. The number of determinants detected on these plasmids is too small to satisfy their large size and it is likely that they may also determine other functions related to bacteria-plant association.

CHAPTER – VI

SUMMARY

SUMMARY

1. Four strains of *Azospirillum brasilense*, two of *A. lipoferum* and one of *A. amazonense* were screened for the presence of plasmids.

2. The number of plasmids varied in species and also within the species. In *A.brasilense* strains the number varied from 2-4 and in *A.lipoferum* it varied from 2-3. In *A. amazonense* strain Y_1 two plasmids were deteced.

3. In *A.brasilense* B25, the two plasmids detected were designated as pAb1 and pAb2. Their approximate molecular weights determined by comparing their relative mobilities in agarose gel were about 90 and 120 Md, respectively.

4. When the plasmids were subjected to RE analysis, it was found that EcoR1 has 6 sites on pAb1 while Bam H1, Kpn1 and Sal1 had no sites on this plasmid. Plasmid pAb2 was not cleaved by any of these enzymes.

5. Using a combination of acridine orange and sodium dodecyl sulphate, *A.brasilense* B25 was cured of either of the two plasmids.

6. By transformation, both pAb1 and pAb2 could be transferred to *E.coli* but by conjugation only pAb1 could be transferred.

7. On the basis of curing, conjugation and transformation studies it was found that plasmid pAb1 codes for Chloramphenicol and cadmium resistance while pAb2 codes for carbenicillin, amoxycillin, streptomycin and erythromycin resistance. Neither nitrogenase, hydrogenase, IAA production nor carbon source utilization is associated with the plasmids in *A. brasilense* B25.

CHAPTER - VII

LITERATURE CITED

LITERATURE CITED

Baldani,V.L.D., Baldani,J.I. and Döbereiner J. 1983. Effects of Azospirillum inoculation, root infection and nitrogen incorporation of wheat. Can.J.Microbiol. 29: 924-29.

Baldani,J.I., Baldani,V.L.D., Sampaio,M.J.A.M. and Döbereiner,J. 1984. A fourth Azospirillum species from cereal roots. An. Acad. Bras. Cien. 56:365-69.

Bani, D., Barberio, C., Bazzicalupo,M., Favilli,F., Ballori,E., and Polsinelli, M. 1980. Mutants of Azospirillum brasilense. J. Gen. Microbiol. 119:239-44.

Barak, R., Nur, I. and Okon,Y. 1983. Detection of chemotaxis in Azospirillum brasilense. J.Appl. Bacteriol. 54:399-403.

Barak, R., Nur, I., Okon,Y. and Henis, Y. 1982. Aerotactic responses of Azospirillum brasilense towards oxygen and organic compounds. J.Bacteriol. 152:643-49.

Barberio,C., Bazzicalupo,M., Gallori,E. and Polsinelli,M. 1982. Regulation of ammonia assimilation and N_2 fixation in Azospirillum brasilense. In:Azospirillum I genetics, physiology, ecology, Ed.Klingmüller, W.Birkhäuser, Verlag. pp.52-58.

Barbieri, P., Zanelli, T., Golle,E. and Zanetti,G. 1986. Wheat inoculation with Azospirillum brasilense Sp6 and some mutatnts altered in N_2 fixation and IAA production. FEMS Microbiol. Lett. 36:87-90.

Bazzicalupo,M., and Gallori,E., 1983. Genetic analysis in Azospirillum In:Azospirillum II, genetics, physiology, ecology Ed. Klingmüller, W. Birkhäuser Verlag pp.24-28

Beijerinck,M.W. 1925. Uber ein Spirillum, Welches freien Stickstoffbinden Kann Zentbl. Bakt. Parasitkde 1163: 353-59. Cited by Day, J.M. and Döbereiner, J. 1976. Soil Biol. Biochem. 8:45-50.

Beringer,J.E., Beynon, J.L., Buchanan-Wollaston,A.V. and Johnston, A.W.B. 1978. Transfer of the durg-resistance transponson Tn5 to Rhizobium. Nature (London)276:633-34.

Berlier,Y.M. and Lespinat,P.A. 1980. Mass spectrometric kinetic studies of the nitrogenase and hydrogenase activities in in vivo cultures of Azospirillum brasilense Sp7. Arch. Microbiol. 125:67-72.

Birnboim,H.C. and Doly,J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-23.

Borovok, I.A., Bakanchikova,T.I., Kortunova, E.Y. and Maisurian, A.N. 1988. Transposon and vector mutagenesis of nitrogen fixing bacterium Azospirillum brasilense. In: Seventh international congress on N Nitrogen fixation held at the University of Cologne in cooperation with the Max Plank Institute of Plant Breeding Koln, FRG, Mar 13-20, 1988, Abstract No.8-08.

Bothe, H., Barbosa,G. and Döbereiner, J. 1983. Nitrogen fixation and nitrate respiration by Azospirillum brasilense. Z. Naturforsch 38:571-77.

Bothe, H., Tennigkeit, J. and Eisbrenner, G. 1977. The hydrogenase nitrogenase relationship in the blue-green alga Anabaena cylindrica. Planta. 133:237-42.

Burris, R.H., Albrecht, S.L. and Okon,Y. 1978. Physiology and biochemistry of Spirillum lipoferum. In: Limitations and Potentials for Biological Nitrogen Fixation in the Tropics, Eds. Döbereiner, J., Burris, R.H. and Hollaender A., Plenum Pub. Corpn. New York. pp.303-15.

Burris, R.H., Okon,Y. and Albrecht, S.l. 1977. Physiological studies of Spirillum lipoferum. In: Genetic engineering for nitrogen fixation. Ed. Hollaender, A. Plenum Press, New York, pp. 445-50.

Cammack, R. and Yates, M.G. 1986. Hydrogenase ; from chemistry to legume growth. Nature (London) 319:182.

Cannon, F.C., Riedel, G.E. and Ausubel, F.M. 1979. Overlapping sequences of Klebsiella pneumoniae nif DNA cloned and characterized. Mol. Gen. Genet. 174:59-66.

Cantrell, M.A., Haugland, R.A. and Evans, H.J. 1983. Construction of R. japonicum gene bank and use in isolation of a hydrogen uptake gene. Proc. Natl. Acad. Sci., U.S.A. 80:181-85.

Casse, F., Boucher, C., Julliot, J.S., Michel, M. and Denarie,J. 1979. Identification and characterization of large plasmids in Rhizobium meliloti, using agarose gel electrophoresis. J. Gen. Microbiol. 113:229-42.

Chan, Y.K., Nelson, L.M. and Knowles, R. 1980. Hydrogen metabolism of Azospirillum brasilense in nitrogen free medium. Can.J. Microbiol. 26:1126-31.

Del-Gallo, M., Gratani, L. and Morpurgo, G. 1985. Mutation in Azospirillum brasilense. In: Azospirillum III, genetics, physiology, ecology. Ed. Klingmüller W. Springer-Verlag pp.85-97.

Del-Gallo M., Martinez - Drets, G., Goebel, E.M., Burris, R.H. and Kreig, N.R. 1984 Carbohydrate matabolism in Azospirillum spp. In: Advances in Nitrogen Fixation Research Eds. Veeger, C. and Newton, W.E., Nijhoff Junk Pudoc, Wageningen, pp.220.

Ditta, G., Stanfield, S., Corbin, D. and Halinski, D.R. 1980. Broad host range cloning system for gram -negative bacteria: Construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. U.S.A. 77:7347

Dixon, R.O.D. 1978. Nitrogenase-hydrogenase inter-relationship in rhizobia. Biochemie. 60:233-36.

Döbereiner, J. 1983. Ten years Azospirillum In:Azospirillum II, genetics, physiology, ecology, Ed. Klingmüller, W. Birkhäuser-Varlag. pp.10-23.

Döbereiner, J. 1987. Isolation and identification of root associated diazotrophs. In: Fourth international symposium on nitrogen fixation with non legumes. Rio de Janeiro, Brazil, Aug. 23-28, 1987. Final abstracts pp.42

Döbereiner, J. and Baldani, V.L.D. 1981. Prospects of inoculation of grasses with Azospirillum spp. In: Associative N_2 fixation Vol.2, Eds. Vose, P.B. and Ruschel, A.P., CRC Press, Baton, F.L. pp.1-9.

Döbereiner, J. and Day,J.M. 1976. Associative symbiosis in tropical grasses : Characterization of microorganisms and dinitrogen fixing sites. Proc. Ist Intl. Symp. Nitrogen Fixation pp.518-38.

Eckhardt, T. 1978. A rapid method for the identification of plasmid DNA in bacteria. Plasmid 1:584-88.

Elmerich, C. 1984. Molecular biology and ecology of diazotrophs associated with non-leguminous plants. Bio/Techology 2: 967-78.

Elmerich, C. 1986. Azospirillum. In: Nitrogen fixation Vol.4. Molecular Biology Eds. Broughton, W.J. and Pühler, S. Oxford Science Publications. pp. 106-26.

Elmerich, C. and Franche, C. 1982. Azospirillum genetics, plasmids bacteriophages and chromosome mobilization. In: Azospirillum I, genetics, physiology, ecology, Ed. Klingmüller, W. Birkhäuser-Verlag, pp. 9-17.

Elmerich, C., Galimand, M., Vieille, C. and Onyeocha, I. 1988. Nitrogen fixation genes of Azospirillum. In: Seventh International congress on N≡ Nitrogen fixation held at the University of Cologne in cooperation with the Max Plank Institute of Plant Breeding, Köln, FRG, Mar 13-20; 1988, Abstract No.9-02.

Fani, R., Bazzicalupo, M., Coianiz, P. and Polisinelli, M. 1986. Plasmid transformation of A.brasilense. FEMS Microbiol. Lett. 35:23-27.

Fani, R., Bazzicalupo, M., Gallori, E., Turbanti, L. and Polsinelli, M. 1985. Construction of a gene bank of Azospirillum brasilense. In: Azospirillum III, genetics, physiology, ecology. Ed. Klingmüller, W. Springer Verlag, pp.1-9.

Francesco, R.D., Zanetti, G., Barbieri, P. and Galli, E. 1985: Auxin porudction by Azospirillum brasilense under different cultural conditions. In: Azospirillum III, genetics, physiology, ecology, Ed. Klingmüller, W.Spring Verlag. pp. 109-15.

Franche, C., Canelo, E., Gauthier, D. and Elmerich, C. 1981. Mobilization of the chromosome of Azospirillum brasilense by plasmid R68-45. FEMS Microbiol. Lett. 10:199-202.

Franche, C. and Elmerich,C. 1981. Physiological properties and plasmid content of several strains of Azospirillum brasilens and Azospirillum lipoferum. Ann. De Microbiol. (Ins Pasteur) 132A:3-17.

Furuichi,T., Masayori, I. and Sumiko, I. 1985. Novel one step cloning vector with a transposable element: application to the Myxococcus xanthus genome. J.Bacteriol. 164: 270-75.

Gauthier, D. and Elmerich, C. 1977. Relationship between glutamine synthetase and nitrogenase in Spirillum lipoferum. FEMS Microbiol. Lett. 2:101-104.

Godson,G.N. and Vapnek,D. 1973. A simple method for preparing large amounts of ϕx 174 RFI supercoiled DNA. Biochem Biophys. Acta 299:516-20.

Gowda, T.K.S. and Watanabe, I. 1983. Autoradiographic technique for screening chemolithotrophic hydrogen utilizing bacteria. Can. J. Microbiol. 29:1081-86.

Gowda, T.K.S. and Watanabe, I. 1985a. Hydrogen supported N_2 fixati of Pseudomonas sp. and Azosporillum lipoferum under free living conditions and in association with rice seedlings. Can. J Microbiol. 31: 317-21.

Gowda, T.K.S. and Watanabe, I. 1985b. Stimulation effect of hydrogen on N_2 fixation in association with field grown wetland rice. Can. J.Microbiol. 31:503-507.

Gupta, R. 1987. Hydrogen metabolism in diaztorophs. Ph.D. dissertic Haryana Agricultural University, Hisar.

Haahtela, K., Wartioraara, T., Sundam, V. and Skujins, J. 1981. Root associated N_2 fixation (acetylene reduction) by Enterobacteriaceae and Azospirillum strains in cold climate spodosols. Appl Environ. Microbiol. 41:203-206.

Haas, D. and Holloway, B.W. 1978. Chromosome mobilization by the R plasmid R68-45: A tool in Pseudomonas genetics. Mol. Gen. Genet. 158: 229-237.

Hartmann, A. 1982. Antimetabolite effects on nitrogen metabolism of Azospirillum and properties of resistant mutants. In Azospirillum I, genetics, physiology, ecology, Ed. Klingmüller, W. Birkhäuser Verlag, pp. 59-68.

Hartmann, A., Singh, M. and Klingmüller, W. 1983. Isolation and characterization of Azospirillum mutants, excreting high amounts of indoleacetic id. Can. J. Microbiol. 29: 916-923.

Haugland, R.A., Cantrell, M.A., Beaty, J.S., Hanus, F.J., Russell, S.A. and Evans, H.J. 1984. Characterization of Rhizobium japonicum hydrogen uptake genes. J. Bacteriol. 159: 1006-

Hegazi,M.A., Khawas, H. and Monib, M. 1981. Inoculation of wheat with Azospirillum under Egyptian conditions. In:Current perspectives in nitrogen fixation, Eds.. Gibson, A.H. and Newton, W.E. Aust. Acad. Sci. Canberra pp.50-55.

Hennecke, H. 1981. Recombinant plasmids carrying nitrogen fixation genes from Rhizobium japonicum. Nature (London) 291:354-355.

Heulin, T., Bally, R. and Balandreau, J. 1982. Isolation of a very efficient N_2 fixing bacterium from the rhizosphere of rice. In Azospirillum I, genetics, physiology, ecology, Ed. Klingmüller, W. Birkhäuser-Verlag. pp.92-99.

Holmes, D.S. and Quigley, M. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114: 193-197.

Horemans, S. and Vlassak, K. 1985. Production of Indole-3-Acetic Acid by Azospirillum brasilense. In: Azospirllum III genetics, physiology, ecology. Ed. Klingmüller, W.Springer Verlag. pp. 98-108.

Hubbel, D.H., Morales, V.M. and Umali-Garica, 1978. Pectolytic enzymes in Rhizobium. Appl. Environ. Microbiol. 35: 210-13.

Ish-Horowicz, D. and Burke, J.F. 1981. Rapid and efficient cosmid vector cloning, Nucleic Acids Res. 9:2989-94.

Jara,P., Quiviger, B., Laurent, P. and Elmerich, C. 1983. Isolation and genetic analysis of A.brasilense nif. mutants Can. J. Microbiol. 29:968-72.

Kado, C.I. and Liu, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-73.

Kapulnik, Y., Kigel, J., Okon,Y., Nur, I. and Henis, Y. 1981. Effect of Azospirillum inoculum on some growth parameters and N-content of wheat, sorghum and panicum. Plant Soil, 61:65-70.

Kapulnik,Y., Sarig, S., Nur,I. and Okon, Y. 1983. Effect of Azospirillum inoculation on yield of field grown wheat. Can.J. Microbiol. 29:895-99.

Katzy, E., Matveev, V. and Panasenko, V. 1988. Use of pSUP5011 for mobilization of Azospirillum plasmids. In:Seventh international congress on N≡Nitrogen fixation held at the University of Cologne in cooperation with the Max Plank Institute for Plant Breeding, Köln, FRG, Mar 13-20, 1988, Abstract No.9-54.

Kennedy, C., Cannon, F., Cannon, M., Dixon, R., Hill, S., Jensen, J., Kumar, S., Mc Lean, P., Merrick, M., Robson, R. and Postgate, J. 1981. Recent advances in the genetics and regulation of nitrogen fixation. In: Current perspectives in nitrogen fixation. Eds. Gibson, A.H. and Newton, W.E. Aust. Acad. Sci. Canberra pp. 146-56.

Klingmüller,W. 1982. Azospirillum I, Genetics, physiology, ecology, Birkhäuser - Verlag, Basel.

Klingmüller, W. 1983. Azospirillum II, Genetics, physiology, ecology. Birkhäuser-verlag, Basel.

Klingmüller, W. 1985. Azospirillum III. Genetics, physiology, ecology, Springer-Verlag, Berlin.

Kondorosi, A., Vincze, E., Johnston, A.W.B. and Beringer, J.E. 1980. A comparison of three Rhizobium linkage maps. Mol. Gen. Genet. 178:403-408.

Kreig,N.R. 1977. Taxonomic studies of Spirillum lipoferum. In: Genetic engineering for nitrogen fixation. Ed. Hollaender, A.Plenum Pub. Corp. New York, PP.463-72.

Kreig,N.R. and Döbereiner, J. 1984. The genus Azospirillum. In: Bergey's Manual of Systematic Bacteriology. Vol.1. Eds. Kreig, N.R. and Holt, J.C. The Williams and Wilkins Co. Baltimore, U.S.A. pp.94-104.

Kundu,B.S. and Tauro,P. 1985. Hydrogen utilization by Azospirillum brasilense from sorghum roots. In: Proc. Soil Biol.Symp. Hisar. Eds. Mishra, M.M. and Kapoor, K.K., H.A.U. Press, pp.241-45.

Kundu,B.S. and Tauro, P. 1987. Chickpea response to dual inoculatic with associative and symbiotic diazotrophs. J.Microbial Biotechnol. 2:35-37.

Lambert, G.R., Cantrell,M.A., Hanus,F.J., Russell,S.A., Hadolad, K.R. and Evans, H.J. 1985. Intra and interspecies transfer and expression of Rhizobium japonicum hydrogen uptake genes and autotrophic growth capability. Proc. Natl. Acad. Sci., U.S.A. 82:3232-36.

Lowry, O.H., Rosenbrough,N.J., Farr, A.L. and Randall,R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-75.

Ludden,P.W., Okon,Y. and Burris,R.H. 1978. The nitrogenase system of Spirillum lipoferum. Biochem. J. 173:1001-1003.

Mac-Neil,T., Mac-Neil,D., Roberts, G.P., Supiano,M.A. and Brill,W.J. 1978. Fine structure mapping and complementation analysis of nif (nitrogen fixation) genes in Klebsiella penumoniae. J. Bacteriol. 136:253-66.

Magalhaes, F.M.M., Baldani,J.I., Souto,S.M., Kuykendall,J.R. and Döbereiner, J. 1983. A new acid tolerant Azospirillum sp. An. Acad. Bras. Cien. 55:417-30.

Maier, R.J., Campbell, N.E.R., Hanus, J., Simpson, F.B., Russell, S.A. and Evans, H.J. 1978. Expression of hydrogenase activity in free living Rhizobium japonicum. Proc.Natl. Acad. Sci., U.S.A. 75:3258-62.

Malik,K.A. and Schlegel,H.G. 1981. Chemolithotrophic growth of bacteria able to grow under N_2 fixing conditions. FEMS Microbiol. Lett. 11:63-67.

Mandel,M. and Higa,A. 1970. Calcium dependent bacteriophage DNA infection. J.Mol.Biol. 53:159-62.

Maniatis,T., Fritsch,E.F. and Sambrook,J. 1982. Molecular coloning : a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Matveev,V., Katzy,E., Petrova, L., Papov ,S. and Panasenko,V. 1988. Use of suicide plasmid vectors for mobilization of Azospirillum plasmids. In: Seventh international contress on N≡Nitrogen fixation held at the University of Cologne in cooperation with the Max Plank Institute for Plant Breeding, Köln, FRG, Mar 13-20, 1988. Abstract No.8-12.

Mazur,B., Rice, D. and Haselkorn,R. 1980. Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridisation probes. Proc. Natl. Acad. Sci., U.S.A. 77:186-90.

Merrick, M., Filser, M., Dixon, R., Elmerich, C., Sibold, L. and Houmard, J. 1980. The use of translocatable genetic elements to construct a fine structure map of Klebsiella pneumoniae nitrogen fixation (nif) gene cluster. J.Gen. Microbiol. 117: 509-20.

Meyers, J.A., Sanchez, D., Elwell, L.P. and Falkow,S. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127: 1529-37.

Michiels, K., Vanstockem, M., Vanderleyden, J. and Van Gool, A. 1985. Stability of broad host range plasmids in Azospirillum Cloning of a 5.9 kbp plasmid from A.brasilense R07. In Azospirillum III, genetics, physiology, ecology. Ed. Klingmüller, W. Springer-Verlag pp. 63-73.

Miller,J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, new York.

Mishra, A.R. and Roy, P. 1979. Transfer of an R-factor (RP_4) from E.coli to Spirillum lipoferum Microbiol. Genet.Bull. 46:11-12.

Nair,S.K., Jara,P., Quiviger, B. and Elmerich,C. 1983. Recent developments in the genetics of nitrogen fixation in Azospirillum. In Azospirillum II, genetics, physiology, ecology, Ed. Klingmüller, W. Birkhäuser-Verlag, pp.29-38.

Nguyen, N.D., Göttfert, Singh, M. and Klingmüller,W. 1983. Nif-hybrids of Enterobacter cloacae: Selection for nif-gene integration with nif-plasmids containing the Mu transposon. Mol. Gen. Genet. 192:439-43.

Nur,I., Okon, Y. and Henis, Y. 1980. Comparative studies on nitrogen fixing bacteria associated with grasses in Israel with Azospirillum brasilense. Can. J. Microbiol. 26:214-20.

Nur, I., Okon, Y. and Henis, Y. 1982. Effect of dissolved oxygen tension on production of carotenoids, Poly-β-hydroxybutyrate succinate oxidase and superoxide dismutase by Azospirillum brasilense Cd grown in continous culture. J.Gen. Microbiol 128:2937-43.

Nur, I., Steinitz, Y.L., Okon, Y. and Henis, Y. 1981. Carotenoid composition and function in nitrogen fixing bacteria of the genus Azospirillum. J. Gen. Microbiol. 122:27-32.

Nuti, M.P., Lepidi, A.A., Prakash, P.K., Schilperoort, R.A. and Cannon, F.C. 1979. Evidence for nitrogen fixation (nif) genes on indigenous Rhizobium plasmids. Nature (London) 282:533-35.

Okon, Y. 1984. Response of cereal and forage grasses to inoculation with N_2-fixing bacteria In: Advances in nitrogen fixation research Eds. Veeger, C. and Newton, W.E. Nijhoff Junk Pudoc Wageningen PP.303-309.

Okon,Y. 1985. The physiology of Azospirillum in relation to its utilization as inoculum for promoting growth of plants. In: Nitrogen fixation and CO_2 metabolism. Eds. Ludden, P.W. and Burris, J.E. Elsevier Science Publishing Co. Inc. pp. 165-74.

Okon,Y., Houchins, J.P., Albrecht, S.L. and Burris, R.H. 1977. Growth of Spirillum lipoferum at constant partial pressures of oxygen and the properties of its nitrogenase in cell free extracts. J. Gen. Microbiol. 98:87-93.

Patriquin,D.C., Döbereiner, J. and Jain, D.K. 1983. Sites and processes of association between diazotrophs and grasses. Can. J. Microbiol. 29:900-15.

Pedresen,W.L., Chakrabarty, K., Klucas, R.V. and Vidaver, A.L. 1978. Nitrogen fixation (acetylene reduction) associated with roots of winter wheat and sorghum in Nebraska. Appl. Environ. Microbiol. 35:129-135.

Pedrosa,F.O., De Souza, E.M., Machado, H.B., Rigo,L.U. and Funayama S. 1987. Regulation of nif genes expression in Azospirillum brasilense and Herbaspirillum seropedicae. In: Fourth International Symposium on nitrogen fixation with non-legumes. Rio de Janeiro, Brazil, Aug 23-28, 1987. Final abstracts pp.62.

Pedrosa, F.O., Döbereiner, J. and Yates, M.G.1980. Hydrogen dependent growth and autotrophic CO_2 fixation in Derxia. J.Gen. Microbiol. 119:547-51.

Pedrosa, F.O., Stephan, M., Döbereiner, J. and Yates, M.G. 1982. Hydrogen uptake hydrogenase activity in nitrogen fixing Azospirillum brasilense. J. Gen. Microbiol. 128:161-166.

Pedrosa, F.O. and Yates, M.G. 1984. Regulation of nitrogen fixation (nif) genes of Azospirillum brasilense by nifA and ntr (gln) type gene products. FEMS Microbiol. Lett. 23:95-101.

Perroud, B., Bhandari, S.K. and Elmerich, C. 1985. The nif HDK operon of Azospirillum brasilense Sp7. In: Azospirillum III, genetics, physiology, ecology, Ed. Klingmüller, W. Springer-Verlag. pp 10-19.

Plazinsky, J., Dart, P.J. and Rolfe, B.G. 1983. Plasmid visualization and nif gene location in nitrogen fixing Azospirillum strains. J. Bacteriol. 155: 1429-33.

Polsinelli, M., Maldanzi, E., Bazzicalupo, M. and Gallori, E. 1980. Transfer of plasmid pRD1 from Escherichia coli to Azospirillum brasilense. Mol.Gen.Genet. 178: 709-711.

Pühler, A. and Klipp, W. 1981. Fine structure analysis of gene region for N_2-fixation (nif) of Klebsiella pneumoniae. In: Biology of inorganic nitrogen and sulfur. Eds. Bothe, H. and Trebst, A. Springer-Verlag, Berlin, pp.276-86.

Quiviger, B., Franche, C., Lutfalla, G., Rice, D., Haselkorn, R. and Elmerich, C. 1982. Cloning of nitrogen fixation (nif) gene cluster of Azospirillum brasilense. Biochemie. 64: 495-502.

Reynders, L. and Vlassak, K. 1979. Conversion of tryptophan to indoleacetic acid by Azospirillum brasilense. Soil Biol. Biochem. 11:547-48.

Rosenberg, C., Boistard, P., Denarie, J. and Casse-Delbart, F. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in Rhizobium meliloti, Mol. Gen. Gent. 184: 326-333.

Ruvukun, G.B. and Ausubel, F.M. 1980. interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci., U.S.A. 77:191-95.

Ruvukun, G.B. and Ausubel, F.M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85-88.

Sarig, S., Kapulnik, Y., Nur, I and Okon, Y. 1984. Response of non-irrigated Sorghum biocolor to Azospirillum inoculat Expl. Agric 20:59-66.

Scott, K.F., Hughes, J.E., Gresshoff, P.M., Beringer, J.E., Rolf *B.G. and Shine*, J. 1982. Molecular cloning of Rhizobiun trifolii genes involved in symbiotic nitrogen fixation. J. Mol. Appl. Genet. 1:315-326.

Shukla, Bela and Kundu, B.S. 1986. Nitrogen transformations by A. brasilense 12S from sorghum roots. Proceedings of the working group meeting, October 9-12, 1986. ICRISAT Centr India pp. 81-84.

Sibold, L. 1982. The polar effect on nifM of mutations in the nifU,-S, -V genes of Klebsiella pnumoniae depends on thei plasmid or chromosomal location. Mol. Gen. Genet. 186: 569-71.

Simon, R. 1984. High frequency mobilization of Gram -negative bacterial replicons by the in vitro constructed Tn5-Mob transposon. Mol. Gen. Genet. 196: 413-20.

Simon, R., Priefer, U. and Pühler, A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram -negative bacteria. Bio/ Technology 1:784-91.

Singh, M. and Klingmüller, W. 1986. Transposon mutagenesis in Azospirillum brasilense: isolation of auxotrophic and Nif⁻ mutants and molecular cloning of the mutagenized nif DNA. Mol. Gen. Genet. 202:136-142.

Singh, M. and Wenzel, W. 1982. Detection and characterization of plasmids in Azospirillum. In: Azospirillum I, genetics, physiology, ecology. Ed. Klingmüller, W. Birkhäuser. Verlag, pp. 44-51.

Smith, L.A., Hill, S. and Yates, M.G. 1976. Inhibition by acetylene of conventional hydrogenase in nitrogen fixing bacteria. Nature (London) 262: 209-210.

Tarrand, J.J., Kreig, N.R. and Döbereiner, J. 1978. A taxonomic study of the Spirillum lipoferum group, with description of a new genus Azospirillum gen. nov. and two species Azospirillum lipoferum (Beijerinck) Comb. nov. and Azospirillum brasilense. sp. nov. Can. J.Microbiol. 24: 967-980.

Tien, T.M., Gaskins, M.H. and Hubbel, D.H. 1979. Plant growth substances produced by Azospirillum brasilense and their effect on growth of pearl millet (Pennisetum americanum L. Appl. Environ. Microbiol. 37: 1016-1024.

Tilak, K.V.B.R., Schneider, K. and Schlegel, H.G. 1986. Autotrophic growth of nitrogen fixing Azospirillum sp. and partial characterization of hydrogenase from the strain. CC. Curr. Microbiol. 13: 291-297.

Umali-Garcia, M., Hubbel, D.H., Gaskins, M.H. and Dazzo, B. 1980. Association of Azospirillum with grass roots. Appl. Environ. Microbiol. 39:219-226.

Vanstockem, M., Michiels, K., Vanderleyden, J. and Van Gool, A.P. 1987. Transposon mutagenesis of Azospirillum brasilense and Azospirillum lipoferum: Physical analysis of Tn5 and Tn5-Mob insertion mutants. Appl. Environ. Microbiol. 53: 410-415.

Vieille, C., Onyeocha, I., Baca, B.E., Flores, M., Palacios, R. and Elmerich, C. 1988. Homology between Azospirillum plasmids and Rhizobium nodulation genes. In: Seventh international congress on N ≡ Nitrogen fixation held at the University of Cologne in cooperation with the Max Plank Institute of Plant Breeding, Köln, FRG, Mar 13-20, 1988, Abstract No.10-58.

Vieille, C., Onyeocha, I., Galimand, M., Paquelin, A. and Elmerich, C. 1987. Genetic analysis of a 90Md plasmid of Azospirillum brasilense Sp7. In: Fourth international syposium on nitrogen fixation with non-legumes. Rio de Janeiro, Brazil, Aug. 23-28, 1987, Final abstracts pp.64

Walker, C.C. and Yates, M.G. 1978. The hydrogen cycle in nitrogen fixing Azotobacter chroococcum. Biochemie. 60:225-232.

Walker, G.C. 1984. E.coli, mutagenesis and inducible responses. Microbiol. Rev. 48: 60-93.

Wang, Z., Zeng, K. and Zhou, Y. 1985. Relation between hydrogen metabolism and nitrogen fixation in Azosprillum spp.1 Studies on H_2 evolution and H_2 uptake hydrogenase activit in A.brasilense. Acta Microbiologica Sinica. 25:54-59.

Wani, S.P., Zambre, M.A. and Upadhyaya, M.N. 1988. Enumeration of nonsymbiotic nitrogen fixing bacteria using Enzyme linked Immunosorbant Assay (ELISA). Ind. J.Microbiol. 28:87-98.

Watanabe, I., Barraquio, W.L. and Daroy, M.L. 1982. Predominance of hydrogen utilizing bacteria among nitrogen fixing bacteria in wet land rice roots. Can. J. Microbiol. 28: 1051-54.

Westby, C.A., Cutshall, D.S. and Vigil, G.V. 1983. Metabolism of various carbon sources by Azospirillum brasilense. J. Bacteriol. 156: 1369-72.

Wood, A.G., Menezes, E.M., Dykstra, C. and Duggan, D.E. 1982. Methods to demonstrate the megaplasmids (or minichromosomes) in Azospirillum. In: Azospirillum I. genetics, physiology, ecology, Ed. Klingmüller, W. Birkhäuser Verla pp. 18-34.

Yates, M.G. and Robson, R.L. 1985. Mutants of Azotobacter chroococcum defective in hydrogenase activity. J. Gen. Microbiol. 131: 1459-66.

Yuan, R., 1981. Structure and mechanism of multifunctional restriction endonucleases. Ann. Rev. Biochem. 50:285-316.

APPENDICES

APPENDIX - I

Calculation for the assay of Acetylene Reduction Activity (ARA)

22.4 L (100% C_2H_4) = 1 mole

22.4 ml = 1 m mole

22.4 ml (110 VPM) = $\frac{110}{1000000}$ m moles

1 ml of C_2H_4 = $\frac{110}{22.4 \times 100}$ m moles

= 4.91×10^{-6} m moles

= 4.91 n moles

Standard

1 ml of C_2H_4 = 40x8x2 C.U. at 10mVx1000 sensitivity

= $\frac{4.91}{40 \times 8 \times 2}$ n moles

ARA = $\frac{4.91 \times \text{C.U.} \times \text{att} \times 2 \times 10}{40 \times 8 \times 2}$ n moles

ARA = $\frac{\text{C.U.} \times \text{att} \times 0.153}{\text{time} \times \text{mg prot.}}$ n moles

APPENDIX -II

Calculations for the assay of hydrogenase

22.4 L of H_2 = 1 mole

22.4 ml (100% H_2) = 1 m mole

1 ml (100% H_2) = $\frac{1}{22.4}$ m mole

0.5 ml (2% H_2) : $\frac{1}{22.4} \times \frac{2}{100} \times \frac{5}{10}$ m moles

Standard

0.5 ml of 2% H_2 = 63.41 x 16 CU at 1mV

in 10 ml = $\frac{1}{22.4} \times \frac{2}{100} \times \frac{5}{10} \times$ 20 n moles

1 CU = $\frac{1}{22.4} \times \frac{2}{100} \times \frac{5}{10} \times \frac{20}{63.41}$ n moles at 16x att & 1mV

= $\frac{1}{22.4 \times 5 \times 63.41}$

= 0.0001408 m moles

= 140.8 n moles at 16 att & 1 mV

or ICU

H_2ase activity = $\frac{C.U \times 140.8}{\text{time} \times \text{mg protein}}$ n moles

APPENDIX -III

Buffers for restriction endonuclease digestion

Buffer	NaCl	Tris.Cl*	$MgCl_2$	Dithiothreitol
Low	0	10 mM	10 mM	1mM
Medium	50mM	10mM	10mM	1mM
High	100mM	50mM	10mM	1mM

* Tris. Cl pH 7.5

APPENDIX - IV

Conditions for restriction enzyme digestion

Enzyme	Buffer	Incubation temperature	Recognition sequence
EcoR1	High salt	37°C	G↓AATTC
Kpn1	Low salt	37°C	GGTAC↓C
Bam H1	Medium salt	37°C	G↓GATCC
Sal 1	High salt	37°C	G↓TCGAC