REVIEW ARTICLE

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Genetics and molecular biology of *Azospirillum*

Abstract Genetic manipulation of *Azospirillum* spp. has facilitated a better understanding of the mode of action of this plant-growth promoting bacterium and should help to improve its ability to stimulate plant growth and development. This review considers and discusses *Agospirillum* plasmids, promoter sequences, the isolation of *Azospirillum* mutants, the genetic transformation of *Azospirillum*, the transfer of foreign genes into *Azospirillum* by conjugation and the *Azospirillum* genes that have been isolated and characterized. The *Azospirillum* genes that are discussed include genes involved in nitrogen fixation, plant root attachment, phytohormone biosynthesis, tryptophan biosynthesis, carbon metabolism and a few other less well characterized processes.

Key words *Azospirillum* · Plant interaction genes· Plasmid p90 · Plant-growth-promoting rhizobacteria· Promoter sequences

Introduction

Azospirillum was first isolated from nitrogen-poor sandy soil in the Netherlands (Beijerinck 1925). The significance of this discovery was not realized, however, for more than 50 years when Döbereiner and Day (1976) reported that grasses associated with *Azospirillum* did not exhibit symptoms of nitrogen deficiency seen in surrounding *Azospirillum*-free grasses. It has since been found that members of this bacterial genera are capable of fixing atmospheric nitrogen and of promoting

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plant growth. At one time it was thought that the counterpart of *Rhizobium,* which is found in legumes, had been found for cereals, and by exploiting the capabilities of *Azospirillum* spp. it would be possible to supply nitrogen to crops of economic importance (Klingmüller 1982).

Inoculation with *Azospirillum* spp. has been known to increase the yield of many cereals in the field by up to 30%, with often even greater increases under greenhouse conditions. However, these results have been difficult to repeat. The factors responsible for these irregularities have not been identified, primarily because the basic features of plant-*Azospirillum* interactions are not well understood. Unlike rhizobia-produced nodules, inoculation with *Azospirillum* does not induce a characteristic morphology in the root system. Therefore, the search for a mechanism by which *Azospirillum* promotes plant growth is somewhat more complicated (Bashan and Levanony 1990; Vande Broek and Vanderleyden 1995).

Among the suggested modes of action for *Azospirillum* are: secretion of phytohormones, nitrogen fixation, production of undefined signal molecules that can interfere with plant metabolism, nitrite production, and the enhancement of mineral uptake by plants (Okon and Itzigsohn 1995). As there is not sufficient evidence to support the notion that one of these mechanisms is solely responsible for plant growth promotion, an additive hypothesis has been proposed (Bashan and Levanony 1990). It suggests that the net beneficial effect to the plant upon *Azospirillum* inoculation is the result of all the above-mentioned mechanisms operating either simultaneously or sequentially. Moreover, soil parameters, bacterial community interactions, plant growth phase, and growth phase of the bacterial inoculum may influence the participation of one or several of these mechanisms (Bashan and Holguin 1997; Okon and Labandera-Gonzalez 1994).

Although the mechanisms involved in the *Azospirillum*-plant interaction ave not clear, it has been repeatedly shown that *Azospirillum* has the potential for

agricultural exploitation (Bashan and Levanony 1990). *Azospirillum*'s versatile metabolism is well suited to competition and to the harsh conditions which exist in the rhizosphere and in soil. *Azospirillum* fixes nitrogen, reduces nitrate, can form cyst-like structures in unfa-vorable conditions, produces plant hormones, vitamins, and siderophores, and efficiently anchors itself to roots with the help of fibrillar material (Patten and Glick 1996; Bashan and Holguin 1997). Besides being a crop rhizosphere bacterium, *Azospirillum* is a natural inhabitant of many non-graminae plants and can sometimes promote their growth (Bashan and Holguin 1997). Briefly, *Azospirillum* can be found in a wide range of habitats in association with many different types of plants.

The genus *Azospirillum* includes five species: *A. brasilense*, *A. lipoferum*, *A. halopraeferens*, *A. amazonense* and *A. irakense*. The differentiation between these species has been supported by physiological, morphological and biochemical characterization (Krieg and Döbereiner 1986), and analysis of DNA restriction profiles (Fani et al. 1991), DNA homology (Falk et al. 1986), 16S ribosomal DNA sequences (Xia et al. 1994) and cellular fatty acid content (Schenk and Werner 1988). Most genetic studies of *Azospirillum* have focused on *A. brasilense* Sp7. Initially, genetic research focused on nitrogen fixation, reflecting the belief that this was the main mechanism for plant growth promotion by *Azospirillum*. It later became apparent that pathogens, symbionts, and plant growth promoting rhizobacteria share similarities with *Azospirillum* spp. with regard to recognition of host plants and affinity phenomena, hormone production, and root morphological modifications (Glick 1995). Therefore, some of the bacterial genes involved in such interactions may be similar (Vieille and Elmerich 1990). Thus, one of the strategies for the identification of *Azospirillum* genes involved in plant interactions has involved comparison of the *Azospirillum* genome with the genome of other bacteria associated with plants.

Plasmids

To date, all strains of *A. brasilense* and *A. lipoferum* examined possess plasmids. Some strains contain as many as six plasmids ranging in size from 4 MDa to over 300 MDa (Elmerich 1983, 1986). All *A. brasilense* and some; *A. lipoferum* strains have 90-MDa plasmids (p90), that share conserved regions and carry several genes involved in the *A. brasilense*-plant root interaction (Onyeocha et al. 1990; Croes et al. 1991). In addition, *A. brasilense* Sp7 contains three other large plasmids, p115 and two plasmids with molecular masses greater than 300 MDa (Crow et al. 1991). Plasmid p115 is frequently lost from *A. brasilense* strains after random transposon Tn5 mutagenesis. Some *A. lipoferum* strains contain a 150-MDa plasmid not present in *A. brasilense* strains. These megaplasmids are present in *Azospirillum* strains at a rate of one copy per cell (Vanstockem et al. 1987).

Croes et al. (1991) demonstrated that p90 carries genes involved in motility, adsorption to roots, colony morphology and growth on minimal media. A p90 DNA fragment was shown to contain two constitutively transcribed open reading frames highly homologous to the *R. meliloti* nodPQ nodulation genes (Vieille and Elmerich 1990), although the function of these loci in *A. brasilense* has not yet been elucidated. Two loci involved in chemotaxis (van Rhijn et al. 1990), as well as two different loci conferring resistance to ampicillin (Onyeocha et al. 1990) have also been localized to p90. Two DNA sequences from *A. brasilense* Sp7 that complemented mutations in the *Rhizobium meliloti* megaplasmid exopolysaccharide synthesis genes (*exo*B and exoC), were also shown to be present on p90 (Michiels et al. 1988, 1989). A Tn5 insertion on an 85-MDa plasmid of *A. brasilense* Sp245, reduced indoleacetic acid (IAA) production in these strains, suggesting that genes located on this plasmid play a role in IAA synthesis in *Azospirillum* (Katzy et al. 1990). The 300-MDa plasmid of *A. lipoferum* was found by Tn5 mutagenesis to encode melanin biosynthesis (Faure et al. 1994). In addition, the 150-MDa plasmid of *A. lipoferum* (Bally and Givaudan 1988) and p85 from *A. brasilense* Sp245 (Katzy et al. 1990) were found by transposon mutagenesis to encode enzymes that produce anthranilic acid, a precursor of tryptophan (Trp).

Promoter sequences

The sigma factor component of bacterial RNA polymerase is responsible for promoter specificity. In general, two families of sigma factors can be distinguished: the sigma-70 family, containing multiple types of sigma factors, and the sigma-54 family, with sigma-54 as its only member. RNA polymerases containing sigma-54 recognize the so-called -24, -12 promoters while RNA polymerases containing a sigma-70 factor recognize the -35, -10 promoters (Merrick 1992). In some cases, *Azospirillum* and *Escherichia coli* promoters are functionally interchangeable (Schipani et al. 1988; Perito et al. 1995).

RpoN-dependent promoter consensus sequences (i.e., sequences that interact with sigma-54) have been found upstream of several *Azospirillum* genes, including *nif*H, *nif*B, *nif*U and *nif*W (de Zamaroczy et al. 1989; Milcamps et al. 1993), the *gln*BA operon coding for P_{II} (a signal transduction protein) and glutamine synthetase (GS) (de Zamaroczy et al. 1993), and the *laf*I gene which is involved in the synthesis of lateral flagella (Moens et al. 1995). An *rpo*N mutant of *A. brasilense* Sp7, was found to be defective in nitrogen fixation, nitrate assimilation, ammonium uptake and motility, indicating that RpoN is involved in controlling diverse cellular functions in *Azospirillum* (Milcamps et al. 1996).

Sigma-70-type promoters in *Azospirillum* have been found associated with some genes involved in nitrogen fixation. For example, the *A. brasilense* *gln*B gene (cod

ing for the PII protein) promoter appears to have a sigma-70 as well as a sigma-54 recognition site (de Zamaroczy et al. 1993). The promoter of the *A. brasilense* DNA fragment equivalent to *phb*B of *Alcaligenes eutrophus* and the promoter of the *A. brasilense carR,* gene involved in carbohydrate assimilation resemble the sigma-70 consensus promoter (Vieille and Elmerich 1992; Chattopadhyay et al. 1994). The promoter regions of the *A. brasilense gln*A and *nif*A genes and of the Open reading frame (ORFl-*ntr*BC operon do not contain sequence elements similar to those of any known promoters (de Zamaroczy 1995; Machado et al. 1995).

Expression of the sigma-54-dependent genes in all organisms studied so far is characterized by the requirement for an activator protein which binds to an upstream activator sequence. The activator proteins for genes related to nitrogen assimilation and nitrogen fixation are either *Nif*A or *Ntr*C (Merrick 1992).

Azospirillum species have a high guanine plus cytosine (G + C) content, i.e., 64-71 mol % (Khammas et al. 1989). Several authors have related the success or failure of the expression of foreign genes in *Azospirillum* to differences in the G + C content between *Azospirillum* and the donor species. For example, the *Streptomyces rochei egl*S gene (encoding endoglucanase) was expressed in *A. brasilense* but not in E. coli cells (Perito et al. 1995). Like *A. brasilense, S. rochei* has a G + C content of about 70% while that of *E. coli* is 48-52%. Failure to express the cry gene of *Bacillus thuringiensis* by *A. brasilense* was attributed to the differences in codon usage between these microbes (Udayasuriyan et al. 1995).

Isolation of mutants and genes

Azospirillum mutants have been induced by chemicals, radiation, Tn5 mutagenesis and gene replacement (also called marker exchange). Using *N*-methyl-N-nitro-*N'*-nitrosoguanadine mutagenesis, Nif $^-$ mutants, defective in nitrogen fixation (Pedrosa and Yates 1984), GltS$^-$ mutants, defective in glutamate synthase (GltS; Mandal and Ghosh 1993), and IAA-overproducing mutants (Hartmann et al. 1983) have been isolated.

Tn5 mutagenesis has been used to obtain auxotrophic and IAA-overproducing mutants of *Azospirillum* (Abdel-Salam and Klingmüller 1987), mutants of *A. lipoferum* 4T devoid of laccase activity and melanization (Faure et al. 1994), IAA-underproducing mutants (Katzy et al. 1990), mutants impaired in chemotaxis and in motility (van Rhijn et al. 1990) and in the ntrBC region (Machado et al. 1995) where NtrB is a histidine kinase that phosphorylates NtrC, which activates the synthesis of GS and the transcription of *Nif*A in enteric bacteria.

A number of *Azospirillum* mutants have been isolated by replacing the functional gene with a marker which disrupts its sequence. These include auxotrophic and Nif mutants (Singh and Klingmüller 1986; Galimand et al. 1989); mutants with defective *nod*PQ genes that are homologous to *Rhizobium meliloti* nodulation genes (Vieille and Elmerich 1990); GlnB $^-$ mutants de-ficient in PII synthesis (de Zamaroczy et al 1993); dini-trogenase reductase ADP-ribosyltransferase mutants (DraT$^-$) and dinitrogenase reductase-activating glyco-hydrolase mutants (DraG$^-$; Zhang et al. 1992); RpoN mutants (sigma-54 factor; Milcamps et al. 1996); NtrBC mutants (Liang et al. 1993); IAA attenuated mutants (Costacurta et al. 1994); and *rec*A mutants (Machado et al. 1995).

Gene replacement, combined with hybridization and/or mutant complementation, has helped in the isolation of nitrogen fixation genes (Liang et al. 1993), Trp biosynthesis genes (Zimmer et al. 1991), two p90 genes that complement mutations in the exopolysaccharide synthesis genes, *exo*B and *exo*C, of *R. meliloti,* a new ampicillin-resistance loci, and a region necessary for the maintenance of p90 as an individual replicon (Onyeocha et al. 1990). By gene replacement Croes et al. (1991) were able to map the loci in p90 involved in the adsorption of *Azospirillum* to wheat roots, its growth on minimal medium, motility and colony morphology.

The identification of *Azospirillum* genes involved in plant interactions has relied on the homology between *Azospirillum* genes and plant interaction genes from other plant-associated bacteria. Hybridization probes have come from a variety of rhizosphere bacteria including: *Rhizobium* (Fogher et al. 1985), *Azorhizobium* (Pelanda et al. 1993), *Bradyrhizobiurn* (Liang et al. 1993), *Agrobacterium* (Vieille and Elmerich 1990), and *nif* genes from diazotrophs like *Klebsiella pneumoniae* (Fahsold et al. 1985), *Rhodospirillum rubrurn* (Fu et al. 1990), and *Azotobacter* (Milcamps et al. 1996). Polymerase chain reaction (PCR) products of exogenous genomic DNA have been used as probes for the isolation of rpoN genes (Milcamps et al. 1996), and for the isolation of *laf*1 which is involved in the synthesis of lateral flagella (Moens et al. 1995).

For the detection and analysis of sequences involved in gene regulation, regulatory regions of A. *brasilense* have been fused to promoterless reporter genes, such as the genes for the enzymes chloramphenicol acetyl transferase (Fani et al. 1989), β-glucuronidasc (Vande Brock et al. 1992) and β-galactosidase (Liang et al. 1993). For example, to analyze the oxygen tolerance of different diazotrophs including *Azospirillum,* Vande Brock et al. (1996) monitored the induction of *nif*H-*gus*A at different oxygen concentrations. Liang et al. (1993) studied the expression of *nif*A in the presence and absence of ammonia and under both aerobic and anaerobic conditions in ntrBC mutants by constructing a *nif*A-*lac*Z fusion (ntrBC are required for nitrate-dependent growth and full nitrogenase activity). For the localization of a promoter region upstream from the ntrBC region, Machado et al. (1995) fused putative promoter regions to a promoterless *lac*Z reporter gene. With the help of a *gln-lac*Z fusion, de Zamaroczy et al. (1993) analyzed the conditions which induced cotranscription of glnBA (coding for PII and glutamine synthetase) and transcription of *gln*A alone in *A. brasi-*

lense. The reporter fusions, *nif*A-*lac*Z, *nif*B-*lac*Z and *nif*H-*lac*Z have been used to locate *A. brasilense* in wheat roots and to study its colonization efficiency (Arsene et al. 1994; Vande Broek et al. 1996).

Transformation

Fani et al. (1986) reported transformation of *A. brasilense* following the induction of competence by heat shock and cations. However, their method was only successful with one strain. Vande Brock et al. (1989) reported transferring plasmid DNA to several *Azospirillum* strains by electroporation, although the transformation efficiency varied considerably with the *Azospirillum* strain used and growth phase.

Arguing that they might thus avoid the repression of nitrogen fixation in *A. lipoferum* by ammonia, Uozumi et al. (1986) transformed *A. lipoferum* with a constitutively expressed *nif*A gene encoding an activator protein required for transcription of *nif*HDK that is normally inactive in the presence of ammonia. They cloned the *nif*A gene of *Klebsiella oxytoca* in a plasmid, downstream of a constitutive Tetr promoter, and delivered it to *A. lipoferum* through the use of calcium chloride and heat-shock treatment. The resultant *A. lipoferum* transformants showed a weak but distinct nitrogenase activity in the presence of ammonium ions

Gene transfer by conjugation

Currently, there is no routine protocol for direct DNA transfer to *Azospirillum.* Generally, exogenous DNA is first transferred to E. coli cells and then to *Azospirillum* by conjugation. Udayasuriyan et al. (1995) attempted to transfer by conjugation the cry gene from *B. thuringiensis*, encoding an insecticidal protein, into *A. brasilense* and *A. lipoferum.* This was accomplished by fusing the *cry* gene to the Tet^r promoter of the vector RSF1010 which has a broad host range. Transformants from *A. lipoferum* were obtained at a frequency of 1.1 x 10^{-7}. However, the *cry* gene was not expressed, although *E. coli* transformants carrying the same plasmid did produce the insecticidal protein, probably as a result of the highly biased codon usage of the cry gene. In this regard, transgenic rice only showed insect resistance when the *cry* gene was chemically synthesized by employing codons favored by rice (Fujimoto et al. 1993).

In Agrobacterium tumefaciens, mutants containing a Tn5 transposon in either the *chv*A or *chv*B loci are impaired in plant-cell attachment and show an avirulent phenotype. These two chromosomal virulence genes are required for β-1-2)-glucan synthesis and secretion, and hence for the attachment of *Agrobacterium tumefaciens* to plants and subsequent tumor formation. After demonstrating that the genome of *A. brasilense* and *A. lipoferum* strains did not hybridize wish these genes from *Agrobacterium tumefaciens*, and that *Azospirillum* strains were unable to form β-glucan, a cosmid containing the

*chv*B and *chv*A genes was introduced by conju-gation into *Azospirillum.* The genes were expressed and β-glucan was synthesized yielding a protein indis-tinguishable from that obtained in *Agrobacterium tumefaciens* (Altabe et al. 1990). Contrary to these results, Waelkens et al. (1987) found DNA homology to chv genes from *Agrobacterium tumefaciens* in all wildtype *Azospirillum* strains tested. They used two separate *Hind*III fragments as *chv*A probes and two *Eco*RI fragments as *chv*B probes, while Altabe et al. (1990) used a 2.3-kb *Bam*HI fragment carrying only the *chv*B locus.

Azospirillum strains use dicarboxylic acids as carbon sources in preference to carbohydrates and are unable to degrade starch and cellulose. Except for *A. lipoferum*, none of the strains can grow on glucose (Khamrnas et al. 1989) and only *A. irakense* can grow with pectin as a sole carbon source. The types of carbon sources in the root exudates constitute a major factor limiting associative nitrogen fixation of *Azospirillum.* Attempting to improve the *Azospirillum*-plant-root association, Keijers et al. (1995) transformed *A. brasilense* with several genes that might have allowed it to use a broader range of carbon sources. Given the similar (high) G+C content of the genomes of *Azospirillum* and *Streptomyces*, a 4.8-kb insert encoding the α-amylase gene of *Streptomyces venezuelae* was mobilized into *A. brasilense* Sp245 and Sp7. The one transformant with aamylase activity that was obtained expressed the gene from the á-amylase promoter. Unexpectedly, the engineered strain was able to grow and fix nitrogen on glucose, maltose or starch as a sole carbon source, as compared to the wild-type, which cannot grow on any of these carbohydrates. A transformant of Sp245 with a tetracycline resistance gene, but without the α-amylase gene, was also able to grow on glucose and fix nitrogen, suggesting that expression of the tetracycline resistance gene facilitated the uptake of this carbohydrate.

A. irakense, unlike other *Azospirillum* strains, can grow and fix nitrogen with pectin as the sole carbon source. When a cosmid library of the *A. irakense* genome prepared in *E. coli* . cells was transferred to *A. brasilense* Sp245 by conjugation, the resultant transformants gained the ability to degrade pectin (Keijers et al. 1995).

Azospirillum genes Isolated and characterized

Nitrogen fixation genes

Researchers have sought to improve plant associative nitrogen fixers like *Azospirillum* to better provide nitrogen to crops of economic importance, under the premise that nitrogen fixation explained improved plant growth when plants were treated with *Azospirillum.* This was inferred from observations of increased levels of nitrogenous compounds and increased nitrogenase activity associated with inoculated plants. However, some researchers believe that the contribution of *Azo-*

spirillum nitrogen fixation to plants is minimal. Alternative avenues of research are currently being developed to improve the transfer of biologically fixed nitrogen to plants. These approaches include the production of ammonia-excreting mutants, para nodule formation and the use of endophytic diazotrophs.

Nitrogen fixation is catalyzed by the nitrogenase complex which includes dinitrogenase (MoFe protein, *nif*DK gene products), containing the active site of dinitrogen reduction, and dinitrogenase reductase (Fe protein, *nif*H gene product) supplies the reducing power to the dinitrogenase (Vande Broek and Vanderleyden 1995). Nitrogenase is subject to elaborate control at the transcriptional and post-translational levels by the concentrations of intracellular nitrogen and oxygen. The genes involved in nitrogen fixation identified in *Azospirillum* are shown in Table 1.

Formation of a functional nitrogenase in *A. brasilense is* primarily controlled at the level of transcription of the nitrogenase structural genes (*nif*HDK operon)

which occurs only under nitrogen-limiting microaerobic conditions. Nitrogenase activity is also regulated at a post-translational level by two mechanisms. The dinitrogenase reductase ADP-ribosyltransferase/dinitrogenase-reductase-activating glycohydrolase (DRAT/DRAG) system, which involves reversible nitrogenase inactivation via ADP ribosylation in response to micromolar concentrations of ammonia (Zhang et al. 1992*;* Fig. 1), has been best described for the photosynthetic bacterium *R. rubrurn*. The *A. lipoferum dra*TG genes were identified by hybridization with the *drat* gene of *R. rubrum* (Fu et al. *1990)* and then used as a probe for the isolation of the *A. brasilense dra*TG genes (Zhang et al. 1992). In *R. rubrum,* when cells are in the dark or treated with ammonia, DRAT carries out the transfer of ADP-ribose from NAD to the dinitrogenase reductase, resulting in inactivation of the enzyme. When light is sufficient or when nitrogen is limiting, DRAG removes the ADP-ribose from the modified enzyme, restoring its activity (Zhang et al. 1992).

Table 1 *Azospirillum* genes involved in nitrogen fixation that have been identified. In some cases, the functions of these genes has been inferred from their function in *Klebsiella pneu-* *moniae* or, in the case of the *fix*ABCX genes, from their functions in *Rhizobium* spp.

| Gene	Product	Putative function	Reference
*nif*H	Dinitrogenase reductase (Fe protein)	Nitrogenase structural gene	Fani et al. (1989)
*nif*DK	Dinitrogenase (FeMo protein)	Nitrogenase structural genes	Galimand et al. (1989); Passaglia et al. (1991)
*nif*Y	Unknown	Allows FeMo cofactor insertion	Passaglia et al. (1991)
*nif*E	Unknown	Synthesis of FeMo cofactor	Galimand et al. (1989)
*nif*N	Unknown	Synthesis of FeMo cofactor	Frazzon et al. (1995)
*nif*X	Unknown	Unknown	Frazzon et al. (1995)
*nif*U	Unknown	Unknown	Galimand et al. (1989)
*nif*S	Unknown	Synthesis of FeS clusters	Galimand et al. (1989)
*nif*V	Homocitrate synthase (tentatively identified)	Processing of FeMo cofactor	Frazzon et al. (1995)
*nif*W	Unknown	Needed for full activity of FeMo cofactor	Milcamps et al. (1991)
*nif*B	Unknown	Synthesis of FeMo cofactor	Elmerich (1994)
*fix*ABCX	Unknown	Electron transport to nitrogenase	Galimand et al. (1989); Prazzon et al. (1995)
*rpo*N	RNA polymerase s^{54} factor	Activation of transcription of some *nif genes*	Milcamps et al. (1995)
*gln*A	Glutamine synthetase	Fixes ammonia into glutamate to from glutamine	de Zamaroczy et al. (1993); Bozouklian and Elmerich (1986)
*gln*B	PII a signal transduction protein	Activation of *Nif*A and glutamine synthetase	de Zamaroczy et al. (1993)
*nif*A	Nif transcriptional activator protein	Required for transcription of *nif*HDK and all other rpoN⁻ dependent promoters	Liang et al. (1991); Singh et al. (1989)
*glt*DB	Glutamate synthase	Structural genes for α end âsubunits	Mandal and Ghosh (1993); Pelanda et al. (1993)
*dra*T	Dinitrogenase reductase ADP-ribosyltransferase	Transfer of ADP-ribose from NAD to dinitrogenase reductase, inactivating enzyme	Fu et al (1990); moue et al. (1996)
*dra*G	Dinitrogenase-reductase-activating glycohydrolase	Removes ADP-ribose from dinitrogenase reductase, restoring enzyme activity	Fu et al. (1990); moue et al. (1996)
*ntr*B	Histidine kinase	Nitrate-dependent growth. Switches off nitrogenase activity	Liang et al. (1993); Zhang et al. (1992)
*ntr*C	Similar to a transcriptional regulatory protein from enteric bacteria	Nitrate-dependent growth. Switches off nitrogenase activity	Liang et al. (1993); Zhang et al. (1992)
*gln*Z	PII-like protein	Unknown	de Zamaroczy et al. (1996)
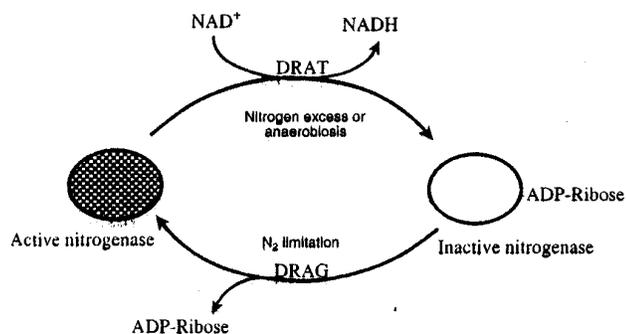
Fig. 1 Schematic representation of the regulation of nitrogenase activity by the dinitrogenase reductase ADP-ribosyltransferase/ dinitrogenase-reductase-activating glycohydrolase (*DRAT/ DRAG*) system in *Azospirillum brasilense* according to Zhang et al. (1992)

A second independent mechanism has been revealed, although it is still not understood. Overall, this second regulatory system has a less dramatic effect on nitrogenase activity in response to ammonia than does the DRAT/DRAG system (Zhang et al. 1996). In *R. rubrum,* the site of ADP ribosylation in dinitrogenase reductase (*nifH* product) is a specific arginine residue. In *Azospirillum,* the replacement of an arginine residue of NifH with tyrosine or phenylalanine by site-directed mutagenesis eliminated ADP ribosylation. However, nitrogenase was still inhibited by ammonium, revealing another post-translational regulatory mechanism of nitrogenase activity independent of the DRAT/DRAG system (Zhang et al. 1996).

Vande Brock et al. (1996) found that the optimal level of oxygen tension for nitrogenase was activity in *A. brasilense* was between 0.3-1.0% , and that nitrogen fixation did not take place above an oxygen tension of 2%, while in *A. irakense* the maximum tolerable oxygen tension was 2.5%. At high oxygen concentrations, nitrogenase is irreversibly inactivated by the oxidation of the metal-sulfur centers of the protein (Vande Broek and Vanderleyden 1995).

The regulation of *nif* gene expression in *Azospirillum,* as well as the identification and organization of these genes, was reviewed by Elmerich et al. (1997). Unlike some other free-living nitrogen-fixing bacteria, *Azospirillum nif* genes are located in the chromosome. In contrast to the; tight clustering of *nif* genes in enteric bacteria, the *nif* genes in *A. brasilense* are located in at least four different genomic regions spanning a minimum of 65 kb of DNA (Singh et al. 1989; Table 1). A 30-kb DNA region contains *nif*HDKY, *nif*ENX, *nifUSV, nifW* and *fix*ABCX (Galimand et al. 1989; Passaglia et al. 1991, 1995; Milcamps et al. 1993; Frazzon et al. 1995). The draTG genes are located upstream of nifHDKY. Another region contains the *nifA* and *nif*B genes (Liang et al. 1991).

Nucleotide sequence analysis of the region upstream of the *nif*H open reading frame shows an RpoN-dependent promoter (Fani et al. 1989; de Zamaroczy et al.

1989). The expression of the *nifH* gene was found to occur only under low oxygen tension arid nitrogen-limiting conditions (Vande Broek et al. 1992). Most RpoN-dependent promoters require transcriptional activator proteins such as NifA whose activity is modulated by physiological signals (Merrick 1992). Similar to other nitrogen-fixing bacteria, transcription initiated from the RpoN-dependent promoter of the *nif*HDK operon in *A. brasilense* depends on the *nif*A gene product (de Zamaroczy 1995). The *A. brasilense S*p7 polypeptide was similar to other NifA proteins (Michiels et al. 1994).

In most bacteria, two enzymes, GS, and GltS, catalyze the uptake of nitrogen. These reactions provide cells with the key intermediates of nitrogen metabolism, i.e., convert á-ketoglutarate to L-glutamate (Moat and Foster 1998; Fig. 2). The GS-G1tS pathway is functional at low concentrations of ammonia (<1 mM) when dinitrogen is the nitrogen source. In the same way as in enterobacteria GS activity in *A. brasilense* is modulated by reversible adenylylation in response to the cellular nitrogen status. In cells growing under conditions of ammonia excess the adenylylation level increases, whereas under nitrogen-limiting conditions, GS is maintained in its non-adenylylated active form (de Zamaroczy et al. 1993).

Under nitrogen-limiting conditions, the synthesis of PII is also increased. This protein has been shown to play a crucial role in the regulation of nitrogen fixation by controlling NifA activity. By constructing deletions in the NifA coding sequence covering the amino terminal domain, and by determining whether NifA proteins could restore nitrogen fixation in the mutants, NifA⁻, GlnB⁻ and NifA⁻/GlnB⁻, Arsène et al. (1996) showed that in the presence of ammonia the N-terminal domain of NifA inhibits the activity of the whole NifA protein, and that PII prevents the inactivation of NifA.
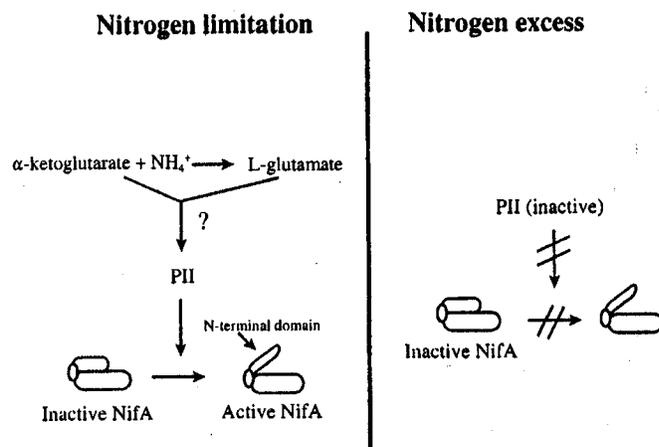

Fig. 2 Model of regulation of NifA activity by PII (a signal transduction protein) in *Azospirillum brasilense* according to Arsène et al. (1996). It has not yet been established whether or not PII is regulated by the ratio of ketoglutarate to glutamate in *Azospirillum*

The mechanism by which P_{II}, blocks the inactivation of NifA is unknown; however, it is believed that only when P_{II}, is present in its uridylylated form can it control NifA activity. In *K. pneumoniae* P_{II}, is uridylyated in response to the ratio between the concentrations of 2-ketoglutarate and glutamine and, although it has not yet been proven for *Azospirillum* spp., this model is nevertheless attractive. *A. brasilense* contains two different but structurally similar P_{II} proteins. The second P_{II} protein, PZ, is encoded by *glnZ* and may be involved in a different regulatory step of nitrogen metabolism in *A. brasilense* than the better characterized P_{II}, (de Zamaroczy et al. 1996).

In *Azospirillum*, *gln*B is located upstream of *gln*A (encoding GS) forming the *gln*B-*gln*A cluster. The transcription of *gln*BA depends on three different selectively used and nitrogen-regulated promoters (de Zamaroczy et al. 1993).

When Mandal and Ghosh (1993) generated GltS⁻ mutants, they found that a GltS deficiency led to pleiotropic nitrogen assimilation defects. The structural genes coding for the α and β subunits of GltS (i.e., *glt*B and *glt*D) were identified by complementation of an *A. brasilense* GltS mutant. The amino acid sequences of the encoded peptides showed considerable homology with GltS from *E. coli* (Pelanda et al. 1993; Mandal and Ghosh 1993), although, in contrast to *E. coli*, the *A. brasilense glt*D is upstream relative to *glt*B. However, the promoter sequences of *glt*B differ in the two bacteria. Whereas the *A. brasilense* gene has sigma-70 as well as potential sigma-54 recognition sites, the *E. coli* gene has only the former.

The *rpo*N gene which codes for the sigma-54 factor was identified in enteric bacteria as a component of a global regulatory mechanism responding to the cellular nitrogen status (de Bruijn and Ausubel 1983). The *Azospirillum rpo*N gene was isolated by PCR amplification using primers derived from three conserved regions found in all RpoN proteins (Milcamps et al. 1996). An *Azospirillum rpo*N mutant was defective in nitrogen fixation, nitrate assimilation, ammonium uptake and motility (Milcamps et al. 1996).

In enteric bacteria, the *ntr*BC gene products, together with the sigma-54 factor, are essential for the transcription of *nif*A. However, in *A. brasilense, ntr*BC genes are not essential for nitrogen fixation, although they are required to turn off nitrogenase activity (Liang et al. 1993) through the regulation of DRAG activity (Zhang et al. 1994). In addition to the requirement of *A. brasilense ntr*B and *ntr*C genes for the regulation of nitrogenase activity, these genes are required for the utilization of nitrate (Machado et al. 1995).

Genes involved in plant root attachment

The secure attachment of *Azospirillum* is essential for a long-term association with a host plant (De Troch and Vanderleyden 1996). It allows the bacteria to anchor it-self to the root for better access to plant exudates And to prevent bacterial cells from being washed away. Attachment helps the plant by allowing substances excreted by bacteria to diffuse into the intercellular spaces of the root cortex and by leaving no empty association sites on the roots for non-beneficial colonizers. Bacterial surface polysaccharide components often play an essential role in plant-microbial interactions. For example, exopolysaccharide (EPS) production by *Rhizobium* has been implicated in the attachment and successful colonization of legume roots (Del Gallo et al. 1989). *Rhizobium* Exo⁻ mutants may be detected by a lack of fluorescence of colonies grown on media con-taining the fluorescent dye Calcofluor, which binds predominantly to β-linked polysaccharides. One approach in the search for *Azospirillum* genes involved in EPS production was to look for polysaccharides which bind Calcofluor. The occurrence in *Azospirillum* of these genes was investigated through complementation studies of *R. meliloti* Exo⁻ mutants with a cosmid library of *A. brasilense* Sp7. Some cosmid clones corrected the deficiency in EPS synthesis as well as the symbiotic effectiveness of the *R. meliloti exo*B, -G, -K, -M, -N, and -P mutants. Interestingly, the *R. meliloti exo*B mutant was shown to be complemented two distinct *A. brasilense* loci, one located on the p90 plasmid and the other on the chromosome (Vande Broek and Vanderleyden 1995). In *R. meliloti*, the *exo*B gene encodes the enzyme UDP-glucose-4-epimerase. or GalE, that is essential for the biosynthesis of lipopolysaccharides, EPS, and cyclic â-glucans. DNA sequence analysis of both *exo*B genes from *A. brasilense* revealed the presence of an ORF encoding a protein with a high homology to the *R. meliloti* ExoB and the *E. coli* GalE proteins. Both *Azospirillum* genes are probably functional, although to date, their role in polysaccharide biosynthesis and plant root association has not been investigated because *A. brasilense* strains carrying mutations at both loci are not yet available.

The *R. meliloti exo*C mutant could be corrected only for its Calcofluor-binding phenotype but not for the formation of nitrogen-fixing nodules. This was because the *A. brasilense exo*C complementing locus, which is located on the p90 plasmid, encodes a different enzymatic activity than the one missing in the *R. meliloti exo*C mutant.

A. brasilense mutants with Tn5 insertions in their *exo*B and *exo*C, loci failed to produce the wild-type high molecular weight EPS, but instead produced EPSs of lower molecular weight. These mutants still fluoresced in the presence of Calcofluor, suggesting that synthesis of Calcofluor-binding polysaccharides is coded for by genes other than exoB and *exo*C. Michiels et al. (1990) obtained other *Azospirillum* Tn5 mutants that could not bind to Calcoflour (Cal⁻), and showed by DNA hybridization that the genetic loci affected were different from those of *exo*B and *exo*C in A. *brasilense*.

Two distinct phases of attachment for *Azospirillum* have been described. The first, or adsorption phase,

consists of reversible weak binding in which hydro-phobic cell wall proteins or polar flagellum are proba-bly involved (De Troch and Vanderleyden 1996). In the second phase of attachment, called anchoring, charac-terized by the production of long fibrils and a large amount of mucigel-like substances (Bashan et al. 1991), the bacteria become irreversibly bound to the roots. Anchoring is probably mediated by a bacterial polysac-charide that binds Calcofluor (Michiels et al.1991). The Cal⁻ mutants, mentioned above, had lost the capability of anchoring to wheat roots (Anc⁻) but retained wild-type adsorption capacity (Ads⁺). These mutants pro-duced comparable amounts of surface polysaccharides to the wild-type, but could not form flocs (cell macroag-gregates in a matrix of polysaccharide material that form large macroscopic clumps; Sadasivan and Neyra 1985). These results indicate that (an) as yet unidenti-fied surface polysaccharide(s) that bind(s) to Calco-fluor, is are involved in both floc formation and anchor-ing to roots. Another mutant, Cal⁺, carrying a deletion in the p90 plasmid, displayed the opposite phenotype (Ads⁻, Anc⁺) confirming the proposal that two steps arc involved in the attachment of *Azospirillum* to plant roots (Michiels et al. 1991). Longer term experiments with plants inoculated with the Cal⁺ and Cal⁻ mutants will help to determine the importance of this *Azospiril-lum* Calcofluor-binding polysaccharide in bacterial col-onization, and its participation in plant growth promo-tion.

Genetic complementation of a spontaneous mutant, impaired in flocculation and colonization of the root surface, led to the identification of a new regulatory gene designated *flc*A in *A. brasilense S*p7 (Pereg-Gerk et al. 1998). The deduced amino acid sequence of the *A. brasilense flc*A gene was homologous to a family of transcriptional activators of the LuxR-UhpA family. This *flc*A gene controls the production of capsular po-lysaccharides, the process of flocculation in culture and the colonization of the root surface in wheat.

In the presence of a suitable carbon and energy source, and under nitrogen starvation, high oxygen ten-sion or water stress conditions, *Azospirillum*'s vibrioid cells can develop into round, nonmotile, highly refrac-tile encapsulated forms (called C forms) rich in poly-β-hydroxybutyrate (PHB; Bastarrachea et al. 1988). Unlike spores and cysts, C forms are not dormant since they are capable of fixing nitrogen, and have high ni-trate reductase and GS activity (Bashan and Holguin 1997). C forms may have a competitive advantage over cells without PHB in soils where water and nutrients are scarce (Fallik and Okon 1996).

The difference in plant growth promoting activity between these two types of *Azospirillum* cells has not been clearly established, although significant growth promotion of maize was observed after inoculation with cells containing high levels of PHB (Fallik and Okon 1996). Bastarrachea et al. (1988) isolated sponta-neous mutants unable to differentiate into encapsulated C forms (Enc⁻) and to synthesize EPS. These results

suggest that *A. brasilense* vegetative cells do not pro-duce surface polysaccharides (De Troch and Vander-leyden 1996) and because of their ability to synthesize EPS, C form cells may have a competitive advantage over vegetative cells by more effectively colonizing plant roots. However, after inoculation of wheat roots, the non-encapsulating mutants adhered to the roots in the same numbers as the wild-type strains, although the colonization pattern was different. The Enc⁻ muta-tions occurred spontaneously at a high frequency, i.e., 10⁻⁵, and the frequency of reversion to the wild-type phenotype was also high. The isolation of the genes re-sponsible for the differentiation and de-differentiation processes from vibrioid to C forms will undoubtedly be helpful in determining the role of vibrioid and C form cells in plant growth promotion. These differentiation processes may occur under natural conditions and may explain some of the variability in results obtained by *Azospirillum* inoculation.

When wheat germ agglutinin (WGA), a graminae lectin, binds to the surface of a bacterial cell it brings about numerous changes in cell metabolism (Mirelman et al. 1975). WGA binds to *A. brasilense* and *A. lipofe-rum* cells (Del Gallo et al. 1989) and, for example, can promote nitrogen fixation, GS activity and ammonia excretion in *A. brasilense* Sp245 (Antonyuk et al. 1993). The latter authors suggest that WGA may function as a signal molecule in the *Azospirillum*-plant association. It is not known which *A. brasilense* cell-surface structures are responsible for the binding of WGA. The receptor may be a capsular polysaccharide component since this fraction was found to have WGA-binding properties (Del Gallo et al. 1989). The indication that vegetative cells do not produce EPSs and hence do not have WGA binding properties, reinforces the sugges-tion that C forms may have competitive advantages over vegetative cells.

The inability of a mutant lacking polar flagella (re-sponsible for swimming motility in liquid medium) to adsorb to wheat roots demonstrates that the adsorption capacity of *Azospirillum* depends on the presence of the polar flagella (Croes et al. 1993). Chemical disintegration of the flagella also eliminated adsorption, and purified polar flagella were able to bind to wheat roots in vitro. In *A. brasilense, A. lipoferum* and *A. irakense,* lateral flagella (encoded by genes designated *laf*) appear when the organisms are grown on solid and semisolid media, suggesting a surface-dependent induction. The *A. brasilense* Sp7 gene *laf*1, encoding the flagellin of the lateral flagella, was isolated using PCR oligonucleotide primers designed from the amino acid sequence of the *A. brasilense* Lafl protein. One PCR product, which hybridized to a *R. meliloti fla*A region (encoding the polar flagella), was then used as a probe to screen a cosmid library of *A. brasilense* Sp7. One positive cosmid clone was selected; its DNA sequence revealed an ORF with a high level of similarity to other bacterial flagellin genes. The degree of wheat root colonization by an *A. brasilense* Sp7 *laf*l mutant, con

structed by gene replacement with a *gus* gene, was not significantly different after 4 days from that achieved by the wild-type. However, the involvement of the Lafl protein in long-term colonization, whereby the lateral flagella would enable the bacteria to move along the root surface (a mechanism suggested for surface colonization by *Vibrio parahaemolyticus*), could not be excluded (Moens et al.1995). The region upstream of lafl did not show the sigma-28 consensus promoter sequence typical for flagellar genes in other strains; a sigma-54 box was, however, present.

Chemotaxis is indispensable for successful root colonization by *Azospirillum* spp. When a wild-type strain of *A. lipoferum* and its motile chemotactic mutant KM105 were inoculated together on roots of Kallar grass and rice, the wild-type bacterium colonized the roots up to 150 times more efficiently than the mutant (Kimmel et al. 1990).

The gene encoding a protein similar to the ChvE protein of *Agrobacterium tumefaciens* was cloned from *A. brasilense* Sp245 using a restriction fragment of the chvE gene of *Agrobacterium tumefaciens* as a hybridization probe and sequenced. This *A. brasilense* protein (which is called SbpA, or sugar-binding protein A) was shown by insertional mutagenesis to be involved in chemotaxis towards the sugars D-galactose, L-arabinose, and D-fucose, and is part of a high-affinity uptake system for D-galactose (Van Dommelen et al. 1997).

Genes involved in phytohormone biosynthesis

In culture, *Azospirillum* produces several phytohormones including IAA, gibberellins, cytokinins and ethylene (Bashan and Holguin 1997). Plant growth hormones produced by bacteria can increase growth rates and improve yields of the host plants (Glick 1995). IAA production in particular has been proposed to account for the beneficial effect of the plant-*Azospirillum* association. Inoculation of wheat seedlings with an *A. brasilense Nif* IAA $^+$ morphotype enhanced the number and length of lateral roots as compared to the wild-type; however, inoculation with a Nif but low IAA producer, did not elicit any response from the plant (Barbieri et al. 1986). The effect of *Azospirillum* inoculation on root elongation of wheat plants (Kolb and Martin 1985) and on branching of wheat root hairs (Jain and Patriquin 1985) was mimicked by the application of IAA; however, in some instances the application of the synthetic hormone did not simulate the effects induced by *Azospirillum* (Yahalom et al. 1990). Other phytohormones may contribute to the effect on plants. For example, the gibberellin GA3 had similar effects to inoculation with *A. lipoferum* on the promotion of root growth, especially in increasing root hair density (Piccoli and Bottini 1994).

The relative specific radioactivities of tryptophan, indoleacetamide (IAM) and IAA formed during ^{3}H-IAM and ^{3}H-Trp feeding experiments suggested the presence of at least three IAA synthetic pathways in *Azospirillum*: one Trp-independent and two Trp-dependent pathways (Fig. 3). One of the Trp-dependent pathways was identified as the IAM pathway. However, when tryptophan was not added to the medium, the IAM pathway accounted for only 0.1% of the total IAA production (Prinsen et al. 1993). Evidence for the IAM pathway in *A. brasilense was* found by Bar and Okon (1993) who detected tryptophan-2-monooxygenase (TMO) activity, an enzyme known to be involved in this pathway (Patten and Glick 1996). They also found partial homology between the iaaM gene (encoding TMO in Pseudomonas syringae) and genomic DNA from *A. brasilense*. Regarding the presence of the tryptamine pathway in *Azospirillum*, Hartmann et al. (1983) found that *A. brasilense* Cd, but not *A. lipoferum*, could convert tryptamine to IAA when it was added to cultures growing on minimal medium (Fig. 3).

Conclusive evidence for the presence of the indole3-pyruvic acid (IPyA) pathway in *A. brasilense was* presented by Costacurta et al. (1994), who isolated the *A. brasilense* gene coding for indole-3-pyruvate decarboxylase (IPDC), a key enzyme in the IpyA pathway. Complementation of an IAA-attenuated Tn5-induced mutant of *A. brasilense* with a fragment from another strain of A. *brasilense,* helped to identify a sequence responsible for the synthesis of IAA. The predicted protein sequence showed some homology with IPDC from *Enterobacter cloacae*. Introduction of the cloned *Azospirillum* gene into R. *meliloti* resulted in increased IAA production. Furthermore, enzymatic activity of IPDC could be detected in cell-free extracts from *A. brasilense and* R. *meliloti*.

Differences between the IPDC arnino acid sequence of *A. brasilense* 245 (Costacurta et al. 1994; Zimmer et al. 1995) and the enterobacterial sequences may indi
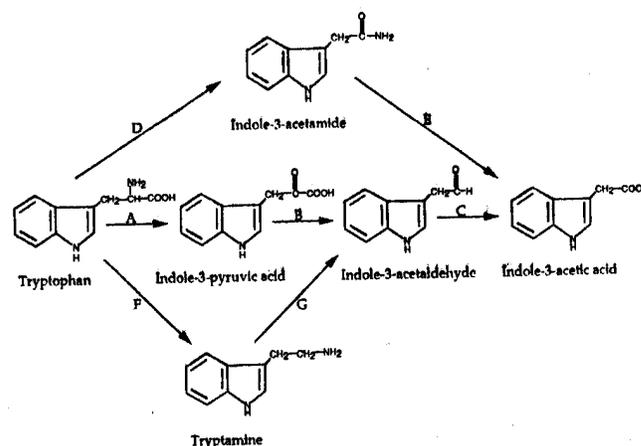

Fig. 3 Formation of indole-3-acetic acid from tryptophan in *Azospirillum* by the indole-3-pyruvic acid pathway (enzymes *A, B* and *C*), the indole-3-acetamide pathway (enzymes D and E) and the tryptamine pathway (enzymes *C, F* and *G*). A Tryptophan transferase, *B* indole-3-pyruvate decarboxylase, C indole-3-acetaldehyde oxidase, *D* tryptophan hydrolase, *E* indoleacetamide hydrolase, *F* tryptophan decarboxylase, *G* amine oxidase

cate that the *Azospirillum* IPDC enzyme has a different substrate specificity than IPDC from other microorganisms, and possibly also has some additional physiological functions. Since the ability to produce IAA is not limited to plant growth promoting bacteria, bacterial IAA might have functions other than promoting plant growth (Zimmer et al. 1995). The *ipd*C gene in *A. brasilense* has been shown to be induced by IAA but not by tryptophan. Induction of this gene by IAA suggests the possible existence of IAA-binding proteins and/or IAA-responsive elements on the DNA, such as has been observed for plants (Dosselaere et al. 1997).

To ascertain the role of IAA in the *Azospirillum*-plant interaction, there is a need for mutants that are totally deficient in IAA production; however, at present such mutants are unavailable (Vande Brock and Vanderleyden 1995). The same is true for the other phytohormones. Making the situation even more complex, a microbe may selectively employ a particular IAA biosynthesis pathway from the multiple pathways of which it is capable, according to its environment (Patten and Glick 1996). For example, experiments with an *ipd*C::gusA gene fusion showed that during the stationary phase, the *A. brasilense ipd*C gene is highly expressed when the bacterium is grown in a nutrient-rich medium; however, when the bacterium is grown in minimal medium IAA production is high while ipdC expression remains low. These results suggest that different IAA biosynthesis pathways are switched on according to the growth medium used (Dosselaere et al. 1997).

Trp biosynthesis genes

The first enzyme in the biosynthesis of L-Trp (TrpL) is anthranilate synthase, which converts chorismate into anthranilate (Fig. 4). By DNA hybridization followed by genetic complementation and DNA sequence analysis, De Troch et al. (1996) found that in *A. brasilense*, anthranilate synthase appears to be a fusion protein consisting of two components coded by trpE and trpG. The *trp*E gene codes for the anthranilate synthase á-subunit, while the *trp*G gene codes for a glutamine amidotransferase, which transfers ammonia from glutamic to chorismate. The trpE(G) gene is preceded by a DNA sequence that encodes a TrpL leader transcript with a secondary RNA structure that suggests attenuation control. Since no additional control mechanisms were found, this indicates that the *trp*E(G) gene is Trp regulated through attenuation (Dosselaere et at. 1997). Another copy of the trpG gene in *A. brasilense* is linked to *trp*DC forming the trpGDC operon which contains two transcription initiation sites, one for *trp*GDC and one for *trp*DC. The *trp*D gene encodes the enzyme phosphoribosyl anthranilate transferase, which adds a phosphoribosyl group to anthranilate in the second step of Trp biosynthesis (Zimmer et al. 1991). TrpC is the indoleglycerolphosphale synthase which catalyzes
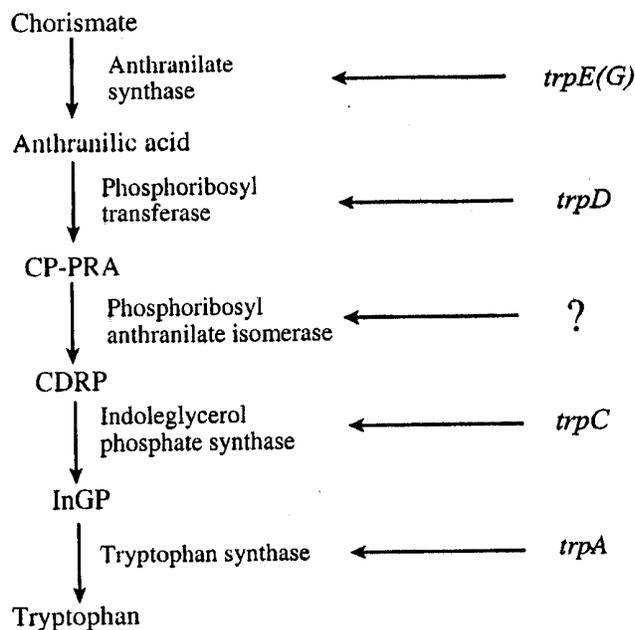

Fig. 4 Organization of the genes of the *Azospirillum brasilense* tryptophan operon. Although the *trpA* gene has only been found in *A. lipoferum,* it is assumed to encode the same protein in A. *brasilense. TrpE(G) A* fusion protein encoded by *trpE* and *trpG, CP-PRA N-(o-*carboxyphenyl)-phosphoribosyla mine, *CDRP N-(o-*carboxyphenylamino)-1-deoxyribulose 5'-phosphate, *InGP* indole-glycerol phosphate

the fourth step in the tryptophan biosynthetic pathway. It has riot been established which growth conditions induce the transcription of *trp*GDC or *trp*DC (Zimmer et al. 1991). In *E. coli*, Trp synthase is a complex formed from the products of *trp*A and *trp*B (Moat and Foster 1988). By complementation of an *E. coli* Trp⁻ mutant, *A. lipoferum trp*A was localized (Ramschütz et al. 1992).

Zimmer et al. (1991) found that *A. irakense* KA3 released 10 times less IAA into the medium than *A. brasilense* Sp7. They were able to enhance *A. irakense* IAA production by transforming the strain with cosmids from a library of *A. brasilense* Sp7 DNA. The region responsible for the enhanced IAA production in *A. irakense* corresponded to TrpD, coding for phosphoribosyl anthranilate transferase which participates in the second step of Trp biosynthesis. The authors proposed that IAA production in *A. irakense* was increased by the insertion of *trp*D because its product, phosphoribosyl anthranilate transferase, allowed the conversion of anthranilate to Trp, followed by IAA synthesis. The activity of TrpD did not allow the intercellular accumulation of anthranilate which can inhibit the conversion of Trp to IAA. In accordance with this proposition regarding the regulatory role of the anthranilate pool in IAA production, Katzy et al. (1990) isolated an IAA mutant which produced very low levels of IAA concomitant with increased anthranilate accumulation (Vande Brock and Vanderleyden 1995).

Genes involved in carbon metabolism

Organic acids are the preferred carbon source of *Azospirillum.* Their availability as root exudates may be decisive in the successful colonization of the bacteria on cereal roots and in associative nitrogen fixation. A DNA probe containing the *R. meliloti* structural gene for dicarboxylate transport (*dct*A) hybridized strongly with fragments of *A. lipoferum* genomic DNA. One cosmid clone from a genomic library of *A. lipoferum,* transferred into a *R. meliloti dct*A, mutant, restored growth of the mutant on succinate. Hybridization of the *R. meliloti dct*A gene with *A. brasilense* and *A. halopraeferens* was positive but weak, suggesting that in these latter two species the dctA gene is less conserved. This work demonstrated the presence of a *Rhizobium*-like dicarboxylate transport system in *A. brasilense* Sp7 and suggested that azospirilla have access to a rather constant supply of dicarboxylates in the rhizosphere (Tripathi and Mishra 1996).

Azospirillum utilizes succinate or malate in preference to carbohydrates, and the synthesis of fructose-inducible enzymes of the fructose (fru) phosphoenol pyruvate phosphotransferase system encoded by the inducible *fru* operon, are subject to repression by succinate in *A. brasilense. In* an effort to understand the regulatory mechanisms of carbohydrate utilization, Chattopadhyay et al. (1993, 1994) identified, cloned, and sequenced a gene responsible for the growth of *A. brasilense* on carbohydrates. DNA sequence analysis of a 2.8-kb genomic fragment of A. *brasilense R*G that complemented a pleiotropic carbohydrate mutant (*car* mutant) of the same strain that was incapable of growth on carbohydrates and that overlapped the *fru* operon, revealed three ORFs; the genes coding for ORF2 and ORF3 have been designated *car*R and *car*S, respectively. The *car*R and *car*S genes are arranged in an operon under the control of a promoter with a sigma-70 recognition site. A complementation study with *car*RS deletion clones showed that only the *car*R gene was required to complement the *car* mutant, which meant that the carbohydrate pleiotropy was due to a lesion within this gene. Comparison of conserved primary structure motifs of some known two-component regulatory proteins with that of the CARR-CARS pair suggests that the protein pair may constitute a novel two-component regulatory system for the utilization of carbohydrates as carbon sources (Chattopadhyay et al. 1993, 1994).

Other genes

A putative *rib*A gene (encoding GTP cyclohydrolase II, which controls the first step in the synthesis of riboflavin) from *A. brasilense was* identified through hybridization with *rib*A-specific oligodeoxyribonucleotides. Sequence analysis of the selected fragment of *A. brasilense* DNA*,* revealed homology to the *E. coli rib*A gene (Van Bastelaere et al. 1995).

Cosmid clones carrying the *A. brasilense rec*A gene were isolated by complementation of an E. coli ReCA⁻ mutant. The recombinational activity of an A. *brasilense* RecA⁻ mutant, constructed by gene replacement, was reduced considerably compared to the wild-typt. The *Rec*A mutant showed severely reduced growth in methyl methanesulfonate as well as increased sensitivity to UV light, suggesting that in *A. brasilense,* as in other bacteria, the main pathways of DNA repair and homologous recombination are mediated by *rec*A (Croes et al. 1990).

The *E. coli lon* gene encodes an ATP-dependent protease (Lon, also called La) that can help to eliminate stress-denatured proteins and catalyzes the turnover of some proteins involved in cell septation and capsule synthesis. The *lon* gene belongs to the *E. coli* heat-shock regulon, and its transcription is controlled by the heat-shock-specific sigma-32 factor. In *A. brasilense* the *lon* gene was identified during the analysis of Tn5-induced mutants defective in iron uptake. Northern blot analysis using an *A. brasilense* Ion-specific probe, showed that transcription of the *Ion* gene was triggered by heat shock. Thus, in addition to its participation in iron uptake, the *lon* gene of *A. brasilense is* also involved in the heat-shock response (Mori et al. 1996).

An *A. brasilense pyr*G gene was identified by complementation of a cytidine-requiring PyrG-deficient *E. coli* mutant. A second open reading frame whose function is unknown was identified downstream of *pyr*G. The deduced amino acid sequence showed homology to the dienelactone hydrolases of *Pseudomonas* and *Alcaligenes,* enzymes involved in the utilization of halogenated aromatic compounds (Zimmer and Hundeshagen 1994).

The genes involved in histidine biosynthesis (his genes) in both *Salmonella typhimurphium* and *E. coli* are arranged in an operon, in a cluster of nine genes. In A. *brasilense* three clustered *his* genes were identified by genetic complementation of his from *E. coli* (Bazzicalupo et al. 1987).

Conclusions

There is little doubt that the isolation and characterization of additional *Azospirillum* genes will contribute to nn increased understanding of the mechanisms that this bacterium uses to promote plant growth. Moreover, the detailed understanding of these mechanisms should permit researchers to genetically engineer new and more effective strains of *Azospirillum.*

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