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Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects

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Abstract

Azospirillum represents the best characterized genus of plant growth-promoting rhizobacteria. Other free-living diazotrophs repeatedly detected in association with plant roots, include *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azoarcus* spp. and *Azotobacter*. Four aspects of the *Azospirillum*–plant root interaction are highlighted: natural habitat, plant root interaction, nitrogen fixation and biosynthesis of plant growth hormones. Each of these aspects is dealt with in a comparative way. Azospirilla are predominantly surface-colonizing bacteria, whereas *A. diazotrophicus*, *H. seropedicae* and *Azoarcus* sp. are endophytic diazotrophs. The attachment of *Azospirillum* cells to plant roots occurs in two steps. The polar flagellum, of which the flagellin was shown to be a glycoprotein, mediates the adsorption step. An as yet unidentified surface polysaccharide is believed to be essential in the subsequent anchoring phase. In *Azoarcus* sp. the attachment process is mediated by type IV pili. Nitrogen fixation structural genes (*nif*) are highly conserved among all nitrogen-fixing bacteria, and in all diazotrophic species of the class of proteobacteria examined, the transcriptional activator NifA is required for expression of other *nif* genes in response to two major environmental signals (oxygen and fixed N). However, the mechanisms involved in this control can vary in different organisms. In *Azospirillum brasilense* and *H. seropedicae* (α- and β-subgroup, respectively), NifA is inactive in conditions of excess nitrogen. Activation of NifA upon removal of fixed N seems to involve, either directly or indirectly, the signal transduction protein P_{II}. The presence of four conserved cysteine residues in the NifA protein might be an indication that NifA is directly sensitive to oxygen. In *Azotobacter vinelandii* (γ-subgroup) *nifA* is cotranscribed with a second gene *nifL*. The *nifL* gene product inactivates NifA in response to high oxygen tension and cellular nitrogen-status. NifL was found to be a redox-sensitive flavoprotein. The relief of NifL inhibition on NifA activity, in response to N-limitation, is suggested to involve a P_{II}-like protein. Moreover, nitrogenase activity is regulated according to the intracellular nitrogen and O_2 level. In *A. brasilense* and *Azospirillum lipoferum* posttranslational control of nitrogenase, in response to ammonium and anaerobiosis, involves ADP-ribosylation of the nitrogenase iron protein, mediated by the enzymes DraT and DraG. At least three pathways for indole-3-acetic acid (IAA) biosynthesis in *A. brasilense* exist: two Trp-dependent (the indole-3-pyruvic acid and presumably the indole-3-acetamide pathway) and one Trp-independent pathway. The occurrence of an IAA biosynthetic pathway not using Trp (tryptophan) as precursor is highly unusual in bacteria. Nevertheless, the indole-3-pyruvate decarboxylase encoding *ipdC* gene is crucial in the overall IAA biosynthesis in *Azospirillum*. A number of genes essential for Trp production have been isolated in *A. brasilense*, including *trpE(G)* which codes for anthranilate synthase, the key enzyme in Trp biosynthesis. The relevance of each of these four aspects for plant growth promotion by *Azospirillum* is discussed.

Keywords: *Azospirillum*; *Azotobacter*; Endophytic diazotroph; Plant root interaction; Nitrogen fixation; Indole-3-acetic acid

Contents

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1. Introduction

Bacteria of the genus *Azospirillum* (α-subclass of proteobacteria) are known for many years as plant growth promoting rhizobacteria (PGPR) [1,2]. They were isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates [3,4]. Both in greenhouse and in field trials, *Azospirillum* was shown to exert beneficial effects on plant growth and crop yields [5,6]. At present, five species have been described: *Azospirillum lipoferum*, *Azospirillum brasilense* [7], *Azospirillum amazonense* [8], *Azospirillum halopraeferens* [9] and *Azospirillum irakense* [10].

Azospirilla are Gram-negative free-living nitrogen-fixing rhizosphere bacteria. They display a versatile C- and N-metabolism, which makes them well adapted to establish in the competitive environment of the rhizosphere. Ammonium, nitrate, nitrite, amino acids and molecular nitrogen can serve as N-sources [11]. In unfavorable conditions, such as desiccation and nutrient limitation, azospirilla can convert into enlarged cyst-like forms [12–14]. This morphological change is accompanied by the development of an outer coat of polysaccharides and by the accumulation of abundant poly-β-hydroxybutyrate granules, which can serve as C- and energy source under conditions of stress and starvation [15,16].

Bacteria belonging to the genus *Azospirillum* are highly motile. *A. brasilense*, *A. lipoferum* and *A. irakense* display a mixed pattern of flagellation [17,18]. One polar flagellum is synthesized during growth in liquid medium and is primarily used for swimming. Additional lateral flagella are induced during growth on solidified media and are responsible for swarming of the bacteria over solid surfaces. *A. halopraeferens* and *A. amazonense* only display the polar flagellum [18]. Motility offers the bacterium the advantage of moving towards favorable nutrient conditions. Azospirilla exhibit positive chemotaxis towards organic acids, sugars, amino acids [19–21] and aromatic compounds [22,23] as well as towards root exudates [24]. Furthermore, migration of azospirilla towards wheat seedlings grown in the soil was demonstrated and was shown to be limited by soil moisture [25]. This indicates that free swimming through water films in the soil rather than swarming plays a major role in chemotactic behavior in natural environments. Another feature of azospirilla is the directed movement towards optimal oxygen concentrations, called aerotaxis [26]. This behavioral response can be advantageous to guide the bacteria to optimal niches for nitrogen fixation [27].

Under certain environmental and soil conditions, *Azospirillum* can positively influence plant growth, crop yields and N-content of the plant. This plant stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms, including biological nitrogen fixation and auxin production. The contribution of biological nitrogen fixation in this plant response, however, has often been questioned [4,5,28–31]. Moreover, azospirilla do not excrete significant amounts of ammonium under diazotrophic growth. Therefore, other factors, such as production of plant growth-promoting substances [32] and increase in the rate of mineral uptake by plant roots [33] have been taken into account to explain the plant yield enhancement and could even be more important in establishing the plant response. Upon *Azospirillum* inoculation an alteration in root morphology was observed, which has been ascribed to the bacterial production of plant growth regulating substances [32,34–37]. An increased number of lateral roots and root hairs enlarges the root surface available for nutrients. This results in a higher nutrient uptake by inoculated roots and an improved water status of the plant, which in turn could be the main factor enhancing plant growth [1,33,38]. Four different aspects of the *Azospirillum*–plant interaction, the natural habitat, plant root interaction, nitrogen fixation and production of plant growth regulating substances, will be highlighted in the following sections.

Other diazotrophic bacteria, which have been repeatedly isolated from plant roots, comprise the microaerobically nitrogen-fixing bacteria, *Acetobacter diazotrophicus* (α-subgroup proteobacteria), *Herbaspirillum seropedicae* (β-subgroup proteobacteria) and *Azoarcus* spp. (β-subgroup proteobacteria). So far, studies concerning these bacteria mainly dealt with establishing their endophytic nature. Less knowledge has been attained at the genetic level. Although the aerobic nitrogen-fixing bacterium *Azotobacter* (γ-subgroup proteobacteria) has been often isolated from the rhizosphere soil of various plants [39,40], re-

Table 1
Bacterial species discussed

Bacterial species		Origin of isolation	Reference
Azospirillum brasilense	Sp245	Surface-sterilized wheat roots, Brazil	[43]
	Sp7	Rhizosphere soil of *Digitaria decumbens*, Brazil	[7]
Azospirillum lipoferum		Roots of wheat and maize, Brazil	[7]
Azospirillum amazonense		Roots and rhizosphere soil of Gramineae, Amazon region, Brazil	[8]
Azospirillum halopraeferens		Roots of Kallar grass, grown in saline soils in Pakistan	[9]
Azospirillum irakense		Roots and rhizosphere of rice, Iraq	[10]
Acetobacter diazotrophicus		Roots and stems of sugarcane, Brazil	[51]
Herbaspirillum seropedicae		Cereal roots (maize, sorghum, rice), Brazil	[61]
Azoarcus		Roots of Kallar grass, grown in saline soils in Pakistan	[64]

search on this genus almost exclusively focused on the nitrogen fixation process [41].

Bacterial species discussed in this review are listed in Table 1.

2. Natural habitat

Azospirilla are generally regarded as rhizosphere bacteria, but display strain-specific differences in the way they colonize roots. They predominantly colonize the root surface and only a few strains are able to infect plants [4,42]. Some *Azospirillum* strains have specific mechanisms to interact with roots and colonize even the root interior, while others colonize the mucigel layer or injured root cortical cells. Using root surface sterilization methods, it was demonstrated that certain strains of *Azospirillum* spp. in fact colonize the root interior of wheat [43]. Monitoring the colonization pattern of *A. brasilense* Sp245 on wheat roots, using a constitutively expressed *Escherichia coli gusA* gene, it was shown that the sites of primary root colonization are the points of lateral root emergence and the root hair zones [44]. By means of strain-specific monoclonal antibodies and immunological techniques, *A. brasilense* Sp245 was detected in the root xylem of a particular Brazilian wheat cultivar, while *A. brasilense* Sp7 only colonized the root surface [45]. The application of fluorescently labelled rRNA-targeted oligonucleotide probes in combination with scanning confocal microscopy confirmed that *A. brasilense* Sp245 enters the interior of root hair cells, which had apparently intact cell walls, whereas the occurrence of strain Sp7 was restricted to the rhizosphere soil, mainly to the root hair zone of that same Brazilian wheat cultivar [46]. The physiological basis for the observed invasiveness of *A. brasilense* Sp245 is not known. Since pectin is a major constituent of the primary cell wall and middle lamellae, and low levels of pectinolytic and cellulolytic activities have been detected in *Azospirillum* cultures, the bacteria may eventually enter the root cortex intercellular spaces via enzymatic degradation of host cell wall middle lamellae [32,36,47]. Except for *A. irakense*, none of the *Azospirillum* species is able to grow on pectin as sole C-source [10]. Recently, genes encoding a pectate lyase (*pelA*) and two aryl β-glucosidases (*salA* and *salB*) have been isolated in *A. irakense* by heterologous expression in *E. coli* [48,49]. Alternatively, *Azospirillum* species may enter the root through lysed root hairs and cracks, disrupted cortical tissues at lateral root junctions [32]. In conclusion, when analyzed for particular properties, such as mode of plant root colonization, strain-specific differences for *A. brasilense* can be observed. Nevertheless, when compared by two-dimensional SDS–PAGE, little differences are observed at the protein level [50].

The microaerobically nitrogen-fixing bacteria *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *Azoarcus* spp. are characterized by a typical endophytic habitat. *A. diazotrophicus* has been found almost exclusively in association with sugar-rich plants, such as sugar cane, sweet potato and Cameroon grass [51–53] and was shown to be completely absent in the soil [54]. However, recently this bacterium was also isolated from surface sterilized stems and roots of coffee plants [55]. *A. diazotrophicus* was isolated from roots, stems and leaves of sugar cane and has even been located in the xylem sap, indicating translocation of the bacteria through the plant tissues via the vascular system [51,56,57]. As could be judged from ultrastructural and immunogold labelling techniques combined with electron microscopy, *A. diazotrophicus* is able to colonize the xylem vessels and penetrate roots intercellularly at the root tip (behind the root cap) and at cracks in the lateral root junctions. It was suggested that xylem sap may act both as a path of infection and a niche for nitrogen fixation (low pO_2 atmosphere) and exchange of metabolites between the bacterium and the plant [58]. The limited genetic diversity of isolates of *A. diazotrophicus* may reflect its predominant endophytic habitat and its restricted host range [59,60].

Hc. seropedicae shows a wider host range specificity, since it has been isolated from many graminaceous plants grown in Brazil, such as maize, sorghum, rice, sugar cane and forage grasses [61]. It has been found in high numbers inside roots, stems and leaves of these graminaceous plants [57], but not within leaves of sugar cane [62]. Electron microscopy studies have shown the colonization of vascu-

lar tissues of sorghum, rice and sugar cane by *H. seropedicae* [42,62]. Like other endophytes, it does not survive well in natural soil without the presence of a host plant [54,62]. Quite recently, experimental data were presented that show intercellular root colonization of *Arabidopsis thaliana* (dicot) by *H. seropedicae*. This colonization appeared to be stimulated by flavonoids [63].

Azoarcus sp. has been isolated from Kallar grass grown in saline soils in Pakistan [64–66] and was repeatedly found inside Kallar grass roots, where it is thought to benefit from a more efficient transfer of metabolites between host and diazotrophs [67]. The sites of primary colonization seem to be the root tips at the zone of elongation and differentiation, and the points of lateral root emergence [67]. By immunocytochemical localization using light and electron microscopy in combination with histochemical localization of reporter gene expression, *Azoarcus* sp. was shown to invade Kallar grass roots inter- and intracellularly, where it infects the cortex region and penetrates the stele of the roots [67,68]. Systemic spreading through the plant shoots probably occurs via the xylem vessels. *Azoarcus* sp. was shown to express two types of cell surface-associated cellulolytic enzymes (exo- and endoglucanase). Since this bacterium cannot grow on cellulose or its breakdown products, it was anticipated that cellulases are involved in the infection process of grass roots [69]. More recently, detection of an *Azoarcus* nitrogenase sequence demonstrated natural occurrence of this bacterium in roots of field-grown rice [70].

Isolation studies that resulted in the identification of the above described different bacterial genera and species could suggest host-specificity. However, to date there are too little data from cross-inoculation studies with isolated strains to support this conclusion. It is very likely that part of the specificity observed reflects the fact that some of the strains are better adapted to specific soil conditions from which the bacteria were originally isolated (e.g. *Azoarcus* in saline soils).

A recent report favors the subdivision of nitrogen-fixing bacteria colonizing graminaceous plants into three categories: rhizosphere diazotrophs, facultative endophytic diazotrophs and obligate endophytic diazotrophs [54]. According to this view, *Azospirillum* would be considered a facultative endophytic microorganism, whereas *A. diazotrophicus*, *H. seropedicae* and *Azoarcus* are in the group of obligate endophytic diazotrophs.

3. Plant root interaction

An *Azospirillum*–plant root association can only be successful if the bacterium is able to survive in the soil and attain significant populations on the host root system. In the rhizosphere, decreasing nutrient gradients from the root to the surrounding soil are generated by plant root exudates. Motility and chemotaxis enable the bacteria to move towards plant roots where they can benefit from root exudates as carbon and energy source, and may therefore contribute to survival and rhizosphere colonization. The exact mechanism of how *Azospirillum* interacts with the plant roots is not yet fully understood. Genes reported in this part are listed in Table 2.

The attempt to isolate plant-inducible bacterial genes of *A. brasilense*, based on analysis of protein patterns of bacteria grown in the presence and absence of plant root exudates, resulted in the identification of an acidic 40-kDa protein, SbpA. This protein is very similar to the ChvE protein of *Agrobacterium tumefaciens*, and is involved in uptake of D-galactose and functions in the chemotaxis of *A. brasilense* towards several sugars (D-fucose, L-arabinose and D-galactose) [71]. This suggests that initial steps of plant interaction are conserved among different bacteria.

Chemotaxis not only requires chemo-attractants, but is also dependent on the motility of the bacteria. *A. brasilense* genes required for motility in liquid medium, for motility on surfaces and for general chemotaxis were found to be located on the chromosome or to reside on a 90-MDa plasmid [72,73]. All *Azospirillum* species possess plasmids of which the sizes range from 4 MDa to over 300 MDa [74]. A 90-MDa plasmid has been commonly observed in *A. brasilense* and *A. lipoferum* [74,75]. In other soil bacteria, megaplasmids are known to carry information essential for plant interaction. The *Agrobacterium* virulence (*vir*) genes, as well as the *Rhizobium* nodulation (*nod*) and host specific nodulation (*hsn*) genes are carried by megaplasmids. The construction of deletion mutants of the 90-MDa plasmid (p90) of *A. brasilense* Sp7 led to the identification of three loci involved in motility: Mot1, Mot2 and Mot3. Since deletions in Mot1 and Mot2 affected motility in semisolid, but not in liquid medium, these loci are most probably involved in the synthesis or functioning of the lateral flagella. The Mot3 deletion mutant is completely non-motile and apparently lacks both polar and lateral flagella [73]. In addition, this latter mutant was severely impaired in adsorption ability to wheat roots [76]. The use of different *A. brasilense* motility mutants in a colonization assay, directly demonstrated the requirement of bacterial chemotactic movement for initiation of wheat root colonization at the root hair zone [77].

The primary function of bacterial flagella involves locomotion and allows the bacteria to move towards the most favorable environment, using environmental information for directing the movement. Furthermore, flagella are designated functions in several attachment processes, including a number of bacteria–plant interactions [78]. Aflagellate mutants of *Pseudomonas fluorescens* were found to be impaired in their ability to colonize potato roots. Motility mutants of *Erwinia carotovora* subsp. *carotovora* exhibited reduced virulence in surface-inoculated tobacco plants and in stem-inoculated potato plants. Mutants of *E. carotovora* pv. *atroseptica*, exhibiting reduced virulence in stem-inoc-

Table 2
Azospirillum loci involved in plant root interaction

Identified as		Function	
Gene	Protein	Homology data	Biochemical data
pelA			Pectate lyase
salA, salB		Genes encoding glycosyl hydrolases of family 3	Aryl β-glucosidases
sbpA		*chvE* of *Agrobacterium tumefaciens*, required for full virulence	Transporter D-galactose; chemotaxis towards sugars
mot1, mot2			Motility, synthesis/functioning lateral flagella
mot3			Motility, synthesis/functioning lateral and polar flagella
laf1			Flagellin lateral flagella
	Fla1		Structural protein polar flagellum
flcA			Regulator which controls CPS production, flocculation, wheat root colonization
	MOMP		Major outer membrane protein
chvB		Chromosomal virulence gene required for plant infection by *Agrobacterium tumefaciens*	
nodPQ		Host-specific nodulation genes of *Rhizobium meliloti*, encoding ATP sulfurylase	
nodG		Host-specific nodulation gene of *Rhizobium meliloti*, proposed to encode a dehydrogenase	
exoB		UDP-glucose-4-epimerase of *Rhizobium meliloti*	UDP-glucose-4-epimerase
exoC		Phosphomannomutase of *Pseudomonas aeruginosa* (AlgC)	

ulated potato plants, were found to be non-motile and affected in genes resembling flagellar biosynthetic genes [78]. The adhesion of *Pseudomonas aeruginosa* to mucin is one of the early steps in the process of colonization of the human airways. A structural component of the flagellum, the flagellar cap protein FliD, participates in the adhesion process [79]. The structural gene encoding the flagellin of the lateral flagella (*laf1*) in *A. brasilense* Sp7 has been isolated [18]. Using a monospecific polyclonal antibody against the polar flagellum and a translational *laf1*::*gusA* fusion, it was shown that hindrance of rotation of the polar flagellum can induce expression of the structural gene of the lateral flagellin [80]. Moreover, *laf1* expression is blocked in an *ntrA* mutant. Root colonization is not altered in a *laf1*::Km mutant compared to the wild-type in the initial stage of plant root interaction. An effect on long-term colonization, whereby lateral flagella enable the bacteria to move along the root surface could not be excluded [18]. The structural protein of the polar flagellum, Fla1, appeared to be a glycoprotein that is suggested to act as a plant root surface adhesin [81]. The glycosylation of the flagellin of the polar flagellum has been demonstrated in several ways: by a decrease in apparent M_r after chemical deglycosylation; by sugar staining after SDS–PAGE; by use of a sugar-specific monoclonal antibody in immunogold labelling coupled with transmission electron microscopy [81].

The attachment of *Azospirillum* to the plant roots is essential for efficient association with the host plant. Using short-term in vitro binding assays, it was demonstrated that this attachment is a biphasic process [82]. In a first step, the bacteria adsorb to the roots as single cells in a rapid, weak and reversible way. Several lines of evidence suggest the involvement of the polar flagellum in this adsorption phase: a mutant of *A. brasilense*, lacking both polar and lateral flagella, as well as three additional and genetically different mutants, lacking the polar flagellum, were shown to be deficient in adsorption to wheat roots; desintegration of the flagella by heat or acid treatments eliminates wheat root adsorption by *A. brasilense*; purified polar flagella of *A. brasilense* were demonstrated to bind to wheat roots, whereas lateral flagella were not [76]. The adsorption phase is followed by the anchoring phase in which bacterial aggregates are formed that are firmly and irreversibly anchored to the roots (Fig. 1). It has been suggested that anchoring depends on bacterial extracellular polysaccharide production [82–85]. The appearance of aggregates of azospirilla entrapped in fibrillar material in association with plant roots has been reported [4,32]. However, the nature of the observed fibrillar material in the *Azospirillum*–plant root association is still obscure [84]. Extracellular polysaccharide production has also been related to the process of flocculation of *Azospirillum* cells and might be similar to the fibrillar material produced during root association [85,86]. A spontaneous

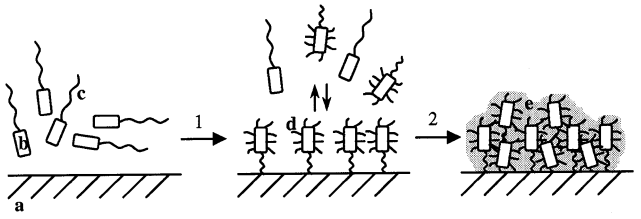

Fig. 1. Biphasic attachment process of *Azospirillum brasilense* to plant root surfaces. Step 1 represents a weak, reversible adsorption mediated by the flagellin of the polar flagellum. Step 2 represents firm, irreversible anchoring, in which extracellular polysaccharides play a role. The letters used refer to the plant root surface (a), *A. brasilense* cells (b), the polar flagellum (c), lateral flagella (d) and extracellular polysaccharides (e). The role of the lateral flagella in the attachment process is not yet clear.

mutant of *A. brasilense* Sp7, impaired in surface polysaccharide production and flocculation, displayed a modified colonization pattern of wheat roots as compared to the wild-type strain [87]. Genetic complementation of the flocculation negative phenotype of this mutant led to the identification of *flcA*, a regulatory gene, which was demonstrated by site-directed Tn*5* mutagenesis to control the production of capsular polysaccharides, the flocculation process and the colonization of wheat roots [88]. Furthermore, it has been hypothesized that root lectins are involved in the process of root colonization [32]. Wheat germ agglutinin (WGA) has been proposed as a signal molecule in the association between *Azospirillum* and wheat roots. Binding of WGA was reported to induce changes in the cellular metabolism of *A. brasilense* Sp245 and promoted N_2 fixation, excretion of NH_4^+ ions and indole-3-acetic acid (IAA) biosynthesis [89,90]. A similar increase of nitrogen fixation capability in the presence of WGA has been demonstrated in *A. lipoferum* [91]. Although binding of WGA results in higher expression of *Azospirillum nif* genes (*nifA*, *nifH*), it is not yet known how specific this effect is. It cannot be excluded that WGA binding induces an overall change in bacterial metabolism, of which enhanced *nif* expression is a consequence. Nevertheless, it will be of interest to identify the initial events leading to the observed changes in gene transcription. Binding of WGA to cell surface components of *Azospirillum* has been demonstrated, but it remains unclear which structures of the cell surface of *A. brasilense* are responsible for binding of WGA [92]. Others report on the presumed presence of lectin-like proteins on the cell wall of *Azospirillum* spp., which might be involved in the recognition and colonization of root surfaces [93]. A major outer membrane protein of about 42 kDa (MOMP) has been identified in *A. brasilense*. It was proposed that, under certain growth conditions, outer membrane proteins interact with exopolysaccharides (EPS) leading to aggregation and flocculation [94]. From the rhizosphere of rice, a motile laccase-negative (4B) and a non-motile laccase-positive (4T) strain of *A. lipoferum* have been simultaneously isolated at the same frequency. Both strains were able to efficiently colonize rice roots, but the motile form 4B (wild-type) remained dominant. After inoculation of rice roots with *A. lipoferum* 4B, spontaneous stable non-motile laccase-negative forms appeared in high numbers [95]. Substantial in vitro evidence has been presented suggesting that a non-motile laccase-positive atypical *A. lipoferum* strain emerged from the motile laccase-negative wild-type strain (4B) via a two-step phenotypic switching event. Generation of an intermediate non-motile laccase-negative variant via a phase variation-like process, is followed by the appearance of a non-motile laccase-positive variant under extremely low O_2 concentrations. This process may represent a strategy for adaptation to the near anaerobic conditions typical of the rice rhizosphere [96].

In a first approach to isolate *Azospirillum* genes involved in plant interaction, sequences homologous to the *A. tumefaciens chv* [97,98] and *Rhizobium meliloti nod* and *hsn* genes [99–101], which are essential for attachment and tumor formation of plant cells or for nodule formation, respectively [102], have been identified. The *A. brasilense chvB* gene could complement a *chvB* mutational defect in *A. tumefaciens* with respect to tumor formation in leaf disks of *Nicotiana tabacum*. It was speculated that the *chvB* locus participates in root adsorption in *Azospirillum* [98]. The *A. brasilense* Sp7 *nodPQ* genes were found to reside on the 90-MDa plasmid [100], whereas the *nodG*-homologous gene appeared to be chromosomally located [103]. However, the function of these genes in the *Azospirillum*–plant interaction remains unclear.

Genetic loci involved in surface polysaccharide synthesis in *A. brasilense* were identified in two independent ways: via random Tn*5* mutagenesis or via genetic complementation of *R. meliloti exo* mutants. *A. brasilense* produces cell-bound capsular (CPS) as well as loosely attached extracellular (EPS) Calcofluor-binding polysaccharides [92,104]. Calcofluor is a dye that specifically binds β-1,4- and β-1,3-linked polysaccharides. Tn*5*-induced *A. brasilense* mutants with altered surface polysaccharide synthesis could be isolated by screening for decreased or increased levels of fluorescence on media containing Calcofluor [105]. Since the *A. brasilense* Cal^- mutants produced slightly higher amounts of EPS, which was shown to bind Calcofluor to the same extent as the parent strain, fluorescence is caused by binding of another surface polysaccharide [106]. These Cal^- mutants had lost the ability to anchor to wheat roots (Anc^-) but retained wild-type adsorption capacity (Ads^+), indicating that a yet unidentified surface polysaccharide is involved in anchoring [82]. Alternatively, *A. brasilense* genes that code for enzymes involved in exopolysaccharide (EPS) synthesis were isolated through complementation of *R. meliloti exoB* and *exoC* mutants for their Calcofluor-dark phenotype. These mutants are deficient in synthesis of succinoglycan, the major acidic EPS of *Rhizobium* spp. and form empty non-fixing nodules on alfalfa. Two *A. brasilense exoB* loci (*exoB1* and *exoB2*) and one *exoC* locus were isolated [107,108]. Both *A. brasilense exoB* loci could complement the *R. meliloti exoB* mutant for production of succinoglycan as well as for the symbiotic phenotype, whereas the *A. brasilense exoC* locus was not sufficient for normal nodule infection [107]. The *A. brasilense exoB* loci are functionally homologous to *R. meliloti exoB*, which encodes an UDP-glucose-4-epimerase, involved in the synthesis of UDP-galactose for incorporation into exopolysaccharides, capsular polysaccharides and lipopolysaccharides [109]. The *A. brasilense exoC* locus encodes a protein that displays significant homology with the *P. aeruginosa algC* gene product. *algC* codes for a phosphomannomutase essential for the conversion of GDP mannose-6-phosphate in GDP mannose-1-phosphate [108]. The *A. brasilense exoC* correcting locus apparently encodes an enzymatic activity different from

the one that is missing in the *R. meliloti exoC* mutant (phosphoglucomutase). This might explain both the inability to restore wild-type EPS production and the symbiotic phenotype in the complemented *R. meliloti exoC* mutant. The *A. brasilense exoB1* and *exoC* loci were mapped on the p90 plasmid [73,75], the *exoB2* locus is chromosomally located [108]. *A. brasilense* strains carrying mutations in the *exoB1* and *exoC* correcting loci fluoresced as brightly as the wild-type strain and produced wild-type levels of EPS, although of a lower molecular mass, indicating that the EPS synthesized by the *A. brasilense exo* loci is not the Calcofluor-binding polysaccharide [107]. No difference in primary wheat root colonization has been observed between the *A. brasilense* wild-type strain and the *exoB1* and *exoC* mutants [77].

Bacterial genes determining properties for plant root interaction by *A. diazotrophicus*, *H. seropedicae* and *Azoarcus* sp. are less well studied. Interestingly, adhesion of *Azoarcus* sp. to plant roots seems to be mediated by type IV pili. Two genes, *pilAB*, involved in pilus formation have been isolated [110]. Type IV pili also play an essential role in the adhesion of some animal and human pathogenic bacteria to eukaryotic cells.

4. Nitrogen fixation

4.1. Characterization of genes involved in nitrogen fixation, ammonium assimilation, ammonium uptake and nitrogen regulation

4.1.1. Nitrogen fixation

Azospirillum can convert atmospheric nitrogen into ammonium under microaerobic conditions at low nitrogen levels, through the action of the nitrogenase complex. This enzyme is built from two components: the dinitrogenase protein (MoFe protein, NifDK), which contains a molybdenum-iron cofactor, is the site of N_2 reduction; the dinitrogenase reductase protein (Fe protein, NifH) transfers electrons from an electron donor to the nitrogenase protein [111]. Most of the genetic work on nitrogen fixation by *Azospirillum* has been carried out with *A. brasilense*. An *A. brasilense nifHDK* operon, encoding both nitrogenase components, has been isolated based on sequence similarity with the *Klebsiella pneumoniae nifHDK* genes [112,113]. A number of additional *nif* and *fix* genes involved in processing of and electron transport to the nitrogenase enzyme complex, FeMo cofactor biosynthesis as well as regulation of nitrogen fixation have been isolated [114–119]. Except for the separately transcribed *nifA* and *nifB* genes, they all reside in the major 30 kb *nif* gene cluster, containing the *nifHDK* genes [120]. *A. brasilense* genes involved in nitrogen fixation and nitrogen assimilation are listed in Table 3.

Table 3
Azospirillum genes involved in nitrogen fixation, nitrogen assimilation and nitrogen regulation

Gene	Function
nifH	Structural gene dinitrogenase reductase (Fe protein)
nifD	Structural gene dinitrogenase (MoFe protein, α-subunit)
nifK	Structural gene dinitrogenase (MoFe protein, β-subunit)
nifA	Transcriptional activator of the nitrogen fixation (*nif*) genes
gltDB	Glutamate synthase structural genes
glnA	Glutamine synthetase structural gene
amtB	Structural gene ammonium transporter
glnB	N-signal transmitter protein P_{II}
glnZ	P_{II} homologue
glnD	Uridylyltransferase/uridylyl-removing enzyme (UTase/UR)
ntrB	Sensor protein of two-component regulatory system involved in general nitrogen control
ntrC	Regulator protein of two-component regulatory system involved in general nitrogen control
ntrA, rpoN	Alternative sigma factor, σ^{54}
draT	Dinitrogenase reductase ADP ribosyl-transferase
draG	Dinitrogenase reductase activating glycohydrolase

Nitrogen fixation in *Azotobacter vinelandii* is complicated by the presence of three biochemically and genetically distinct nitrogenase enzymes, synthesized under different conditions of metal supply [121]. The conventional molybdenum nitrogenase is encoded by the *nifHDK* genes. Similarly to *Azospirillum*, the *A. vinelandii nifA* and *nifB* genes are not linked to the major *nifHDK* gene cluster [122–124]. The *H. seropedicae* and *A. diazotrophicus nifHDK* genes have been isolated by hybridization with an *A. brasilense nifHDK* probe [125,126]. Complementation of an *A. brasilense nifA* mutant led to the isolation of the corresponding *H. seropedicae nifA* gene [127]. Additional *A. diazotrophicus nif* genes, including *nifA*, were isolated by complementation of *A. vinelandii* mutants [126,128]. Unlike the genetic organization in the other three bacterial species, the *A. diazotrophicus nifA* and *nifB* genes are clustered with the *nifHDK* genes [126,127].

4.1.2. Ammonium assimilation

Ammonium fixed by nitrogenase is assimilated by *Azospirillum*, mainly through the glutamine synthetase (GS)/glutamate synthase (GltS) pathway [129]. Two genes, *gltDB*, were found to encode the subunits of glutamate synthase in *A. brasilense* [130]. Glutamine synthetase is encoded by *glnA*, which is part of an operon with *glnB* [131,132]. *glnA* can be cotranscribed together with *glnB* from a σ^{70}- or a σ^{54}-dependent promoter according to the cellular N-status, or can be expressed from its own, as yet unidentified, N-regulated promoter [133]. Moreover, GS activity is regulated in response to ammonium by reversible adenylylation. During N-excess, GS is highly adenylylated and inactive, whereas removal of the adenylyl groups of GS activates the enzyme in N-limiting conditions. Contrary to the situation in enteric bacteria, this regulation of the adenylylation level of GS is not mediated through the *glnB* gene product (P_{II}). The gene corresponding to the adenylyltransferase (ATase, GlnE) has not yet

been characterized in *Azospirillum*. *A. brasilense* derivatives impaired in GS activity and nitrogen fixation have been isolated and show two phenotypes: Gln^-Nif^- and Gln^-Nif^c (fixing nitrogen in the presence of ammonium). Both mutant types could be complemented by the *A. brasilense glnA* gene, suggesting a central role for GS in the regulation of nitrogen fixation [131,134].

The structural organization of the *A. brasilense* GS encoding region differs substantially from the *glnA–ntrBC* operon of enteric bacteria and *Azotobacter* [41,121,135]. The *A. vinelandii glnA* gene is expressed from a σ^{70}-dependent promoter [135].

4.1.3. Ammonium uptake

The finding that, in nitrogen-limiting conditions, *Azospirillum* can take up trace amounts of NH_4^+, suggested the existence of an ammonium-repressed energy-dependent uptake mechanism [136]. An additional role in re-uptake of small amounts of NH_4^+, which might leak out of the cell by NH_3 diffusion through the membrane, has been assigned to this transport system [137]. An *A. brasilense* gene encoding this N-regulated ammonium transporter (*amtB*) has been characterized [138]. Mutants with an interrupted *amtB* gene are impaired in both $^{14}CH_3NH_4^+$ uptake and growth on low ammonium concentrations. The observation that the latter is not completely abolished in an *amtB* mutant, suggests the existence of a second NH_4^+ transport system [138]. The AmtB protein was predicted to have 12 transmembrane domains, which is consistent with the overall structure of other membrane carrier proteins. Unlike the situation in enteric bacteria, the *A. brasilense* gene encoding the ammonium transporter (*amtB*) is not located in the vicinity of the *glnB* or *glnZ* gene. The *amtB* promoter region is characterized by a σ^{54} consensus sequence and transcription requires the NtrBC two-component regulatory system [138,139]. The second *A. brasilense* P_{II}-like protein (P_Z, encoded by *glnZ*) was shown to negatively regulate (methyl)ammonium uptake. Since P_Z is not required for NtrC-dependent regulation in *A. brasilense*, it is proposed that this protein is involved in modulating AmtB activity [140].

A similar ammonium transporter gene (*amtB*) has been found in *A. vinelandii* downstream of *glnK* (*glnB* homologue) [141,142]. Both genes are cotranscribed and their levels of expression are not significantly influenced by the fixed N supply. This is in agreement with the lack of σ^{54} and NtrC recognition motifs.

4.1.4. Nitrogen regulation

In enteric bacteria the global nitrogen regulatory system (*ntr*) depends on four gene products (GlnD, GlnB, NtrBC) and controls the expression of several N-regulated operons, including the nitrogen fixation operon *nifLA* [143]. The uridylyltransferase/uridylyl-removing enzyme (GlnD) is considered the primary sensor of the N-status of the cell and reversibly affects the uridylylation-status of the P_{II} protein (GlnB), which in turn modulates the activity of NtrBC. The *ntrBC* genes encode a two-component regulatory system. In contrast to the situation encountered in enteric bacteria, the *A. brasilense ntrBC* genes seemed to be located distantly from the *glnA* gene and are not essential for nitrogen fixation, as an *ntrC* mutant does not result in a Nif^- phenotype [144,145].

In *K. pneumoniae*, the P_{II} protein has been shown to play a central role in the regulation of nitrogen metabolism by coordinating the response of the *ntr* system to the cellular N-status and by regulating GS biosynthesis and activity. However, the *glnB* gene product is not essential for nitrogen fixation [41]. The *A. brasilense* N-responsive signal transduction protein (P_{II})-encoding *glnB* gene is adjacent to the *glnA* gene. The *glnBA* operon is characterized by three different, selectively used N-regulated promoters [132,133]. In conditions of N-excess, *glnBA* are cotranscribed at a low level from the *glnBp1* (σ^{70}) promoter and *glnA* is transcribed at a high level from its own, as yet unidentified, promoter. Maximal transcription of *glnBA* occurs in N-limiting conditions and is initiated from the *glnBp2* (σ^{54}) promoter. Under these conditions, *glnA* is poorly expressed from *glnAp*. As a consequence, *glnB* expression is increased under N-limiting conditions, which correlates with the essential role of the corresponding protein in the nitrogen fixation process. Although clearly N-regulated, expression of *glnB-glnA* does not involve NtrC, but requires an alternative transcriptional activator. The P_{II} protein, however, was shown to negatively regulate its own synthesis in conditions of N-excess, presumably by acting on this unknown activator [133]. Recently, a second P_{II}-like protein (P_Z) has been identified in *A. brasilense*. It is encoded by the *glnZ* gene [140,146]. Although structurally similar to P_{II}, the P_Z protein seems to be functionally different. Both P_{II} and P_Z were demonstrated to be uridylylated under N-limiting conditions, most probably at a conserved tyrosine residue (Tyr-51). A clear phenotype could be ascribed to an *A. brasilense glnB* mutant, which could not be compensated by P_Z. The P_{II} protein is required for N_2 fixation and is involved in swarming motility. P_{II} nor P_Z seemed to be involved in control of GS activity by adenylylation [140,146]. The role of the P_Z protein in the nitrogen regulation cascade remains unclear [140,146]. As for *glnB*, *glnZ* transcription is initiated from a σ^{54}-dependent promoter. Contrary to *glnB*, *glnZ* expression requires the transcriptional activator NtrC in conditions of nitrogen fixation as well as of ammonium assimilation. A weak transcription of *glnZ* was assumed to start from a second unidentified promoter [140].

Two *glnB*-like genes have been reported in *H. seropedicae*, of which one has been sequenced. The location of this *H. seropedicae glnB*-like gene resembles the organization in enteric bacteria and is different from that in *A. brasilense*, in that *glnB* is not adjacent to *glnA*. It is constitutively expressed from a σ^{70} promoter and it is apparently

independent from *ntrBC* [147]. A *H. seropedicae glnB* mutation did not affect GS synthesis nor activity. Likewise, *A. diazotrophicus* harbors two copies of *glnB*-like genes. Sequence analysis revealed a *glnA* gene downstream of *glnB*, and an *amtB*-like gene adjacent to *glnZ* (*glnK*) [148]. Recently a *glnB*-like gene (*glnK*) has been characterized in *A. vinelandii*. This gene was suggested to be the only P_{II}-encoding gene present in this organism. Attempts to isolate *glnK* mutant strains were unsuccessful, indicating an essential role for *A. vinelandii glnK* [141,142]. As in enteric bacteria, the *A. vinelandii* P_{II}-like gene is probably involved in control of GS adenylylation level [141,142]. In *Azoarcus*, even three *glnB* homologues (*glnB*, *glnK*, *glnY*) have been isolated and sequenced, of which two are preferentially transcribed under conditions of nitrogen fixation [149].

The *rpoN* or *ntrA* gene encoding the alternative σ^{54} factor has been characterized in *A. brasilense*. An *rpoN* mutant displays a pleiotropic phenotype, revealing involvement of the *rpoN* gene product in diverse cellular functions, including nitrogen fixation, nitrate assimilation, ammonium uptake and flagellar biosynthesis [139].

4.2. Regulation of the nitrogen fixation process

Azospirillum only fixes nitrogen in microaerobic N-limiting conditions. Ammonium, glutamine, nitrate and nitrite have all been shown to repress N_2 fixation in *A. brasilense* [150]. Since the reduction of N_2 into ammonium is a highly energy-demanding process and the nitrogenase enzyme complex is very sensitive to oxygen, biological N_2 fixation is a tightly regulated process. It is indeed subject to elaborate control in response to the intracellular concentration of fixed N and to the oxygen tension. This regulation is situated at two levels and involves transcriptional as well as posttranslational control (Fig. 2).

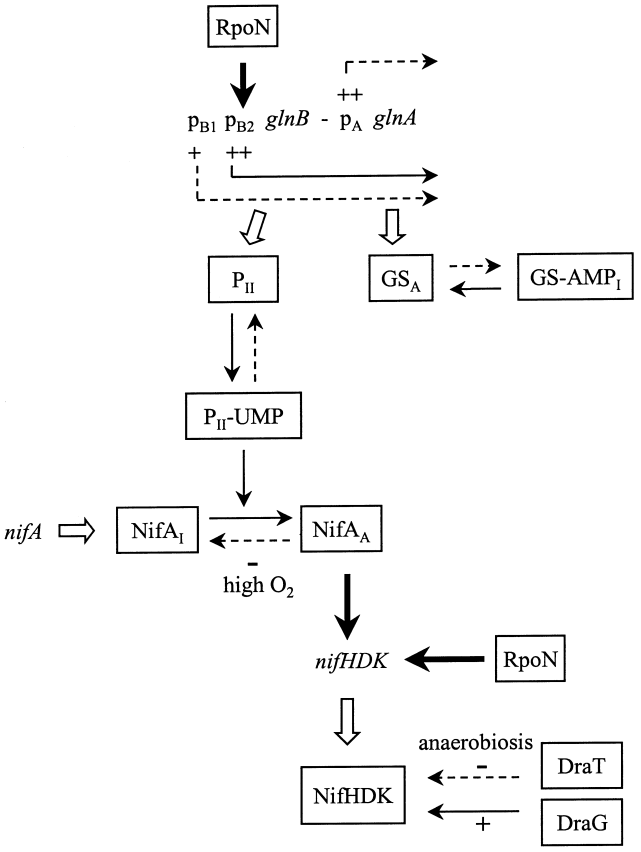

Fig. 2. Proposed model for the regulation of nitrogenase biosynthesis and activity in *Azospirillum brasilense*. Symbols: transcription (open arrows), positive (+) or negative (−) regulation of transcription (thick arrows) and activity (thin arrows), N-limitation (full lines) and N-excess (dashed lines), relative efficiencies of transcription of the promoters (+, ++). GS, glutamine synthetase; AMP, adenosine monophosphate; UMP, uridine monophosphate; $_A$, active form; $_I$, inactive form.

4.2.1. Transcriptional regulation of nif gene expression

As for all Gram-negative nitrogen-fixing bacteria studied thus far, expression of the *Azospirillum* and *Azotobacter nif* structural genes generally requires NtrA and the transcriptional activator, NifA [120,143,151–154]. In accordance, σ^{54} type (−24/−12) promoters and upstream activating sequences (UAS) typical for the binding of NifA have been found upstream of several *A. brasilense* and *A. vinelandii nif* genes [116,123,124,155,156]. Upon binding of NifA at the upstream activator sequences, NifA interacts with the bound RNA polymerase holoenzyme and concomitant hydrolysis of ATP results in the formation of an open promoter complex and initiation of transcription. Similarly, sequences identical to the σ^{54} consensus sequence and NifA binding sites in the promoter region of *H. seropedicae nifB* and *nifH* and *A. diazotrophicus nifB*, suggested that *nif* gene expression in these organisms is likely to require NtrA and NifA [125–127,157]. All characterized NifA proteins have the three-domain structure characteristic of the family of σ^{54}-dependent activator proteins. It consists of three domains interconnected by two linker regions. The C-terminal domain contains a helix-turn-helix motif, characteristic for DNA binding, whereas the central domain is involved in interaction with the $E\sigma^{54}$ RNA polymerase holoenzyme and contains a nucleotide binding site necessary for NifA nucleotide phosphotransferase activity. The N-terminal domain of all NifA proteins shows a low degree of sequence similarity [120,122,126,157].

In *A. brasilense nifA* is constitutively expressed independently of the presence of combined N or of the oxygen concentration. However, its level of expression is lower under conditions incompatible with N_2 fixation [120,158]. As in the case of *Azotobacter*, *nifA* expression in *A. brasilense ntrBC* mutants indicated no major role for the regulatory NtrBC proteins in the regulation of *A. brasilense nifA* transcription [120,153,154,158]. This coincides with the absence of a σ^{54} consensus sequence in its promoter region. However, NtrC is required for optimal nitrogen fixation and maximal expression of the *A. brasilense nifA*

gene under N_2 fixation conditions, although probably not as the result of a direct action of NtrC on the *nifA* promoter [145]. *nifA* gene expression is somewhat different in *H. seropedicae*. The presence of a −24/−12 consensus sequence and an NifA binding site in the *nifA* promoter region, favors the idea that NifA may autoregulate its own synthesis. Furthermore, an additional σ^{54} consensus sequence and an NtrC binding site further downstream of *nifA*, suggest a possible role for NtrC in *nifA* transcription [157].

However, in response to the cellular N-content and the oxygen tension, *A. brasilense* NifA activity is modulated [158]. By assaying the ability of truncated NifA proteins to drive *nifH–lacZ* transcription in the absence or presence of ammonium in wild-type and mutant strains, it has been proposed that the N-terminal domain of NifA is involved in the negative regulation of its own activity in the presence of ammonium. Upon removal of fixed nitrogen, this inhibition is assumed to be relieved through the direct or indirect action of the P_{II} protein (*glnB* gene product) [133,140,159]. An *A. brasilense glnB* mutant was demonstrated to be Nif$^-$, although transcription of *nifA* was not affected in this background [133,158]. A recently characterized *glnD* (UTase) null-mutant strain exhibits a Nif$^-$ phenotype, confirming that P_{II}-UMP is the active form required for NifA activation [160]. Moreover, a similar inhibitory effect of the N-terminal domain of NifA on NifA activity by ammonium has been demonstrated in *H. seropedicae*, by monitoring the activity of a truncated form of NifA in different genetic backgrounds with or without ammonium [161,162]. The Nif$^-$ phenotype of a *H. seropedicae glnB* mutant resembled that of an *A. brasilense glnB* mutant, suggesting that the P_{II} protein might possibly participate in signalling the nitrogen status to NifA [147,161]. Furthermore, four conserved and similarly spaced cysteine residues in the *A. brasilense* and *H. seropedicae* NifA proteins, as in *Bradyrhizobium japonicum* NifA, have been postulated to be involved in oxygen sensitivity of NifA activity [120,157,161,163].

In the γ-subdivision of proteobacteria, an additional gene, *nifL*, is required to regulate the activity of NifA in response to oxygen and fixed nitrogen [164]. In accordance to the situation in *K. pneumoniae* [143], *A. vinelandii nifA* is cotranscribed with a second gene *nifL* [165,166]. However, *nifLA* transcription does not involve NtrBC regulation in *A. vinelandii* [153,166]. From studies with transcriptional *nifA–lacZ* and *nifL–lacZ* fusions, *A. vinelandii nifLA* appears to be constitutively expressed [165,166]. On the other hand, NifA activity is controlled by the inhibitor protein NifL, both in response to oxygen and to fixed nitrogen. The mechanisms involved in NifL inhibition of NifA activity, in response to both environmental stimuli, are clearly separate [167,168]. Although *A. vinelandii* NifL resembles the histidine protein kinases of two component regulatory systems, modification of NifA by phosphorylation has not been detected [169]. Inhibition of NifA activity by NifL apparently requires stoichiometric amounts of both proteins, implying a direct protein–protein interaction between NifL and NifA [169]. Since nucleoside triphosphatase activity of NifA decreases when the inhibitory complex between NifL and NifA is formed, NifL may block NifA activity by inhibiting its catalytic function [170]. An *A. vinelandii nifL* mutant produced nitrogenase constitutively and allowed *nifH–lacZ* expression even in the presence of ammonium [165,166]. Nitrogen control by NifL on the transcriptional activator NifA was demonstrated to be dependent on the *glnD* (originally named *nfrX*) gene product (UTase) since a *nifL* mutation suppressed the Nif$^-$ phenotype of an *nfrX* mutant [154,171]. Recently, a *glnK* gene, which encodes a second P_{II} protein, has been characterized in *K. pneumoniae*. In a *glnK* null mutant strain, NifA activity is inhibited by NifL even in the absence of fixed N, suggesting a role for the second P_{II}-like protein, either directly or indirectly, in relieving NifL inhibition [172]. The recent discovery of a P_{II}-like protein encoded by *glnK* in *A. vinelandii*, supports a similar mechanism of regulation of NifL activity in response to fixed N [141,142]. Additionally, NifL activity is altered according to the oxygen tension. *A. vinelandii* NifL has been characterized as a flavoprotein, containing FAD as the prosthetic group. The ability of NifL to inhibit NifA activity is not influenced by oxygen, but is responsive to the oxidation state of the chromophore, indicating that NifL is a redox-sensitive regulator [173,174]. Moreover, inhibition of *A. vinelandii* NifA by the NifL protein is stimulated in vitro by the presence of adenosine nucleotides, particularly ADP, suggesting that formation of the inhibitory NifL–NifA complex might be regulated by the ATP/ADP ratio [170]. Therefore, it seems that in *A. vinelandii*, besides the nitrogen and oxygen status, the energy status is an important determinant of *nif* gene regulation. Studies with truncated NifL proteins have indicated that conserved regions in the N-terminal domain of NifL are the determinants for flavin binding. Conserved glycine rich motifs in the C-terminal part might be involved in nucleotide binding [168].

4.2.2. Posttranslational regulation of nitrogenase activity

An additional level of N_2 fixation regulation in *A. brasilense* involves the posttranslational control of nitrogenase activity. In conditions incompatible with nitrogen fixation the nitrogenase reductase subunit is reversibly inactivated by ADP-ribosylation. This switch-off mechanism has been described in *A. brasilense* and *A. lipoferum*, but was not found in *A. amazonense* [175,176]. Two genes (*draT* and *draG*) involved in this posttranslational regulatory process were found upstream of and divergently oriented with respect to *nifHDK* in both *A. brasilense* and *A. lipoferum* [177,178]. DraT encodes a dinitrogenase reductase ADP ribosyl-transferase, whereas DraG is a dinitrogenase reductase activating glycohydrolase. In the presence of high ammonium concentrations, DraT catalyzes

the transfer of an ADP-ribose of NAD^+ to Arg-101 of one subunit of dinitrogenase reductase, whereby the covalently modified nitrogenase enzyme is inactivated. When ammonium concentrations are low, this inactivation is reversed by the action of DraG, which removes the ADP-ribosyl moiety [179]. Similarly, the inactivation of the nitrogenase resulting from a shift to anaerobiosis is mediated by the DraT/DraG system [179,180]. However, posttranslational switch-off in response to high oxygen concentrations was found to be independent of an Fe protein modification [180]. The physiological signals responsive to anaerobiosis or fixed N and acting on DraT/DraG, however, still remain to be elucidated.

Both *A. brasilense* DraT and DraG themselves are subject to posttranslational regulation [179]. DraT is inactive under nitrogen-fixing conditions and is activated following a negative stimulus. DraG is active under nitrogen fixation conditions and is inactivated by a negative stimulus. *A. brasilense ntrBC* mutants escape nitrogenase switch-off by ammonium. The primary effect of *ntrBC* mutations probably involves changes in DraG activity [144,145,181]. However, nitrogenase was shown to be normally regulated in response to anaerobiosis in *ntrBC* mutants [181]. Therefore, different environmental stimuli (NH_4^+, anaerobiosis) probably use independent signal transduction pathways to affect the reversible ADP-ribosylation system.

An alternative mechanism of posttranslational regulation of nitrogenase in response to ammonium has been suggested based on the finding that strains, expressing a dinitrogenase reductase that could not be ADP-ribosylated, still showed partially NH_4^+ inhibited nitrogenase activity [182].

Small amounts of exogenous NH_4^+ rapidly and reversibly inhibited nitrogenase activity in *H. seropedicae*. However, the mechanism for this inhibition appears to be different from ADP-ribosylation [183]. Under oxygen stress conditions in *Azotobacter* spp., the nitrogenase components and an Fe_2S_2 protein (Shetna protein) form a stable complex, that is protected against oxygen damage, but that shows no nitrogenase activity. Reconversion to the catalytically active form occurs in conditions favorable for nitrogen fixation [184].

5. Production of plant growth regulating substances

One of the alternative explanations for the observed plant growth stimulation by *Azospirillum* inoculation, involves the production of plant growth regulatory substances by the bacterium. Three types of plant growth promoting substances could be detected in the supernatant of *Azospirillum* cultures (auxins, cytokinins and gibberellines) [34,185,186]. The quantitatively most important phytohormone produced by *Azospirillum* is the auxin indole-3-acetic acid (IAA). Bacterial phytohormone production is assumed to cause the detected changes in root morphology after *Azospirillum* inoculation, which in turn may be related to enhanced mineral uptake [35,36,187]. Inoculation experiments with *Azospirillum* mutants altered in indole-3-acetic acid production support this view [28,188,189].

The isolation of mutants that completely lack IAA biosynthesis has been unsuccessful, so far. Therefore, it was suggested that this auxin is being synthesized through at least two biosynthetic pathways in *Azospirillum* [190,191]. Tryptophan (Trp) is generally regarded as a precursor of IAA, because addition of this amino acid to cultures of IAA-producing bacteria, results in higher IAA production. Three main pathways are known for the conversion of tryptophan into IAA: the indole-3-pyruvic acid (IPyA) pathway; the indole-3-acetamide (IAM) pathway; and the tryptamine (TAM) pathway [192]. Feeding experiments with ^{3}H-Trp and ^{3}H-IAM confirmed the existence of two Trp-dependent pathways. Moreover, these studies provided evidence for the presence of an additional Trp-independent pathway of IAA biosynthesis in *A. brasilense* (Fig. 3) [193]. In medium without supplementation of exogenous Trp, only 10% of the specific radioactivity of ^{3}H-Trp is detected in IAA, and less than 0.1% of the radioactivity label of ^{3}H-IAM is incorporated in IAA. Therefore, it was concluded that 10% of IAA is produced from Trp in an non-IAM-dependent pathway, whereas Trp-independent IAA synthesis accounts for about 90%, in case no exogenous Trp is supplied. The specific activity recovered in IAM from ^{3}H-Trp confirmed that all IAM was produced from Trp. Addition of Trp to bacterial cultures remarkably enhances the Trp-dependent pathways for IAA production (approximately 20-fold increase). Biosynthesis of IAA without tryptophan as precursor has previously been shown in plants, but seems to be highly unusual in bacteria [194,195].

A first pathway for the production of IAA starting from Trp, involves the oxidative decarboxylation of Trp into IAM and the subsequent hydrolysis of IAM to obtain IAA (Fig. 3). Two different enzymes are involved in this two-step conversion, respectively tryptophan-2-monooxygenase (TMO) and indole-3-acetamide hydrolase (IAM-hydrolase). The genes encoding these enzymatic activities in *A. tumefaciens* and *Pseudomonas syringae* have been

Table 4
Azospirillum genes (proteins) involved in indole-3-acetic acid production

Identified as		Function
Gene	Protein	
ipdC		Indole-3-pyruvate decarboxylase
trpE(G)		Anthranilate synthase
trpG		Glutamine amido transferase (GATase)
trpD		Phosphoribosyl anthranilate transferase
trpC		Indole-3-glycerol phosphate synthase
	AATs	Aromatic amino acid aminotransferases

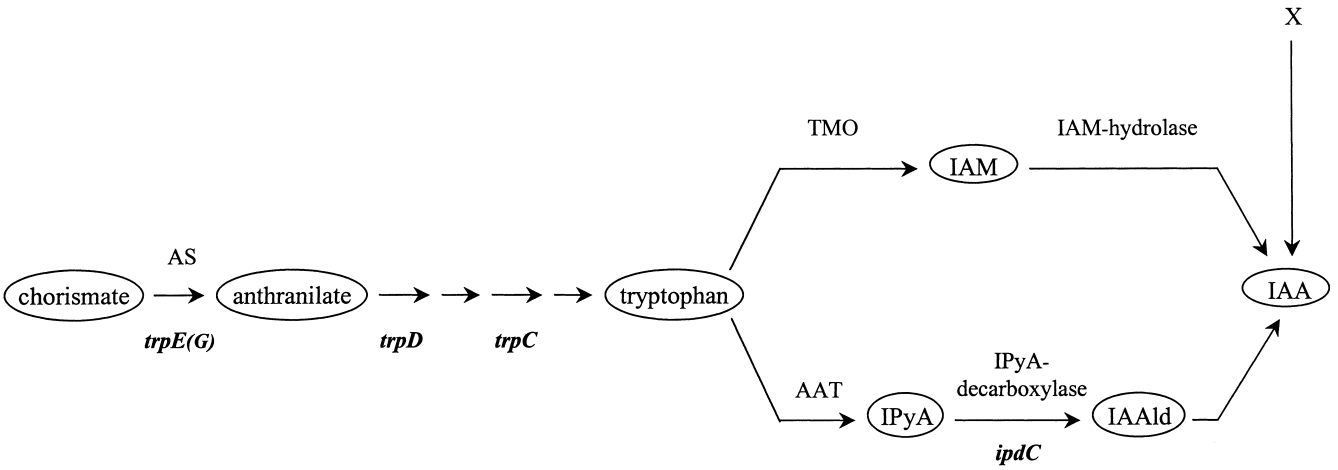

Fig. 3. Biosynthetic pathways for the production of tryptophan and indole-3-acetic acid in *Azospirillum brasilense*. IAM, indole-3-acetamide; IPyA, indole-3-pyruvic acid; IAAld, indole-3-acetaldehyde; IAA, indole-3-acetic acid; X, unknown precursor; AS, anthranilate synthase; TMO, tryptophan-2-monooxygenase; AAT, aromatic amino acid aminotransferase. The genes mentioned are those already isolated and characterized in *A. brasilense*.

cloned and sequenced [192]. The occurrence of an IAM-dependent pathway for IAA biosynthesis in *Azospirillum* was proposed upon the detection of TMO activity in crude cell extracts from *A. brasilense* and the observation of partial homology of an *A. brasilense* genomic DNA fragment with the *iaaM* gene of *P. syringae* [196]. *A. brasilense* genes and proteins known to be involved in IAA synthesis are listed in Table 4.

The second Trp-dependent pathway operating in *A. brasilense* has been identified as the IPyA pathway through genetic characterization of a low IAA producing mutant strain [197]. This is a major biosynthetic pathway for IAA production in plants. It involves the transamination of Trp to yield IPyA, followed by a decarboxylation reaction to form indole-3-acetaldehyde (IAAld). The subsequent oxidation reaction results in IAA formation (Fig. 3). Additional indications for the occurrence of this pathway in *Azospirillum* came from the identification of *A. brasilense* aromatic amino acid aminotransferases (AAT) [198]. One of these has been purified and was shown not to be exclusively specific for transamination of Trp. The only isolated and sequenced gene, so far, involved in IAA production in *A. brasilense* is the *ipdC* gene, encoding indole-3-pyruvate decarboxylase, which mediates the conversion of IPyA into IAAld [197]. Although the *ipdC* gene is preceded by a consensus sequence for a σ^{54}-dependent promoter, the functionality of this promoter element has not been established. DNA hybridization experiments suggested that the corresponding sequence is widespread in *A. brasilense*, *A. lipoferum*, *A. halopraeferens*, but absent in *A. irakense*. The nucleotide sequence of the *ipdC* gene has previously been reported for *Enterobacter cloacae* [199] and, more recently, for *Erwinia herbicola* [200]. An *A. brasilense ipdC* knock-out mutant was found to synthesize less than 10% of the level of wild-type IAA production, indicating that IPyA decarboxylase is a key enzyme for IAA biosynthesis in this bacterium [193]. Assaying β-glucuronidase activity from a translational *ipdC–gusA* fusion, showed that expression increased with cell density to reach a maximum level in the stationary growth phase [201]. This is in agreement with the highest production levels of IAA obtained in stationary grown cells. In the *ipdC* mutant maximum β-glucuronidase activity was reduced to 60% of that of wild-type cells. This finding made it tempting to speculate that the end product of the biosynthetic pathway (IAA) could be involved in a positive feedback regulation responsible for the increasing *ipdC* transcription levels during growth of an *A. brasilense* culture. Indeed, upregulation of *ipdC–gusA* expression was observed by exogenously added IAA, as well as by three synthetic auxins, and was most obvious in the *ipdC* mutant. The upregulation by IAA of *ipdC* transcription was further confirmed by Northern analysis [201]. These findings and the recognition of a TGTCCC element, reminiscent of auxin responsive elements (AuxRE) in plants, make the presence of proteins for IAA binding and signal transduction likely [202].

Since Trp is a precursor for IAA synthesis, research has focused on elucidating the biosynthetic pathway for the production of this amino acid. Trp is produced from chorismate in five consecutive steps (Fig. 3). Anthranilate synthase, which catalyzes the first step, namely the conversion of chorismate into anthranilate, has been considered a key enzyme in Trp biosynthesis. In *A. brasilense* anthranilate synthase is formed by a single polypeptide chain. It is encoded by the *trpE(G)* gene, deriving from the fusion of the *trpE–trpG* genes found in other bacteria. *trpE* encodes a protein similar to Component I of the well-characterized *Escherichia coli* anthranilate synthase and contains the site of anthranilate synthesis. *trpG* codes for Component II of the anthranilate synthase, which contains the glutamine amido transferase site of the enzyme [203]. Fusion of the *trpE* and *trpG* genes apparently took place, not only in *A. brasilense*, but also in *R. meliloti*

[204]. Upstream of the *trpE(G)* coding region, a short leader peptide-encoding sequence was detected, which contains three consecutive Trp codons. DNA sequences presumably involved in the formation of terminator and antiterminator stems are present as well. These findings suggest that Trp-directed regulation of synthesis of the anthranilate synthase of *A. brasilense* proceeds through attenuation control by the leader peptide TrpL, as was also found in *E. coli* and *R. meliloti* [203,205]. Of the two motifs known to be essential for feedback inhibition of anthranilate synthase by Trp, only one has been located in *A. brasilense* TrpE(G). However, feedback inhibition cannot be excluded. Previously, a *trpGDC* gene cluster was isolated in *A. brasilense* [206]. The physiological meaning of the presence of two *trpG* copies in the *Azospirillum* genome is still not clear. The *trpD* gene encodes a phosphoribosyl-anthranilate transferase, which adds a phosphoribosyl group to anthranilate in the second step in the biosynthesis of Trp. Introduction of this gene in *A. irakense*, which is a low IAA producer, and which releases high levels of anthranilate into the medium, enhanced IAA production. To explain this observation, it was proposed that regulation of IAA biosynthesis involves inhibition of IAA synthesis by anthranilate. According to this model, it was hypothesized that the phosphoribosyl-anthranilate transferase enzyme is lacking or inactive in *A. irakense*. As a result, accumulation of anthranilate takes place, leading to inhibition of IAA biosynthesis [206]. The characterization of an *A. brasilense* mutant, carrying a Tn*5*-Mob transposon in its 85-MDa plasmid, as being suppressed in IAA production and concomitantly enhanced in anthranilate production, could support this hypothesis of repression of IAA synthesis by anthranilate [207].

Although *Azotobacter* spp., *H. seropedicae*, *A. diazotrophicus* and *Azoarcus* spp. were found to produce plant growth regulatory substances, no genes involved in the biosynthesis of these phytohormones have yet been isolated [53,208–210].

6. Conclusions

Extensive genetic, biochemical and ecological studies have ranked *Azospirillum* as one of the best characterized genera among associative plant growth promoting rhizobacteria. However, despite the considerable amount of experimental data concerning *Azospirillum* stimulation of plant development, the mechanisms underlying the association process are still not well understood. This is mainly due to the absence of a clear plant phenotype indicative of a successful interaction, which makes a direct screening of large numbers of mutants not feasible. To get around this difficulty, genes possibly involved in plant interaction have been traced indirectly by characterization of mutants defective in a phenotype that is thought to be involved in plant association, or by screening for conserved structural or functional homology between *Azospirillum* genomic DNA and specific plant interaction genes of other plant associative bacteria. Numerous genes involved in chemotaxis, motility, extracellular polysaccharide production, nitrogen fixation and IAA biosynthesis have been identified. To facilitate the molecular analysis of the plant association, a strategy to visualize the *Azospirillum*–plant interaction was designed by using *Azospirillum* strains constitutively expressing the *E. coli gusA* gene [44]. In addition, the expression of the *A. brasilense* structural nitrogenase *nifH* gene has been analyzed on the plant roots by means of a *nifH–gusA* fusion. Many factors appear to affect the association between *Azospirillum* and the plant. Monitoring the expression of putative *Azospirillum*–plant interaction genes during plant association by the use of reporter genes, and the application of strains carrying mutations in those genes, may lead to a better understanding of the bacterial determinants controlling this association, and may therefore constitute an important step towards engineering and exploitation of more efficient plant growth-promoting *Azospirillum* strains.

The genus *Azospirillum* comprises predominantly rhizosphere colonizing bacteria, with only a few strains that can enter the root system. However, the plant root interaction by other associative nitrogen-fixing bacteria, like *A. diazotrophicus*, *H. seropedicae* and *Azoarcus*, clearly displays an endophytic character [42]. The efficient plant root association and the invasive potential of a plant growth-promoting rhizobacterium might be of great importance for establishing a beneficial bacterium–plant interaction. The organism escapes competition with other rhizosphere bacteria and achieves close contact with the host, which could facilitate the mutual exchange of nutrients and provide a protective environment for instance against oxygen-mediated damage of the nitrogenase. Interestingly, the endophytic diazotrophs *Azoarcus* sp., *A. diazotrophicus* and *H. seropedicae*, show some particular features regarding regulation of nitrogen fixation. Nitrogen-fixing *Azoarcus* cells developed into a hyperinduced state under certain conditions in empirically optimized batch cultures, in response to a shift to extremely low oxygen concentrations [211]. This physiological state of high respiration and N_2 fixation rates is paralleled by the induction of complex intracytoplasmic membrane stacks, referred to as 'diazosomes' [212,213]. Immunohistochemical studies with Fe protein specific antibodies, which revealed that the Fe protein of the nitrogenase (NifH) is mainly associated with the newly formed membranes in those conditions, as opposed to a cytoplasmic localization in standard N_2-fixing conditions, and mutational analysis of nitrogenase structural genes confirmed their relationship with N_2 fixation [212]. In *A. diazotrophicus* and *H. seropedicae* nitrogenase is only partially inhibited by ammonium, permitting nitrogen fixation at low levels of NH_4^+ [183,214]. Moreover, *A. diazotrophicus* can fix nitrogen even at high levels of nitrate, because of the absence of assimilatory nitrate reductase activity

[51], which may allow the complementation of biological nitrogen fixation and N-fertilization. No evidence has been found for the presence of a switch-off process involving covalent modification of nitrogenase in these bacteria [183,214]. In addition, half of the fixed N_2 by *A. diazotrophicus* can be excreted into the medium [215]. These characteristics, together with the endophytic nature of the *A. diazotrophicus* and *H. seropedicae* plant interactions holds promise, as nitrogen fixed by these bacteria might be far more available to the plant than in the rhizosphere associations. Studies dealing with the regulation of nitrogen fixation in these bacteria and the way these endophytic diazotrophs internally colonize plant roots, can significantly contribute to the development of improved plant growth promoting rhizobacteria, including *Azospirillum*.

Bacterial phytohormone production and nitrogen fixation are recognized as processes potentially involved in plant growth promotion by *Azospirillum*. However, a number of obstacles remain for ascribing them a fundamental role in the plant response. Although *Azospirillum* is known to produce the auxin indole-3-acetic acid in free-living cultures, it is still not clear to what extent this occurs in the rhizosphere and how this process would be regulated. The main obstacle for a significant contribution of nitrogen fixation by *Azospirillum* is the lack of transfer of fixed nitrogen to the plant. With the present knowledge of the nitrogen fixation process in *Azospirillum* and the large number of isolated genes, it is now theoretically possible to construct improved N_2-fixing and NH_4^+-excreting strains. Interference with the regulatory mechanisms for NH_4^+ inhibition of nitrogenase synthesis and activity might lead to higher rates of N_2 fixation, even in the presence of ammonium. Enhanced NH_4^+ excretion could be achieved by preventing ammonium assimilation. Since glutamine synthetase appears to be a key enzyme in nitrogen fixation, the *glnA* gene provides a potential target for genetic engineering of this process. However, one cannot completely abolish GS activity, as the obtained mutants are affected in their growth capacity (glutamine auxotrophs). On the other hand, creating NH_4^+-excreting mutants might be accomplished by manipulating the ammonium transporter-encoding *amtB* gene [138].

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