REVIEW / SYNTHÈSE

Azospirillum-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003)

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Abstract: This review presents a critical and comprehensive documentation and analysis of the developments in agricultural, environmental, molecular, and physiological studies related to *Azospirillum* cells, and to *Azospirillum* interactions with plants, based solely on information published between 1997 and 2003. It was designed as an update of previous reviews (Bashan and Levanony 1990; Bashan and Holguin 1997*a*), with a similar scope of interest. Apart from an update and critical analysis of the current knowledge, this review focuses on the central issues of *Azospirillum* research today, such as, (*i*) physiological and molecular studies as a general model for rhizosphere bacteria; (*ii*) co-inoculation with other microorganisms; (*iii*) hormonal studies and re-consideration of the nitrogen contribution by the bacteria under specific environmental conditions; (*iv*) proposed *Azospirillum* as a non-specific plant-growth-promoting bacterium; (*v*) re-introduction of the "Additive Hypothesis," which suggests involvement of multiple mechanisms employed by the bacteria to affect plant growth; (*vi*) comment on the less researched areas, such as inoculant and pesticide research; and (*vii*) proposes possible avenues for the exploitation of this bacterium in environmental areas other than agriculture.

Key words: Azospirillum, plant–bacteria interaction, plant-growth-promoting bacteria, PGPB, PGPR, rhizosphere bacteria.

Résumé : Cette revue présente une analyse et une documentation critique et compréhensive des progrès récents dans les études agricoles, environnementales, moléculaires et physiologiques liées aux cellules de *Azospirillum* et aux interactions de *Azospirillum* avec les plantes, en s'appuyant seulement sur des informations publiées entre 1997 et 2003. Elle a été conçue comme une mise jour de revues antérieures (Bashan et Levanony 1990; Bashan et Holguin 1997*a*) couvrant les mêmes champs d'intérêt. Outre une mise à jour et une analyse critique des connaissances actuelles, cette revue souligne les questions d'actualité dans la recherche sur *Azospirillum*, tel que les études physiologiques et moléculaires en tant que modèle général pour les bactéries de la rhizosphère, la co-inoculation avec d'autres microorganismes, les études hormonales et la reconsidération de l'apport en azote par la bactérie dans des conditions environnementales spécifiques, la suggestion que *Azospirillum* soit une bactérie non-spécifique favorisant la croissance végétale, la réintroduction de « l'Hypothèse Additive », qui suggère que plusieurs mécanismes sont employés par la bactérie pour influencer la croissance de plante, des commentaires sur des domaines moins étudiés, comme la recherche sur les inoculants et les pesticides. La revue propose des pistes pour l'exploitation de cette bactérie dans des domaines environnementaux autres que l'agriculture.

Mots clés : Azospirillum, interactions plante–bactérie, bactérie favorisant la croissance des plantes, BFCP, RFCP, bactéries de la rhizosphère.

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Introduction

Azospirillum is a free-living, plant-growth-promoting bacterium (PGPB), capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance. The leading theory concerning its growth promotion capacity lies in its ability to produce various phytohormones that improve root growth, adsorption of wa-

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ter and minerals that eventually yield larger, and in many cases, more productive plants (Dobbelaere et al. 2001). Yet, its mode of action is still under discussion, as recent information regarding contributions of N_2-fixation and affects on membranes accumulate. Since its re-discovery in the mid-1970s, by the late J. Döbereiner, it has consistently proven to be a very promising plant-growth-promoting bacterium (PGPB). It is not surprising that, in several developing and developed countries, *Azospirillum* is used as the bacterial inoculant of choice, alone or together with other PGPB and vesicular arbuscular mycorrhizal (VAM) fungi, for many crops. It also serves as a potential agent to solve environmental problems. Substantial advances in exploring the genetic basis of the beneficial effects of *Azospirillum* and other PGPBs on plants have been made (Bloemberg and Lugtenberg 2001). From the extensive genetic, biochemical, and applied studies, *Azospirillum* is considered one of the best-studied PGPB (Vande Broek et al. 2000).

Our last 2 comprehensive reviews of the agricultural, environmental, and physiological aspects of *Azospirillum* interactions with plants were published in 1990 (Bashan and Levanony 1990) and 1997 (Bashan and Holguin 1997*a*), and will serve as general references for this update. Commercial field applications, critical analysis of particular sub-fields, and genetic aspects of *Azospirillum* with plants, were the subjects of a few recent reviews (Holguin et al. 1999; Dobbelaere et al. 2001, 2003; James 2000; Kennedy and Islam 2001), and therefore, these issues will be discussed in less detail. This review concentrates on reports published since 1997; earlier studies are cited only for the purpose of enhancing clarity of the current review, or providing a better perspective.

Two new species and unique strains

There are currently 7 species within the genus *Azospirillum*. *Azospirillum brasilense*, *A. lipoferum*, *A. amazonense*, *A. halopraeferens*, and *A. irakense* were described earlier (Bashan and Levanony 1990; Bashan and Holguin 1997*a*). *A. doebereinerae* was found in association with roots of the gramineaous plant *Miscanthus*. Strains of the new species are curved rods or S-shaped, 1.0–1.5 µm wide and 2.0–3.0 µm long, Gram-negative, and motile with a single polar flagellum. Nitrogen-fixation occurs under microaerobic, nitrogen-limited conditions. All these features are very similar to other *Azospirillum* spp. The trait that differentiates this species from others is its ability to use (or not) several sugars and some minute genetic details. Optimum growth occurs at 30 °C, and at pH values between 6.0 and 7.0, but not at 37 °C, as is the case for the other species (Eckert et al. 2001). *A. largomobile* was technically transferred, on the basis of some phylogenetic relationships, from the genus *Conglomeromonas* to *Azospirillum* (Dekhil et al. 1997).

Azospirillum strains are routinely isolated from agricultural lands and crop plants, including traditional isolations from grasses and cereals (Nath et al. 1997; Weber et al. 1999). In recent years, using a combination of traditional isolation and in situ molecular detection, exemplified by phylogenetic oligonucleotide probes and PCR-fingerprinting techniques, strains of *Azospirillum* or sequences identical to sequences of *Azospirillum* have been found in [illegible] previously unstudied, locations. Endophytic *A. lipoferum* was isolated from *Pennisetum purpureum*, *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and *Spartina pectinata*, which are used for energy (Kirchhof et al. 1997). PCR-amplified *nifH* sequences from sediment in oligotrophic sea grass beds in the Bahamas, revealed several *nifH* sequences nearly identical to *A. brasilense* (Bagwell et al. 2002). Using analyses of PCR amplicons containing the ribosomal intergenic spacer and partial 16S rRNA gene, thermo-tolerant *Azospirillum* strains were isolated from an aerated lagoon, where pulp and paper mill effluent are treated (Y. and Mohn 2001). A high cell density strain of *Azospirillum* was found on the roots of black mangrove *Avicennia marina*. This saline tolerant bacterial strain has potential to improve beans and rice in coastal agricultural fields (Ravikumar et al. 2002). Nitrogen-fixing microbial populations from leaf litter in an Oregon Douglas fir forest, assessed by restriction fragment length polymorphism (RFLP) of PCR products, showed that all the *nifH* sequences obtained from the forest litter were characterized by the *nifH* sequences of members of *Rhizobium*, *Sinorhizobium*, and *Azospirillum* genera (Widmer et al. 1999).

Isolation and detection methods

Isolation

Apart from direct isolation of potential new strains of *Azospirillum* on various N-free, semi-solid media (for review, see Bashan et al. 1993), a new, simple method for isolating *Azospirillum* strains from the roots and the rhizosphere of rice, based on the capacity of *Azospirillum* to grow on soil extract medium, was described. Soil extract medium repressed the most abundant bacterial populations and facilitated isolation of azospirilla from a population representing <0.001% of the total microflora (Van et al. 1997).

An immuno-magnetic isolation technique was developed to isolate *Azospirillum* from soil. The procedure uses antibody recognition, and physical separation of bacteria from soil samples using magnets. For detecting and counting azospirilla in natural soils, this procedure was claimed to be more efficient than common culturing in N-free, semi-solid medium (Han and New 1998*a*, *b*).

Detection

Most detection methods described recently are either: immunological, molecular, or combinations thereof. To detect known inoculated strains in roots, the most common approach is to tag it with a gene that produces a visible feature or produces a specific antibody against a unique antigen of the bacterial cell.

The *gfp* gene, encoding the green fluorescent protein, was incorporated into the chromosome of several *Azospirillum* spp., allowing easy detection of the inoculated bacteria on maize roots (Liu et al. 2003). The *gusA* gene, encoding for the β-glucuronidase enzyme, was used in *A. brasilense* Sp7 to evaluate the influence of O_2 on gene expression (Sun et al. 2001), and to study the colonization of rice and wheat roots by *A. lipoferum* (Chebotar et al. 1999; Steenhoudt et al. 2001*a*). Double-tagging of the *gfp* gene, combined with

the *gusA* gene in the mini-*Tn5* transposon, was inserted into several PGPBs, including *A. brasilense*. Double-tagging constitutes a potentially powerful tool for studying gene expression of *Azospirillum* in its environment and during colonization of roots (Ramos et al. 2002; Xi et al. 1999). A stable chromosome marker encoding the β-galactozidase enzyme, *Tn5-lacZ*, was inserted into *A. brasilense* strains capable of growing at low temperatures. Mutants screened for plant growth promotion and root colonization were found to be isogenic with their respective wild *Azospirillum* strains (Kaushik et al. 2000). Similarly, the *lacZ-nifA* marker was inserted into *A. brasilense* Cd to study wheat root colonization under salt stress (Fischer et al. 2000) and endophytic colonization of 2,4-D-treated wheat seedlings (para-nodule colonization) (Kennedy et al. 1998).

Some approaches to identifying bacteria are based on restriction fragment length polymorphism (RFLP) and PCR-fingerprinting techniques (Kirchhof et al. 1997). These involve probes obtained from random amplification of DNA fragments (RAPD), allowing specific detection of *A. lipoferum* strain ATCC 29731 (Fancelli et al. 1998), *A. lipoferum* CRT1 (Jacoud et al. 1998), or indigenous populations of *A. brasilense* under highly fertilized corn cultivation (Ceccherini et al. 2001). Similarly, the AZO 23S-rRNA probe, specific for *Azospirillum*, can identify members of this genus in soil samples (Jofré et al. 2000). Some probes were specifically designed for the *Azospirillum–Skermanella–Rhodocista* genus cluster, Azospirilli subcluster I (*A. lipoferum*, *A. doebereinerae*, *A. largimobile*, *A. brasilense*, *A. halopraeferens*), Azospirilli subcluster II (*A. amazonense*, *A. irakense*), and the genus *Skermanella*. When these probes were fluorescently labeled, in situ identification of isolates and localization of *A. brasilense* on maize roots by confocal laser scanning microscopy was possible (Stoffels et al. 2001).

Antibodies, sometimes coupled with species- and genus-specific oligonucleotide probes or fluorescence-labeled antibodies, were used to study taxonomic identity and ecology of diazotrophic bacteria (including *Azospirillum* spp.) associated with roots of non-legume plants (Kirchhof et al. 1997), and to monitor root colonization (Pinheiro et al. 2002). Rat monoclonal antibodies directed against lipopolysaccharides of *A. brasilense* Wa5 have detection sensitivity of about 100 bacteria/mL. These monoclonal antibodies were suitable for in situ immunofluorescence detection and a sensitive, direct quantification of the strains in rhizosphere extracts (Schloter et al. 1997; Schloter and Hartmann 1998). Strain-specific monoclonal antibodies against *A. brasilense* strains Sp7, and Sp245 were used to study the colonization of 2 wheat cultivars under axenic conditions and in soil microcosms (Schloter and Hartmann 1998). Fluorescence-labeled monoclonal antibodies and rRNA-targeted, fluorescence-labeled oligonucleotides were used simultaneously for in situ identification of specific strains in mixed cultures of *A. brasilense*, as well as for detection of *Azospirillum* spp. in the rhizosphere of inoculated plants. High-resolution images from scanning confocal laser microscopy or epifluorescence microscopy were used for detection of individual cells in root samples of inoculated wheat plantlets. The bacterial populations were quantified by chemi-luminescence ELISA. This relatively complex strategy gave new insight into the relative abundance of an inoculated strain within the background of the species and the total rhizosphere microflora. It also enabled precise localization of single cells of a specific strain on roots, and enabled localization and quantification of inoculated bacteria in different parts of the rhizosphere (Assmus et al. 1997; Kirchhof et al. 1997).

In summary, these methods and those described earlier (Bashan and Holguin 1997*a*) provide a variety of possible tools for precise detection at the strain level and location of small populations of many useful *Azospirillum* strains. With no shortage of detection methods, the method of choice depends primarily on the setup and infrastructure of the laboratory.

The *Azospirillum* genome

As perhaps the most studied PGPB, *Azospirillum* serves as a model for genetic and molecular studies of rhizosphere associated bacteria. Since its genetics and molecular biology, up to the last decade, have been reviewed (Holguin et al. 1999; Steenhoudt and Vanderleyden 2000), we review only recent findings, and cite some of the older studies to facilitate comprehension.

The genome of the genus *Azospirillum* varies in overall size, from 4.8 Mbp in *A. irakense* to 9.7 Mbp in *A. lipoferum* strain Sp59b (Martin-Didonet et al. 2000). The genomes of 5 *Azospirillum* species (*A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. irakense*, and *A. halopraeferens*) analyzed by pulsed-field gel electrophoresis, possessed several mega-replicons, with molecular sizes ranging from 0.2 to 2.7 Mbp, and hybridized with a 16S rDNA probe from *A. brasilense* (Martin-Didonet et al. 2000). Horizontal Eckhardt-type gel electrophoresis showed that rhizosphere and endophytic *A. brasilense* isolates, recovered from sugarcane plants and the reference strains *A. brasilense* Sp7 and Cd, harbored 5 to 8 replicons, ranging from 0.65 to over 1.8 Mbp (Caballero-Mellado et al. 1999). The 1.7 Mbp mega-plasmid replicon of the *A. brasilense* isolates and some replicons around 0.6 Mbp, strongly hybridized to 16S rDNA genes. Replicons of *Azospirillum*, around 0.6 Mbp with chromosomal characteristics, are the smallest bacterial chromosomes described up to now (Caballero-Mellado et al. 1999). These results suggest that the genome of *A. brasilense* consists of multiple and mini-chromosomes instead of a single circular chromosome. Multiple chromosomes have also been described for *Rhodobacter sphaeroides*, *Agrobacterium tumefaciens*, *Burkholderia cepacia*, *Brucella* spp., *Vibrio parahaemolyticus*, and *Phyllobacterium* (Jumas-Bilak et al. 1998). The functions of these replicons are unknown, but might support the exceptional ecological distribution and metabolic flexibility of this genus (Caballero-Mellado et al. 1999; Martin-Didonet et al. 2000). Contrary to previous work suggesting that *nif* genes are located only in the chromosome, DNA hybridization studies with an *A. brasilense nifHDK* probe showed the *nifHDK* operon to be present in a 2.5 Mbp mega-plasmid of *A. brasilense* FP2 and *A. brasilense* Sp7 (Martin-Didonet et al. 2000).

The interior of the sugarcane root constitutes a stable environment for *A. brasilense*. After passing 4 times through the root interior, no changes in the genome of the bacterium were observed (Pedraza and Ricci 2003).

Attachment to roots

Physiological mechanisms

Attachment of *Azospirillum* to roots was determined to be a two-step process comprised of adsorption and anchoring. The primary adsorption phase is rapid (reaching a maximum within 2 h of exposure of the bacteria to roots), weak, reversible, and probably governed by bacterial proteinaceous compounds. The anchoring phase is stronger, takes several hours to form, is irreversible, and is based on bacterial extracellular surface polysaccharides involving a network of fibrillar material that permanently connects the bacteria to the surface and prevents removal from the site (for previous articles: Bashan and Holguin 1997). This attachment sequence in wheat was recently reconfirmed (Egorenkova et al. 2000).

Adsorption was evaluated by measuring the cell-surface charge and cell-surface hydrophobicity of 10 strains of *Azospirillum* spp. *Azospirillum* spp. had moderate cell-surface hydrophobicity and charge, lower than known values for human pathogens. Cell-surface hydrophobicity and charge of *Azospirillum* can be affected by external treatments of the bacterium cell, but a general pattern of modifying hydrophobicity and charge by external treatments of the cells of all strains was not found. Various chemicals, temperatures, and enzymatic treatments changed the cell-surface hydrophobicity and charge (Castellanos et al. 1997). *A. brasilense* Sp245 and its lipopolysaccharide-defective mutants were compared. Despite the similarity of adsorption dynamics of the parent strain and this mutant, attachment capacity of the mutant was lower than that of the parental strain. The mutant strain had a considerable decrease in hydrophobicity of the *Azospirillum* cell surface (Fedonenko et al. 2001). This shows that hydrophobicity and charge may play a role, perhaps small, in the primary adsorption of *Azospirillum* spp. to root surfaces. When bacterial cells were subjected to starvation periods, many of the cell-wall characteristics changed. Nevertheless, no change was considered major, and apparently starvation is not a major limiting factor in the attachment of *Azospirillum* to surfaces (Castellanos et al. 2000). The effect of pH and cations was also studied. The presence of significant concentrations of Ca^{+2} or $(PO_4)^{-3}$ in the incubation medium reduced bacterial adsorption. The effect of the incubation medium's pH on adsorption of *Azospirillum* showed that strains from roots or rhizosphere of wheat had optimal adsorption at pH 6.0, while all other strains preferred pH 7.0 (Pinheiro et al. 2002). Additionally, lipopolysaccharides extracted from the outer membrane of *A. brasilense* Sp245 and its mutant cells induced deformation of the wheat seedling root hairs after exposure (Egorenkova et al. 2000; Fedonenko et al. 2001). As some lectins were identified in the cell wall of *A. brasilense* and *A. lipoferum*, perhaps these compounds play a role in the adsorption phase (Castellanos et al. 1998). Adsorption of *A. brasilense* to roots of spring wheat was affected by the bacterial concentration and possible involvement of lectins. The number of attached cells increased with increasing inoculum size and time of contact. Saturation of root surface adsorption was observed after 3–24 h of incubation, depending on the strain. The longer the incubation period, the firmer was the attachment. Adsorption to roots was partially inhibited when the roots were treated earlier with *N*-acetyl-D-glucosamine, indicating the possible involvement of lectins. Root hair deformation induced by polysaccharides from *Azospirillum* capsular material was inhibited by *N*-acetyl-D-glucosamine and chitotriose, specific haptens of the wheat germ lectin agglutinin (Yegorenkova et al. 2001).

A major outer membrane protein, acting as an adhesin and possibly involved in root adsorption and cell bacterial aggregation, was purified from *A. brasilense* Cd. This protein binds to root extracts of various plant species, but with stronger adhesion to extracts of cereals, in comparison with legumes and tomatoes. It also binds to roots of different cereal seedlings in an in vitro adhesion assay (Burdman et al. 2001). These studies indicate the participation of lectin molecules in the attachment process.

Genes involved in chemotaxis and plant root attachment

Azospirillum spp. attach to and colonize plant root surfaces, and this process depends on active motility and chemotaxis toward root exudates. The β-glucuronidase (GUS) reporter system in nonflagellated mutants and a Tn*5* mutant with low chemotactic motility towards various amino acids, sugars, and organic acids, revealed that the mutants colonized wheat roots much less than the wild-type (Vande Broek et al. 1998). Genes that encode the central signal transduction pathway for chemotaxis in *A. brasilense* were identified by phenotypic complementation of chemotactic mutants (Hauwaerts et al. 2002). Sequencing of a DNA fragment, which complemented 2 of the chemotactic mutants, revealed a region of 5 open reading frames encoding homologs of known genes (*cheA*, *cheW*, *cheY*, *cheB*, and a partial *cheR* gene) comprising excitation and adaptation pathways for chemotaxis in other bacterial species (Hauwerts et al. 2002). The gene encoding a protein similar to ChvE in *Agrobacterium tumefaciens* is necessary for *vir* gene induction when responding to sugars. It was cloned from *A. brasilense* Sp245. Insertional mutagenesis showed this *A. brasilense* sugar-binding protein A (SbpA) is involved in chemotaxis towards D-galactose, L-arabinose, and D-fucose, and is part of a high-affinity uptake system for D-galactose (Van Dommelen et al. 1997).

Genetic complementation of an *A. brasilense* Sp7 spontaneous mutant, Sp7S (Katupitiya et al. 1995), that did not flocculate, swarm, or form C cells, lacked capsular polysaccharides, reduced acetylene, and colonized the root surface to a lesser extent than the wild-type. This led to the identification of a new regulatory gene in *A. brasilense*, *flcA*, that restored these phenotypes (Pereg-Gerk et al. 1998). Characterization of *A. brasilense flcA* mutants showed the key role of *flcA* in cellular processes related to adhesion: production of capsular polysaccharides, flocculation in culture, and wheat root colonization (Pereg-Gerk et al. 1998). Restoration of acetylene reduction in the Sp7S mutant by complementation with *flcA* to wild-type levels suggested that

flcA influences the rate of nitrogenase activity directly (Pereg-Gerk et al. 2000). Genetic complementation of *A. brasilense* Sp7S with defective swarming motility, with a gene bank of strain Sp7, led to the identification of 2 loci that control swarming motility in *A. brasilense*. The deduced translation product of the first locus was highly similar to a phosphoribosyl-aminoimidazol carboxylase, encoded by *purK*, and involved in purine biosynthesis (Carreño-Lopez et al. 2002).

The wealth of previous chemotactic studies, together with the few done recently, and the recent molecular data, show that chemotaxis is a major force leading to colonization of roots. This is practical information for selecting strains as inoculants; in so far as the strains should be motile and this feature is easy to detect and allows for fast screening of a large collection of strains.

Involvement of polysaccharides and lectins in attachment

The role of cell-wall components, including lectin recognition between the plant and the bacterium, has been studied for decades without definite conclusions, contrary to the situation in rhizobia-legume symbiosis. Some advances in that topic have been made in Russia. *Azospirillum* is capable of secreting capsular polysaccharides, which is possibly involved in the adhesion of bacteria to surfaces and several other exopolysaccharides (EPS). Involvement of EPS in the aggregation phenomenon has been recently documented (see related section in this review). Capsular polysaccharides, exopolysaccharides, and *O*-specific polysaccharides in *A. brasilense* strains Sp7 and Sp245, showed close antigenic identity. These extracellular polysaccharides (EPS) are likely to represent O-antigenic lipopolysaccharide fragments excreted by the bacteria into the culture medium. Identification as a capsule or EPS depends on the strength of the attachment of these polysaccharides to the cell surface. This could explain the absence of a specific capsular antigen in *A. brasilense* (Matora and Shchegolev 2002). Additionally, a new *O*-specific polysaccharide was isolated and characterized from the lipopolysaccharide of *A. brasilense* Sp245 (Fedonenko et al. 2002). High-molecular-weight carbohydrate-containing complexes (lipopolysaccharide–protein [LP] complex and polysaccharide–lipid [PL] complex) that were isolated from the capsular material of *A. brasilense* Sp245, are possibly responsible for protecting them from extreme conditions, such as high (46–48 °C) and low (–20 °C and –70 °C) temperatures, extreme pH values (2 and 10), and desiccation, which are otherwise detrimental. Before exposure to extreme conditions, addition of these complexes to a suspension of decapsulated cells significantly increased survival rates (Konnova et al. 2001). Maintaining the pH at 7.0 during incubation favors accumulation of LP and PL complexes in the capsule of *A. brasilense* (Kannova et al. 2003). In vitro, some *Azospirillum* produced significant quantities of exocellular and capsular polysaccharide. After depletion of the carbon source from the culture medium, polysaccharides were consumed, especially by *A. lipoferum* strains, and this significantly increased nitrogenase activity, although oxygen uptake was low during this period. This allowed a mathematical model predicting biochemical activities of *Azospirillum* to be developed (Kefalogianni and Aggelis 2002).

Interaction of EPS, LP complexes, PL complexes, lipopolysaccharides, and *O*-specific polysaccharides from *Azospirillum* with plant lectins is a promising avenue to explore (Skvortsov and Ignatov 1998). Also to be considered are bacterial lectins interacting with polysaccharides of plants. Cell-surface lectins (agglutination with fucose and glucose, and to a lesser extent, with mannose) were detected in 7 strains of *A. brasilense* and *A. lipoferum*. Cell-wall proteins extracted from 2 *Azospirillum* strains exhibited lectin-like activities (Castellanos et al. 1998). A strong interaction between the *A. lipoferum* 59b lectin and 3 glucosidases was detected, but their exact role was not determined (Alenkina et al. 2001). Under saline conditions, the normal composition of the EPS of *A. brasilense* Cd dramatically changed from 7 monosaccharides to mainly galactose. Root exudates induced changes in the EPS only under regular growth conditions, and led to higher amounts of arabinose and xylose (Fischer et al. 2003).

The hypothesis that the lectin, wheat germ agglutinin (WGA), also found in wheat roots, is a signal molecule in the *Azospirillum*-plant interaction that diverts bacterial metabolism in the direction favorable for growth and development of the host plant and is essential for effective associative arrangement has been suggested. WGA induced modifications of the phospholipid fraction of *A. brasilense* membranes. Exposure to WGA decreased the total content of phosphatidylglycerol and phosphatidylinositol, increased phosphatidylethanolamine and cardiolipin levels, but did not induce a change in phosphatidic acid content. These specific changes in the phospholipids are thought to mediate transmembrane signaling and cell response to WGA (Antonyuk et al. 1999). WGA, when added to *A. brasilense* Sp245 culture (final concentration 10^{-8} to 10^{-9} mol/L), enhanced IAA production, N_2-fixation, and ammonium excretion by the cells. Additionally, WGA promoted synthesis of proteins, both new and those already present in bacterial cells (Antonyuk and Ignatov 2001). Changing the medium's C-source from malate to gluconate increased the content of glucosamine, which increased the affinity of polysaccharides for WGA (Konnova et al. 2003). Under in vitro conditions, N_2-fixation capability of *A. lipoferum* was efficiently increased in the presence of WGA, and a putative WGA-binding receptor was detected in the cell capsule. The stimulatory effect required the molecule *N*-acetyl-D-glucosamine dimer (GlcNAcdi), and was dependent on the number of GlcNAcdi links involved in the receptor-WGA interface. The effect on N_2-fixation was attributed to the generated WGA-receptor complex stimulus leading to elevated transcription of the *nifH* and *nifA* genes and *glnBA* gene cluster (Karpati et al. 1999). This indirect evidence is not, at the moment, strong enough to demonstrate significant involvement in primary attachment, and influence of this attachment on plant–bacteria interaction. However, these results provide a direction for future research.

Root colonization

If a positive effect of inoculation with *Azospirillum* sp. is

expected, significant root colonization should happen first. Inappropriate root colonization usually resulted in marginal or no effects on plant growth (Hecht-Buchholz 1998; Benizri et al. 2001). Early studies with *Azospirillum* revealed a small cluster type of colonization when the bacteria were located on small roots, and in particular, in the root hair zone, and bacteria were anchored to the root surface with numerous fibrilar materials and the partially digested mucigel layer (for reviews: Bashan and Levanony 1990; Bashan and Holguin 1997*a*).

Some strains of *A. lipoferum* and *A. brasilense*, but not others, are capable of colonizing the interior of wheat roots. This strain affinity is not present during the initial stages of root colonization (anchoring stage), since isolates from the root interior or an isolate with a proven ability to colonize the interior of wheat roots showed no greater ability to anchor to the roots than other *Azospirillum* strains isolated from the wheat rhizosphere or from the rhizosphere of other grasses. Thus, previous studies of this type of colonization were confirmed. Cell aggregates and single bacteria were visible on the surface of young roots (root hairs and lateral roots). Large cellular clumps were observed at the emergence points of lateral roots or at intercellular spaces of root epidermal cells (Ramos et al. 2002). Sometimes, the majority of the colonizing bacteria resided on the root surface and only a minority appeared in the root interior (Liu et al. 2003). In the root hair zone, bacteria were more numerous, but principally located in depressions between epidermal cells. In all root zones, mucilage was present, and near the tip it appeared to have been partially digested, forming "halos" around the bacteria and revealing fibril-like strands attached to the bacteria (Pinheiro et al. 2002). *A. brasilense* Cd grown under standard conditions was distributed over the entire wheat root system, except the elongation zone, as is well-known. However, bacteria under saline stress were mainly localized at the root tips and lateral roots (Fischer et al. 2000). Similarly, inoculation of black mangrove seedlings in seawater with either *A. halopraeferens* or *A. brasilense* produced high density colonization of the root surface. The colonization pattern was different for the 2 species. *A. halopraeferens* yielded mainly single cells embedded in a thick sheath, whereas *A. brasilense* produced primarily micro-aggregates. *A. brasilense* cells were anchored to the root surfaces and to themselves by a network of fibrillar material. *A. halopraeferens* was a better root surface colonizer, whereas the *A. brasilense* population was greater in the entire root (Puente et al. 1999). Differential wheat root colonization among different strains of *A. brasilense* (Sp7, Sp245, and Wa5) was evaluated. All strains colonized the root tip in large numbers. *A. brasilense* Sp245 showed the highest colonizing potential, and could be found in other parts of the root, including the inner root tissue, forming micro-colonies in intercellular spaces. The other 2 strains formed micro-colonies exclusively on the root surface. Following colonization, from cotyledon to flowering stage, Schloter and Hartmann (1998) demonstrated that, while colonization of the total root by *A. brasilense* Sp7 and Wa5 dropped continuously, inside the roots, Sp245 remained constant during these growth stages. Competition among *Azospirillum* strains for root colonization was demonstrated. Co-inoculation of all 3 *A. brasilense* strains resulted in similar colonization patterns, but the population of strain Wa5 decreased on the roots. Assmus et al. (1997) found that following co-inoculation of strains Sp7 and Wa3, Sp7 performed better than Wa3 for colonization niches. Competition with naturally occurring endophytic PGPB (*Corynebacterium flavescens* and *Bacillus pumillus*) on rice excluded root colonization with inoculated *A. brasilense* (Bacilio-Jiménez et al. 2001).

As reported earlier, wheat, maize, and rice can develop tumors (para-nodules) along primary and secondary roots when treated with low concentrations of various auxins, the most well-known is the herbicide 2,4-dichlor-phenoxy-acetic-acid (2,4-D). As a consequence, they can develop an endophytic diazotrophic "symbiosis". Histologically, auxin-induced tumors appear as cancerous root meristems. Auxin-affected root meristems do not recover and further develop into large nodule-like structures. Introduced diazotrophs (*Azospirillum* spp., *Azorhizobium caulinodans*, *Rhizobium* spp.) potentially inhabit para-nodules as a major colonization niche. Colonizing bacteria follow a "crack entry" at sites where developing tumors have emerged through the root cortex and epidermis, and therein establish high cell density inside intercellular spaces of cortical and meristematic tissues. Infection of tumor cells occurs with bacteria found inside cell-cytoplasm surrounded by membrane-like structures. Once they inhabit para-nodules, inoculated diazotrophs colonize endophytically with high cell numbers (Christiansen-Weniger 1997, 1998). Not all diazotrophs are capable of effectively colonizing para-nodules. In wheat, only *Herbaspirillum seropedicae*, *Azorhizobium caulinodans*, and the mutant *A. brasilense* Sp7S strain displayed significant endophytic colonization of para-nodules, while *Acetobacter diazotrophicus*, *Azotobacter vinelandii*, *Derxia gummosa*, and other *Azospirillum* strains colonized the rhizoplane and not the interior of the para-nodules (Kennedy et al. 1997, 1998). The effect of saline stress on the colonization of wheat para-nodules by *A. brasilense* Cd established that para-nodules acted as bacterium-protected niches supporting higher bacterial populations than in plants without para-nodules. In para-nodules, most of the bacteria were present around the basal surface of the modified lateral root structures (Fischer et al. 2000).

No major breakthrough has occurred in the study of the attachment process and subsequent root colonization. Only refinements of previous studies have been reported. Perhaps a better understanding of the plant genes involved in the interaction will shed additional light on these crucial steps.

Cell aggregation as a factor of root colonization

Aggregation is one of the most basic phenomena of *Azospirillum* colonization of roots. In vitro aggregation/flocculation phenomena have been extensively studied (for reviews: Bashan and Levanony 1990; Bashan and Holguin 1997), and some factors were recently summarized (Burdman et al. 2000*b*). The objective was to produce a superior inoculant for plants, based on this typical characteristic of *Azospirillum*. Growth of *A. brasilense* Cd in a high C:N medium, with fructose and ammonium chloride as C and N sources, induced visible flocculation after 24 h. Suspending aggregates in urea disrupted the aggregates. No cell aggregates were formed after 72 h when grown in low C:N

medium. Aggregating cells, but not cells grown in a low C:N medium, accumulated large amounts of poly-β-hydroxybutyrate, and the cell envelope contained a well-defined electron-dense layer outside the outer membrane. The concentration of EPS produced by 4 different strains of *A. brasilense*, differing in their capacity to aggregate, was strongly correlated with the extent of aggregation (Burdman et al. 1998). For cells grown in aggregate-induced medium, outer-membrane protein fractions exhibited high aggregation-specific activities that were strongly correlated with protein content. This suggests that this protein is also involved in the aggregation process of *A. brasilense* cells (Burdman et al. 1999). The EPS and capsular polysaccharide of *A. brasilense* showed a positive correlation between aggregation and the relative quantity of arabinose. Arabinose was not detected in polysaccharides from mutant strains that were aggregation-impaired or in bacteria grown in non-aggregation-inducing medium. The only monosaccharides able to significantly inhibit aggregation at low sugar concentrations were arabinose and, to a lesser extent, galactose. It is possible that residues of arabinose present in the EPS are involved in aggregation of *A. brasilense* (Burdman et al. 2000*a*). Mutants that lack part of the surface polysaccharide, characteristic of wild-type cells, are impaired in flocculating activity (Blaha and Schrank 2003). Some evidence that lectins are involved in cell aggregation was presented. A mutant strain of *A. brasilense* Sp7 with impaired lectin activity exhibited poorer cell aggregation than its parent strain. Pretreatment of bacterial cells with haptens (L-fucose and D-galactose) of a lectin located at the cell surface of the mutant inhibited aggregation of azospirilla. Interaction of lectins of several *Azospirillum* strains (*A. brasilense* 75, *A. brasilense* Sp7, and *A. lipoferum* 59b) with polysaccharide-containing complexes isolated from these strains was not specific, and no interstrain cross-interaction between the EPS and lectins of azospirilla was found (Nikitina et al. 2001).

These interactions show that the aggregation phenomenon is mediated by proteins and polysaccharides, but how this works is not clear. As aggregation can be manipulated easily, this may provide an opportunity to construct better *Azospirillum* inoculants.

Physiology of the bacterium and possible mechanisms by which *Azospirillum* affects plant growth

Despite intensive studies on physiology and molecular biology of these bacteria, the exact mode of action of the bacteria on plants is not much clearer than it was a decade ago. There are several facts that are beyond dispute; the bacteria fix nitrogen and produce several phytohormones in culture and in association with the plant, but the transfer of these products is limited and not always detected. Yet, the growth response is evident. The bacteria affect several plant metabolic pathways, including cell membrane activity. The most apparent outcome of inoculation are changes in the morphology of the root system (both positive and negative). Inoculated plants absorbed minerals and water better.

Several possible mechanisms were suggested to explain these phenomena, some with more experimental data than others. Yet, there is no definite agreement on exactly how the bacteria affect plant growth, what the major mechanisms are, or if there is one major mechanism responsible for the observed effects on plant growth, and in particular, on plant yield. These questions are the driving force in the *Azospirillum* research field. The Additive Hypothesis suggested 13 y ago is apparently still valid. The hypothesis considers multiple mechanisms rather than 1 mechanism participating in the association. These mechanisms operate simultaneously or in succession, the contribution of an individual mechanism being less significant when evaluated separately. The sum of their activities, when induced under appropriate environmental conditions, results in the observed changes in plant growth (Bashan and Levanony 1990). Today, the most common explanation for the effect of the bacteria on plants is the production of phytohormones that alter the metabolism and morphology of the plant, yielding better mineral and water absorption. The contribution of N_2-fixation is more controversial (see below). Finally, direct effects of the bacteria on plant cell membranes also have been proposed.

Production of phytohormones

Azospirillum spp. are known mainly for their ability to produce plant hormones as well as polyamines and amino acids in culture (Thuler et al. 2003). Among these hormones, indoles, mainly indole acetic acid (IAA), and gibberellins may play a major role.

IAA

Confirmatory studies on IAA production by several strains of *Azospirillum* showed that production depended on the type of culture media and availability of tryptophan as a precursor. *A. brasilense* Cd produced the highest level of IAA among the strains tested (approx. 380 μmol/L) (El-Khawas and Adachi 1999; Radwan et al. 2002). The pH has a significant effect on the amount of IAA produced (Ona et al. 2003). Assessment by chemical methods and with HPLC of possible precursors (indole, anthranilic acid, and tryptophan) for IAA formation in *A. brasilense* Sp245 revealed a high motive force for tryptophan synthesis from chorismic acid and for IAA synthesis from tryptophan, and this makes it unlikely that anthranilic acid and indole act as the precursors to IAA in a tryptophan-independent pathway (Zakharova et al. 1999). Vitamins may also play a role in the regulation of IAA synthesis in *A. brasilense*. Very low levels of the B vitamins, especially pyridoxine and nicotinic acid, increased production of IAA in *A. brasilense* (Zakharova et al. 2000).

Auxin production by *Azospirillum* sp. is believed to play a major role in plant growth promotion, although little new evidence with plants has been published in recent years. *A. brasilense* produced high quantities of extracellular IAA and tryptophol in culture medium supplemented with tryptophan, a precursor of IAA. Addition of filter-sterilized culture supernatants to rice roots grown in hydroponic tanks increased root elongation, root surface area, root dry matter, and development of lateral roots and root hairs, compared with untreated roots. Higher concentrations of the supernatant strongly inhibited root elongation, lateral root development, and caused nodule-like tumors on the roots (El-Khawas and Adachi 1999). Similarly, a cell-free

supernatant of *A. brasilense* Cd applied to soybean plants induced the highest number of roots and increased root length (Molla et al. 2001*b*). Inoculation of wheat with *A. brasilense* Sp245 and Sp7 wild strains led to a strong decrease in root length and increase in root hair formation, as is common for such inoculations. The effect on root morphology was further enhanced by adding tryptophan, and this could be mimicked by replacing *Azospirillum* cells with IAA (Dobbelaere et al. 1999). A mutant of *A. brasilense* with low production of phytohormones, but with high nitrogenase activity did not enhance root growth over uninoculated controls. In contrast, a mutant with increased phytohormone production significantly affected root morphology. In general, increased plant biomass and N_2-fixation were recorded in strains having increased production of indole compounds (Kundu et al. 1997).

Induction of para-nodules in rice roots by the auxins 2,4-D, naphthalene acetic acid (NAA), and IAA enhanced polygalacturonase activity in rice roots during formation of the para-nodules and endophytic colonization by *Azospirillum*. While inoculation with *Azospirillum* could augment polygalacturonase activity of rice roots to a small extent without any visible effect on root morphogenesis; auxin application, together with *Azospirillum* inoculation, enhanced polygalacturonase activity of rice roots to a high level, thus, yielding root change into para-nodules that later were colonized by *A. brasilense* (Sekar et al. 1999).

One effect of *Azospirillum* inoculation on the metabolism of maize and common bean seedlings was increased respiration rates. In vitro inoculation of detached root segments reduced kinetic values (*K*m and *V*max) of β-glucosidase, an enzyme that may be involved in phytohormone release from conjugates, and yielded lower total activity of β-glucosidase, but higher affinity to the substrate of specific β-glucosidases (Vedder-Weiss et al. 1999).

Genes involved in IAA synthesis

Costacurta et al. (1994) isolated the *ipdC* gene in *A. brasilense* Sp245, encoding indole pyruvate decarboxylase, a key enzyme in the pathway of IAA synthesis by *A. brasilense* that mediates conversion of indole-3-pyruvic acid into indole-3-acetaldehyde and presented conclusive evidence for the indole-3-pyruvic acid pathway in *A. brasilense*. Zimmer et al. (1998) isolated the *ipdC* gene from *A. brasilense* Sp7. A *lacZ-K*m cartridge introduced into the *ipdC* gene showed tryptophan-dependent stimulation of gene expression in this bacterium.

Expression of the *A. brasilense ipdC* gene with the translational fusion *ipdC-gusA* gene in an *A. brasilense* Sp245 *ipdC* mutant and in the wild-type (Vande Broek et al. 1999) showed that mutant *gus* expression was approximately 60% less than that of the wild-type. To test if an extracellular product (an auto-inducer, such as an *N*-acyl homoserine lactone) induced *ipdC*, the authors added supernatant from stationary grown cells of wild-type Sp245 to exponentially grown cells and observed induction in *ipdC* expression. When they repeated the experiment with the supernatant from the IAA mutant, no induction in *ipdC-gusA* expression was observed. These results suggested that auto-inducers in the supernatant did not activate the *ipdC* promoter, but that IAA might. Analyses of *ipdC-gusA* expression demonstrated that IAA and other synthetic auxins induce transcription of the *A. brasilense ipdC* gene (Vande Broek et al. 1999). Contrary to these results, in *A. brasilense* Sp7 having a chromosomal *ipdC-lacZ* fusion, addition of IAA did not induce expression of *lacZ*, and a *ipdC-lacZ* fusion was fully expressed during the stationary phase, with no IAA present (Carreño-Lopez et al. 2000).

Comparison of the *ipdC* structural genes isolated from *A. brasilense* Sp7 and Sp245 revealed that they were highly homologous. However, their upstream flanking regions were different (Costacurta et al. 1994; Zimmer et al. 1998). *A. brasilense* Sp245 is able to colonize superficial layers of the root cortex, while strain Sp7 is essentially a root surface colonizer. This preference for distinct ecological niches might have led to differences in the regulation of the indole-3-pyruvic acid pathway and would explain the differences in their *ipdC*-regulatory regions (Zimmer et al. 1998).

A consensus sequence for a σ^{54}-dependent promoter, which partly overlapped at the 3′ end with a TGTCCC element reminiscent of auxin-responsive elements (AuxRE) in plants, was found in *A. brasilense* Sp245 upstream of the *ipdC* gene. AuxREs in plants have been identified in the promoter region of some auxin-regulated genes and are involved in their regulation. It was proposed that a combination of the σ^{54} consensus sequence and the TGTCCC element is responsible for conferring auxin inducibility to the *ipdC* promoter in *A. brasilense* Sp245 (Lambrecht et al. 1999).

No typical bacterial promoter sequence was found, but inverted repeat sequences were observed around 90 bp upstream of the *ipdC* gene in *A. lipoferum* FS (Yagi et al. 2001). A gel mobility-shift assay showed 2 DNA-binding proteins that might be involved in regulation of *ipdC* expression. Also, a TGTCCC sequence found at the inverted repeated sequences in the putative promoter region of the *ipdC* gene of *A. lipoferum* FS might function as an auxin-responsive *cis*-element for the *ipdC* gene (Yagi et al. 2001).

Disruption of *ipdC* in *A. brasilense* Sp7 resulted in a mutant strain that produced IAA with lactate or pyruvate as the C source. However, with gluconate as a C source, IAA synthesis by the *ipdC* mutant was inhibited. This suggested a route for IAA biosynthesis, other than the indole-3-pyruvic acid pathway, regulated by catabolite repression (Carreño-Lopez et al. 2000). A tryptophan auxotroph carrying the *ipdC* mutation produced IAA if tryptophan was present, but did not synthesize IAA from indole, suggesting that the alternate route was tryptophan dependent. Using permeabilized cells, the authors observed enzymatic conversion of tryptamine and indole-3-acetonitrile to IAA by both the wild-type and the *ipdC* mutant. IAA production from tryptamine strongly decreased with gluconate as the carbon source, suggesting that tryptamine is an intermediate in the alternate pathway. Conversion of indole-3-acetonitrile into IAA was also observed, and might constitute another pathway. In summary, *A. brasilense* Sp7 appears to possess 2 differently regulated tryptophan-dependent routes for IAA synthesis, via indole-3-pyruvic acid and via tryptamine, as well as an alternate route that uses indole-3-acetonitrile as an intermediate (Carreño-Lopez et al. 2000).

Morphological changes in plant roots following *Azospirillum* inoculation were mimicked by applying a combination of plant growth substances, pointing to the involvement of an auxin produced by *Azospirillum* for root proliferation and consequent plant growth promotion (review, Bashan and Holguin 1997). Further evaluation of the contribution of auxin biosynthesis by *A. brasilense* in altering root morphology showed that inoculation of wheat seedlings with an *A. brasilense* Sp245 strain, carrying a mutation in the *ipdC* gene, did not decrease root length nor stimulate root hair formation, in contrast to inoculation with the wild-type. These experiments indicate the role of IAA in root proliferation when inoculated with *Azospirillum* (Dobbelaere et al. 1999).

Gibberellins

A beneficial effect of *Azospirillum* spp. on plants has been suggested to be partially caused by the production of gibberellins. Application of gibberellins had effects similar to *Azospirillum* inoculation in increasing root hair density.

When *A. lipoferum* USA 5b, a gibberellin-producing strain, was cultured in the presence of glucosyl ester or glucoside of gibberellin A_{20}, both conjugates were hydrolyzed. These in vitro results support the hypothesis that growth promotion in plants induced by *Azospirillum* inoculation results from a combination of both gibberellin production and gibberellin-glucoside/glucosyl ester deconjugation by the bacterium (Piccoli et al. 1997). The effect of water potential or O_2 concentration on growth and gibberellin A_3 (the main gibberellin identified from *Azospirillum*) production in *A. lipoferum* showed that gibberellin A_3 produced by each culture was reduced severely at high water potentials or low O_2 concentrations. At the highest water potential concentration, gibberellin A_3 was reduced by only 50%, despite a 90% reduction in cell numbers. This indicates an increase in the amount of gibberellin A_3 produced per cell with increasing water potential (Piccoli et al. 1999). The involvement of gibberellin A_3 produced by *Azospirillum* spp. in promoting maize growth was also suggested (Lucangeli and Bottini 1997).

A. brasilense Cd and *A. lipoferum* USA 5b promoted elongation of root sheaths with 2 single genes in GA-deficient dwarf rice mutants, dy and dx, when the inoculated seedlings were supplied with [17, 17-$^2H_2$] gibberellin A_{20}-glucosyl ester. This growth resulted from gibberellin metabolism by the bacteria in the dx mutant, and by both the rice plant and microorganism in the dy mutant. In the dy mutant, inoculation by both bacterial strains reversed dwarfism in seedlings incubated with [17, 17-$^2H_2$] gibberellin A_{20}, forming [17, 17-$^2H_2$] gibberellin A_1. It was possible that the bacterial enzyme responsible for these phenomena is 2-oxoglutarate-dependent dioxygenase, similar to those of plants (Cassan et al. 2001*a*, *b*).

Ethylene

During most phases of plant growth, ethylene production is minimal. Ethylene plays a major role in germination by breaking the dormancy of seeds, however, a high level of ethylene concentration inhibits subsequent root elongation. High levels of ethylene may be synthesized as a response to biological or environmental stresses, causing wilting and senescence (Glick et al. 1999). Controlling ethylene levels, often by lowering them, prevents significant economic losses in agriculture. One of the precursors of ethylene synthesis is 1-aminocyclopropane-1-carboxylic acid (ACC). ACC deaminase is a key enzyme, commonly found in many soil microorganisms and PGPBs, capable of degrading ACC. Thus, lowering ethylene levels in plants can be considered as having potential for promoting growth (Glick et al. 1999). Wild *Azospirillum* spp. do not have ACC deaminase, but some strains can produce ethylene, nevertheless.

Modulation of ethylene by PGPB can occur through degradation of the ethylene precursor ACC via ACC deaminase, yielding ammonia and α-ketobutyrate as by-products.

The ACC deaminase structural gene (*acdS*) of the PGPB *Enterobacter cloacae* UW4 was cloned in the broad host range plasmid pRK415 under control of the *lac* promoter, and then transferred into *A. brasilense* Cd and Sp245. Roots of canola and tomato seedlings, plants sensitive to ethylene, were significantly longer in plants inoculated with the *A. brasilense* transformants than plants inoculated with nontransformed strains of the same bacterium (Holguin and Glick 2001).

Speculating that a construct with the ACC deaminase gene under control of a constitutive promoter weaker than the *lac* promoter, might impose less metabolic load on *Azospirillum*, the *acdS* gene was cloned under the control of a tetracycline resistance gene promoter: *A. brasilense* Cd transformants holding *acdS* fused to the Tet^r gene promoter showed lower ACC deaminase activity than transformants with *acdS* controlled by *lac* promoter. However, *acdS* controlled by the Tet^r gene promoter exerted less metabolic load on *A. brasilense* Cd transformants than *acdS* controlled by *lac*, resulting in increased IAA synthesis, growth rate, survival on tomato leaf surfaces, and ability to promote growth of seedlings (Holguin and Glick 2003).

Nitrogen metabolism

Nitrogen cycle

Since N_2-fixation was the original proposed mechanism by which *Azospirillum* affects plant growth, considerable information has already been published (for reviews: Bashan and Levanony 1990; Bashan and Holguin 1997*a*; Kennedy and Islam 2001). In recent years, only a few studies have focused on the nitrogen cycle within the cell, apart from the genes involved.

Mutants of common *A. brasilense* strains Sp7 and Sp245, defective in flocculation, differentiation into cyst-like forms, and colonizing roots, had a higher nitrogenase expression than wild strains when associated with wheat. Apparently, the ability of Sp7 and Sp245 mutant strains to remain constantly in vegetative forms (spirillum and rods) improved their ability to express exceptional nitrogenase activity rates. Restoring cyst formation and a normal colonizing pattern to the spontaneous mutant Sp7S reduced nitrogenase activity rates to the level of the wild Sp7. This suggests that retention of bacterial cells in the vegetative state provides faster metabolism, which directly affects N_2-fixation of the bacterium (Pereg-Gerk et al. 2000). The efficiency of N_2-fixation and denitrification in *A. lipoferum* can be regulated by varying the concentration of oxygen, nitrate, and molybdenum.

The maximum growth rate in 2 strains was observed under microaerobic conditions (5% O_2), minimal nitrate (2 g/L), and the maximum allowable concentration of molybdenum (0.5 g/L). These conditions also were conducive for the maximum efficiency of denitrification (nitrate reduction to molecular N_2) (Furina et al. 1999).

Microaerobic conditions favor N_2-fixation. In *A. brasilense*, cytochrome *c* oxidase is required under microaerobic conditions when a high respiration rate is needed. However, under N_2-fixing conditions, respiration rates do not seem to be a growth-limiting factor. Evidence for this was provided when a wild-type *A. brasilense* was compared with a *cytN* mutant *A. brasilense*. Under aerobic conditions, growth during the log phase was similar between the 2 types. Under microaerobic conditions (with NH_4^+ supplied; no N_2-fixation), low respiration of *A. brasilense cytN* decreased its growth rate compared with the growth rate of the wild-type *A. brasilense*. Under N_2-fixing conditions (without NH_4^+ supplied), growth and respiration rates of the wild-type were significantly diminished, and the differences in growth and respiration rates between the wild and mutant forms were smaller. Yet, the N_2-fixing capacity of the mutant was still approximately 80% of the wild-type (Marchal et al.1998).

Capability to withstand low temperatures and low oxygen concentrations could depend on the capacity of the bacterium to use nitrate to form nitrite more efficiently. *A. lipoferum* JA03 showed better growth at low temperature (28 °C), while *A. brasilense* Sp245 and JA04 grew better at 32 and 37 °C. Additionally, *A. lipoferum* JA03 showed remarkably high nitrite accumulation and more intense growth at low oxygen tension, compared with *A. brasilense* Sp245 and JA04. Perhaps the greatest growth rate at low oxygen tension was partly a result of efficient utilization of nitrate in respiration. These *A. lipoferum* JA03 traits might be adaptive characteristics leading to better acclimation in waterlogged and highly compacted subtropical soils in Brazil (Didonet and Magalhães 1997). Dissolved oxygen was also found to be a limiting factor when ammonium concentrations limit growth of *A. lipoferum* (Tsagou et al. 2003). Out of 40 thermo-tolerant mutants developed from a mesophilic *A. lipoferum*, only 14 mutants could grow and fix nitrogen at 45 °C. These mutants excrete ammonia only as very old cultures (maximum production after 12 d under stationary conditions). Finally, from an *A. brasilense* that is normally capable of reducing NO_3^-, a spontaneous mutant was isolated that was defective in both assimilatory and periplasmic dissimilatory nitrate reductase activity. It was also significantly reduced in its capability to colonize wheat and rice seedling roots. As the rhizosphere is poor in nitrates, functional periplasmic nitrate reductase might be essential for the survival and efficient colonization by *A. brasilense* (Steenhoudt et al. 2001*b*).

N_2-fixation was the first mechanism suggested to promote the growth of plants by *Azospirillum*. The majority of evidence collected during the last 3 decades concerning this mechanism has generated controversy. Some greenhouse and field experiments have shown repeatedly that the transfer of nitrogen (measured as transfer of $^{15}N_2$) fixed by *Azospirillum* spp. to the plant is minimal (Kennedy et al. 1997, 1998; for earlier studies Bashan and Holguin 1997*a*, *b*). Yet, other studies showed that the bacteria cannot fulfill all of the nitrogen requirements of plants, but nevertheless contribute significant amounts of nitrogen. Furthermore, inoculation commonly and significantly reduced the required doses of nitrogen fertilization in numerous greenhouse and field experiments of many plant species (Bashan and Levanony 1990; Bashan and Holguin 1997*a*, Tables A1 and B1).

Several confirmatory reports about the contribution of fixed nitrogen by *Azospirillum* to plants, similar in nature to reports of previous years, illustrate the controversy. The ^{15}N isotope dilution technique indicated that there were significant biological N_2-fixation contributions to 2 genotypes of maize that showed similar increases in grain yield when they were inoculated with a mixture of *Azospirillum* spp. strains or fertilized with the equivalent of 100 kg N/ha. These genotypes showed a large increase in total N in the plant. This suggests that the yield response resulted from increased nitrogen acquisition, but not from bacterial nitrate reductase; NR^- mutants generally caused plant responses similar to those of the parent strains (Garcia de Salamone et al. 1997). The ability of the bacteria to transfer fixed nitrogen from the atmosphere to wheat plants was tested using $^{15}N_2$-enriched atmosphere. Labeled fixed nitrogen was detected in plant growth media, in roots and shoots of wheat grown 26 d in $^{15}N_2$-enriched atmosphere, but the highest levels of ^{15}N were detected in wheat shoots. Ammonia or nitrate supply to plants did not repress $^{15}N_2$-fixation (Ruppel and Merbach 1997). Bacterial strain-wheat cultivar relations were studied using 12 *A. brasilense* strains collected from wheat roots. They were compared for responses in root colonization, growth stimulation, and nitrogen supply to the plant. All strains colonized the surface and interior of roots. Most strains stimulated plant growth, but with variable degrees of stimulation. Some strains increased the total nitrogen of roots and leaves up to 80% over uninoculated plants, while others produced no effect on nitrogen content. Inoculation of different wheat cultivars with the most efficient strain for N_2-fixation resulted in increased growth and nitrogen to the 5 cultivars tested, but the effects varied among the cultivars. These results suggest that a potential exists for *A. brasilense* to supply considerable nitrogen to wheat plants, probably dependent on bacteria-cultivar interaction (Saubidet and Barneix 1998). Para-nodules induced by low concentrations of the auxin 2,4-D in rice seedlings were the preferential sites for colonization by a NH^+_4-excreting *A. brasilense* mutant. Nitrogenase activity in tumor structures inhabited by bacteria significantly increased, compared with untreated control plants (Christiansen-Weniger 1997). It is probable that within the para-nodule bacterial nitrogenase activity is less sensitive to increased oxygen tension in the roots. Host plants benefit from enhanced N_2-fixation in their roots with para-nodules because fixed nitrogen is incorporated into host plant material. Host plants probably stimulate nitrogenase activity of endophytic azospirilla by providing a carbon source as energy (Christiansen-Weniger 1998). The effects of inoculating wheat with a highly efficient *A. brasilense* strain under 3 nitrogen regimes revealed that inoculation stimulated plant growth, nitrogen accumulation, and nitrogen and NO_3^- accumulation in the tissues. At maturity, inoculated plants showed higher biomass, grain yield, and nitrogen content than the uninoculated ones, as well as

higher grain protein concentration. It was concluded that *A. brasilense* increased plant growth by stimulating nitrogen uptake by the roots (Saubidet et al. 2002).

On the other hand, using an in vitro model (*A. brasilense* and wheat) within 70 h after inoculation, insignificant amounts of newly fixed N_2 were transferred from an ammonia-excreting strain of *A. brasilense* to the shoot tissue of wheat. By the addition of malate (a preferred carbon source for *Azospirillum*), transfer of nitrogen to the shoots increased 48-fold, indicating that 20% of shoot nitrogen had been derived from N_2-fixation. Apparently, the inability of the host plant to release sufficient carbon into the rhizosphere is a significant constraint on the development of the *A. brasilense*-wheat association. Perhaps wheat plants with an increased release of photosynthate to the rhizosphere should be a priority for improving the association (Wood et al. 2001).

N_2-fixation by aerobic bacteria is a very energy-demanding process, requiring efficient oxidative phosphorylation, while O_2 is toxic for the nitrogenase complex. Azospirilla and other well-known N_2-fixing soil bacteria have evolved a variety of strategies to deal with and overcome this apparent "O_2 paradox". The question is whether the specific environmental adaptations of azospirilla are sufficient to allow optimal proliferation and N_2-fixation in their natural habitat. Could improving O_2 tolerance of the N_2-fixing process contribute to the development of more efficient strains for inoculation of plants? (Marchal and Vanderleyden 2000). This is left for future research.

N_2-fixation genes

In *A. brasilense*, 3 *nif* operons have been identified: *nifHDK*ORF1*Y*, *nifENX*ORF3, and ORF2*nifUSV*ORF4 (Frazzon and Schrank 1998; Galimand et al. 1989; Passaglia et al. 1991). The nucleotide sequence of *nifUSV* genes and 2 flanking, open reading frames (ORF2 and ORF4) were determined to constitute an operon apparently transcribed from a promoter upstream of ORF2. Consensus σ^{54} and NifA binding sites were present in the putative promoter region (Frazzon and Schrank 1998).

Expression of 15–20 genes involved in nitrogenase synthesis and activity in many diazotrophs require the transcriptional activator NifA. In enteric bacteria, NtrC-P positively regulates synthesis of NifA (for review, Steenhoudt and Vanderleyden 2000). In *Azospirillum*, however, *nifA* expression is not regulated by NtrC, and is transcribed under conditions incompatible with N_2-fixation, i.e., with oxygen or ammonia. Independent of nitrogen status, NifA remains inactive from self-inhibition by its N-terminal domain. The P_{II} protein (*glnB* product) changes NifA conformation, and relieves this self-inhibition (for reviews, Holguin et al. 1999; Steenhoudt and Vanderleyden 2000). Investigation showed that P_{II} interacted directly with the N-terminal domain of NifA in *A. brasilense* Sp7 and did not interact with the central and C-terminal domains (Du et al. 2002).

Sequencing of a region upstream of *nifA* in *A. brasilense* revealed putative –35/–10 sequences for σ^{70} recognition that are candidates for the *nifA* promoter (Fadel-Picheth et al. 1999). A series of sequentially deleted *nifA* promoters showed that deletions between nucleotides –67 and –46 upstream of *nifA* decreased promoter activity significantly, suggesting that the enclosed region is essential for *nifA* promoter activity. This region might bind regulatory proteins responsive to oxygen or ammonia. Repression by oxygen may be through a *Fnr*-like protein; 2 sequences resembling a *fnr* consensus sequence were found downstream from the transcription initiation start of *nifA* (Fadel-Picheth et al. 1999).

The *A. lipoferum nifA* region was cloned and sequenced as a homologue of the *Klebsiella oxytoca nifA* gene (Shigematsu et al. 1997). The amino acid sequence showed 91% identity to that of *A. brasilense* Sp7 NifA. A conserved cysteine motif responsible for oxygen-dependent regulation of NifA activity was found in *A. lipoferum* NifA, suggesting that, like NifA of *A. brasilense* Sp7 (Arsène et al. 1996), NifA in *A. lipoferum* is regulated by oxygen concentration (Shigematsu et al. 1997).

In *Azorhizobium caulinodans, nifA* expression is regulated partly by the two-component regulatory system NtrYX under symbiotic conditions. An *A. brasilense* spontaneous mutant, constitutive for N_2-fixation in the presence of ammonium and deficient in nitrate-dependent growth, was complemented for nitrate-dependent growth and ammonium regulation of nitrogenase by a plasmid containing the *ntrYX* genes of *A. brasilense*. These results suggest that in *A. brasilense*, NtrY and NtrX proteins also regulate N_2-fixation (Vitorino et al. 2001).

P_{II} in *A. brasilense* is essential for N_2-fixation (Liang et al. 1993). An analogue of P_{II} was found and named P_Z (encoded by *glnZ*) (de Zamaroczy et al. 1996). P_{II} and P_Z have similar structures, and are uridylylated under nitrogen-limiting conditions, but are not functionally equivalent (de Zamaroczy 1998; de Zamaroczy et al. 1996), since P_Z does not relieve inhibition of NifA activity upon removal of fixed nitrogen, as the P_{II} protein does. P_{II} negatively regulates *glnZ* expression and might be involved in nitrate-dependent growth. P_Z protein, on the other hand, negatively modulates ammonium uptake. The severely abrogated growth of an *A. brasilense* P_{II}–P_Z double mutant suggests that both P_{II} and P_Z are necessary for optimizing nitrogen and carbon metabolism under any condition (de Zamaroczy 1998). P_{II} and P_Z seem to be involved in the dinitrogenase reductase ADP-ribosyl transferase (DRAT) / dinitrogenase reductase-activating glycohydrolase (DRAG) regulatory system that regulates nitrogenase activity at a post-translational level, in response to changes in nitrogen status (Klassen et al. 2001). Klassen et al. (2001) found that the signal pathways for ADP-ribosylation and removal of ADP-ribosyl are different. Studies with a *glnZ* mutant showed that GlnZ is involved in the reactivation mechanism of the ADP-ribosylated iron protein, but is not essential for nitrogenase switch-off by ammonium ions. Possibly, GlnZ in *A. brasilense* is required for DRAG enzyme reactivation (Klassen et al. 2001). Perhaps as in *Rhodobacter capsulatus* and *Rhodospirillum rubrum* (Hallenbeck 1992; Johansson and Nordlund 1997), *A. brasilense* P_{II} forms part of the signal pathway leading to the reversible ADP-ribosylation of the iron protein (Klassen et al. 2001).

In *A. brasilense*, P_{II} and P_Z occur in 2 forms, a native form when there is excess nitrogen and an uridylylated form under nitrogen-limiting conditions (de Zamaroczy 1998).

GlnD catalyzes uridylylation and de-uridylylation of the P_{II} and P_Z proteins that function as uridylyltransferase and uridyl-removing enzymes (Van Dommelen et al. 2002). The isolated and sequenced *A. brasilense glnD* gene was very similar to *glnD* in the family Rhizobiaceae. Nitrogen regulation of glutamine synthetase was not altered in a *glnD*-Tn*5*-B30 insertion mutant. However, with limited nitrogen, GlnD was necessary to fully de-adenylate glutamine synthetase. The mutant could not take up (methyl) ammonium or grow with nitrate as the sole nitrogen source, and was Nif$^-$. The effect of *glnD* on P_{II} might explain the last characteristic, since an uridylylated P_{II} is required to activate NifA. Possible σ^{54}-dependent promoter sequences were found upstream of the *glnD*-coding region, but they poorly matched the consensus σ^{54}-promoter sequence (Van Dommelen et al. 2002).

In *A. brasilense*, *glnB* and *glnA* are clustered in an operon regulated by 3 different promoters: 2 located upstream of *glnB* (*glnBp1*-σ^{70} and *glnBp2*-σ^N); and an unidentified promoter in the *glnBA* intergenic region. With limited nitrogen, *glnB* and *glnA* are co-transcribed from the σ^N dependent promoter (de Zamaroczy et al. 1993). Deletion of 2 putative NtrC activation sites upstream of *glnBp2*-σ^N showed that NtrC binding sequences were essential for *glnB* expression with nitrogen limitation (Huergo et al. 2003). In vitro studies also indicated that the *glnB* promoter region bound the NtrC protein. Additionally, the *A. brasilense* NtrC protein activated transcription of *glnB-lacZ* fusions in an *Escherichia coli* genetic background. Contrary to de Zamaroczy et al. (1993), these results show that NtrC activates *glnBglnA* expression in *A. brasilense* under conditions of limited nitrogen.

In *A. brasilense*, glutamine synthetase (GS) catalyzes ammonium and glutamate to yield glutamine. Nitrosoguanidine-induced mutants with reduced GS activity and the ability to excrete ammonium, a key trait for agronomically useful diazotrophic bacteria, showed alterations of the ammonium and glutamate-binding site of GS. These results suggest that *Azospirillum* GS protein engineering could generate ammonium-excreting strains (Van Dommelen et al. 2003).

Genes involved in nitrogen metabolism

Sequencing of the *A. brasilense* ammonium transporter gene *amtB* showed homology among known ammonium transporter genes, and classified AmtB as an integral membrane protein (Van Dommelen et al. 1998). The *ntr* mutants did not take up [^{14}C]methylammonium, suggesting that nitrogen regulates transcription of the *amtB* gene in *A. brasilense* by the Ntr system. Mutants lacking the AmtB protein take up ammonium, inferring a second ammonium transport mechanism (Van Dommelen et al. 1998).

A. brasilense Sp245 *napABC* genes coding for periplasmic nitrate reductase were isolated and sequenced (Steenhoudt et al. 2001*a*). Based on whole cell nitrate reductase and periplasmic cell-free extract assays, Steenhoudt et al. (2001*a*) concluded that *napABC*-encoded enzyme activity in *A. brasilense* Sp245 corresponds to a periplasmic dissimilatory nitrate reductase expressed under anoxic or oxic conditions.

Nitrite is reduced in denitrifying bacteria by 2 enzymes entirely different in structure and prosthetic metal: NirS, the tetraheme protein cytochrome cd_1, and NirK, the copper-containing nitrite reductase (Zumft 1997). *A. brasilense*, *A. lipoferum*, and *A. halopraeferens* strains possess cytochrome cd_1-containing nitrite reductases with low sequence similarities, while *A. irakense* and *A. doebereinerae* have Cu-containing nitrite reductases, and *A. amazonense* does not denitrify (Kloos et al. 2001).

Proton efflux from roots

Improving plant growth by affecting proton and organic acid extrusion mechanisms of plants by inoculation with *Azospirillum* sp. was proposed over a decade ago (for review, Bashan and Levanony 1990 and Bashan and Holguin 1997*a*), and recently was shown to occur in cardon cactus plants (Carrillo et al. 2002). Lowering the pH of the rhizosphere increases the availability of phosphorus and iron to plants, especially in arid lands with high calcium content and soil pH. A recent confirmatory study of the proton extrusion phenomenon in wheat showed that inoculation enhanced proton efflux and root elongation of the roots. Although the evidence is circumstantial, perhaps these 2 phenomena are related. This effect was directly dependent on the bacterial strain-plant combination, suggesting that compatible strains are necessary to induce this phenomenon (Amooaghaie et al. 2002).

Phosphate solubilization, rock weathering, mineral uptake and content, and degradation of siderophores

Improved mineral uptake by plants was suggested as a major contribution of *Azospirillum* inoculation, therefore, bacterial cells weathering minerals have received recent attention.

A. halopraeferens, a non-glucose using bacterium, and consequently non-acid producer, is reported to solubilize insoluble inorganic phosphate in vitro by unknown mechanisms (Seshadri et al. 2000). Sugars, like glucose, that are part of the root exudates of pea plants grown in P-deficient substrates enhanced the capacity of *Azospirillum* sp. to solubilize normally insoluble $Ca_3(PO_4)_2$. The relative proportion of glucose in pea exudates decreased under P deficiency, while the content of galactose, ribose, xylose, and fucose increased. The shift in sugars under P deficiency increased the capability of *Azospirillum* spp. to mobilize phosphate (Deubel et al. 2000). Similarly, inoculation of cardon (a giant columnar cacti) with *A. brasilense* Cd enhanced phosphate solubilization (Carrillo et al. 2002). These observations can partly be explained by acidification of the nutrient medium by protons, since bacteria can produce different organic acids that assist in P solubilization, depending on the sugar supplied in the root exudates. Yet, *Azospirillum* can solubilize P by itself without addition of root exudates. Three *Azospirillum* strains were isolated from the ectomycorrhizal sporocarps (*Rhizopogon vinicolor*) that colonized Douglas-fir trees. In vitro, they were able to degrade limestone, marble, and calcium phosphate (Chang and Li 1998).

Uptake by *A. brasilense* of the essential elements Mg, Ca, Mn, and Fe, and trace elements V, Co, Ni, Cu, Zn, and Pb (which do not essentially suppress growth of the bacterial culture), showed that essential elements are accumulated by the cells. Zinc and Cu were accumulated in the bacterial bio-

mass in relatively significant amounts, but uptake of Co and Ni was significantly smaller, and Pb and V were apparently not assimilated by azospirilla. In particular, Cu cations were effectively absorbed by the bacterium, and this addition increased the rate of uptake of other metals, however, the process takes time. Short exposures have only a limited effect on Cu absorption. The effect of trace elements on the absorption level of the 4 essential elements may provide evidence that they compete with them for the formation of biologically active cell components (Kamnev et al. 1997*a*; Ignatov et al. 2001). Additionally, the bacteria are capable of producing structural modifications of the magnesium-ammonium orthophosphate molecule when added to the medium (Kamnev et al. 1999*a*). Fourier transform infrared spectroscopy is a powerful tool for non-destructive identification and characterization of cell components, and it was applied to molecular structure studies of *A. brasilense*, its essential element content (Kamnev et al. 1997*b*, 2001), heavy metal-induced metabolic changes in the cells (Kamnev et al. 2002*b*), and membrane composition and structure (Kamnev et al. 1999*b*). Finally, *A. irakense*, but not *A. lipoferum*, *A. brasilense*, or *A. amazonense*, was capable of degrading desferrioxamine-type siderophores by desferrioxamine hydrolase. The products of the enzymatic hydrolysis were monohydroxamate and dihydroxamate metabolites. In addition to desferrioxamine B, several other linear and cyclic desferrioxamines and derivatives were degraded, but not desferricoprogen and desferri-ferrichrome. This shows a high substrate specificity of the desferrioxamine hydrolase in *A. irakense* (Winkelmann et al. 1999). This relatively new emerging field of mineral solubilization and transformation in *Azospirillum* research is potentially useful for studying interactions and survival of the bacteria in the soil environment.

In a comprehensive overview of what is known about the physiology and molecular biology of the bacteria and their mode of action on plants, it is apparent that phytohormones, especially IAA, play a role in promoting plant growth. However, to attribute a phenomenon of nonspecific growth-promotion of numerous plant species by *Azospirillum* inoculation to one (or a few) substance(s) produced in abundance, mainly in vitro, is an oversimplified, but useful research tool for probing the mode of action of the bacteria. For a more accurate determination of the role of phytohormones in general, and IAA in particular, in growth-promotion, there is a need for a mutant that is totally deficient in IAA production, but otherwise identical to the parental strain. Although some mutants that are partially IAA deficient were constructed in recent years, this goal has not yet been achieved. The same is true for other phytohormones. Additionally, to clearly state that hormones are the main mechanism for growth promotion, there is a need to demonstrate that other proposed mechanisms have a minor role. Yet, there is evidence to the contrary, such as the importance of N_2-fixation under specific circumstances, including the post-para-nodule colonization mentioned earlier and under field conditions. The overall accumulated evidence that N_2-fixation plays a role in the association reconfirms that dismissal of N_2-fixation as a mechanism for plant growth, as reported in several reviews in recent years, is premature, and that N_2-fixation should be reconsidered as a plausible mechanism. Additionally, the importance of signal molecules, like WGA (one of the most studied plant lectins), in initiating the cascade of events that yielded a plant response should be considered. Perhaps root membranes (the main area responsible for mineral uptake detected in numerous plant-*Azospirillum* associations) are the primary targets for *Azospirillum* colonization on plant roots. Finally, the multitude of options for affecting plant growth should lead to a detailed evaluation of the Additive Hypothesis as the actual mechanism/mode of *Azospirillum* action. These may include mineral uptake and partitioning of carbon compounds, phenomena well-recognized as being multi-parametric. Indirectly, this evidence suggests that the mode of action of *Azospirillum* sp. is probably composed of multiple mechanisms.

Production of enzymes and enzymatic studies

Several enzymes in *Azospirillum* have been studied in vitro in recent years. *A. brasilense* glutamate synthase, a complex iron-sulfur flavoprotein that catalyzes the reaction of glutamine to L-glutamate, was characterized and transferred to *E. coli* for the overproduction of glutamate synthase holoenzyme. Recombinant *A. brasilense* glutamate synthase was purified to homogeneity after overproduction in *E. coli*, and the purified enzyme was indistinguishable from the original enzyme prepared from *A. brasilense* (Stabile et al. 2000). The effect of Mg^{+2}, Mn^{+2}, and Co^{+2} on kinetic properties of GOGAT, a key enzyme of nitrogen metabolism in *A. brasilense* Sp245, revealed that the enzyme level and kinetic behavior of GS depend essentially on the concentration of the ions and their ratios (Bespalova et al. 1999; Antonyuk et al. 2001). Cobalt metabolism in *A. brasilense* Sp245 was studied with emission Mossbauer spectroscopy of Co-57(II)-doped bacterial cells. This technology allowed elements in biological samples to be monitored at their physiological (trace) concentrations. It showed that Co-57(II)-activated glutamine synthetase had 2 different Co (II) forms at its active sites (Kamnev et al. 2002*a*).

Laccase, a *p*-diphenol oxidase that is most typical of plants and fungi, was detected in *A. lipoferum* (Diamantidis et al. 2000). Laccase activity was also detected in a nonmotile, in vitro-grown variant that originated from the motile laccase-negative wild-type isolated from rice cultivated under extremely low oxygen concentrations. This stable, atypical variant acquired laccase activity and was capable of producing melanin (Alexandre and Bally 1999). During the exponential growth phase under fully aerobic conditions, the laccase-positive variant lost a respiratory branch that terminated in a cytochrome *c* oxidase, probably from a defect in the biosynthesis of a heme component essential for the oxidase. The laccase-positive variant was far less sensitive to the inhibitory action of quinone analogs, apparently caused by rearrangements in its respiratory system. It is possible that the loss of the branch containing cytochrome *c* oxidase in the variant is an adaptation to the presence of intracellular, oxidized quinines produced by laccase (Alexandre et al. 1999). The ecological significance of these azospirilla is yet to be discovered.

Eukaryote and prokaryote microorganisms contain β-glucosidases that catalyze the hydrolysis of cellobiose

(biodegradation product of cellulose) and chemically related β-glucosides. *A. irakense* KBC1 grows on pectin and β-glucosides, such as cellobiose, arbutin, and salicin. From an *A. irakense* cosmid library expressed in *E. coli*, 3 β-glucosidases, SalA, SalB (required for growth of *A. irakense* on salicin) (Faure et al. 1999), and CelA (required for optimal growth on cellobiose) (Faure et al. 2001), were characterized genetically and biochemically. An *A. irakense salA-salB* insertion mutant did not grow on salicin, but did on arbutin, cellobiose, gentiobiose, and other carbon sources, such as glucose and malate. Complementation with either *salA* or *salB* restored *salA-salB* mutant growth on salicin, suggesting functional redundancy of these genes in salicin utilization. Glucose released after biological cleavage of aryl-β-glucosides is a suitable carbon source for many bacteria, and could contribute to the competitiveness and survival of bacteria in the rhizosphere (Faure et al. 1999).

A. irakense isolated from surface-sterilized, field-grown rice roots suggested that the bacterium can penetrate plant roots and use cell wall-degrading enzymes (Khammas et al. 1989). Two new pectate lyase enzymes were isolated and characterized from *A. irakense* (Bekri et al. 1999; de Armas et al. 2002), the sole source of these enzymes in the genus *Azospirillum*. The *A. irakense pelA* gene that encodes a pectate lyase was isolated by heterologous expression in *E. coli* (Bekri et al. 1999). Pectin stimulated *pelA* transcription significantly. An *A. irakense pelA*-Tn*5* mutant still displayed pectate lyase activity, suggesting multiple pectate lyase genes in *A. irakense* (Bekri et al. 1999).

Use of Tn*5* in mutant construction

Although it is generally accepted that Tn*5* transposition does not involve replication, there is evidence that Tn*5* can have a replicative mechanism of transposition. Characterization of a DNA fragment with a Tn*5* insertion in a regulatory *nifA* type gene of *A. brasilense* (Singh et al. 1989) revealed that duplication of IS50 occurred at the Tn*5* insertion site (Tripathi et al. 1998). Nucleotide sequencing of the Tn*5* insertion region proved that IS50 was duplicated, and that, together with the insertion event, a 484 bp sequence was deleted from the outside end of the internal IS50. This kind of IS50 duplication, accompanied by partial deletion, has not been described so far, and suggests that Tn*5* might transpose by replication, as well as by the conservative "cut and paste" mechanism (Tripathi et al. 1998).

Disruption of an open reading frame of 840 bp and 280 amino acids (ORF280) in an *A. brasilense* Tn*5* mutant resulted in a pleiotrophic phenotype that is 4 times better at fixing N_2 and less able to grow on glutamate and arginine than the wild-type. Analysis of *nifH-gusA* fusion in the mutant and the wild-type showed that the mutation in ORF280 gave rise to increased levels of *nifH-gusA* expression (Revers et al. 2000). ORF280 sequencing revealed that its C-terminus exhibited a striking similarity to a range of hypothetical proteins recently classified as universal stress proteins in eukaryotes, prokaryotes, and archaea (Makarova et al. 1999). The only member with a defined phenotype, the *E. coli* phosphoprotein UspR, is essential for survival in the stationary growth phase, and its phosphorylated form increases during starvation. A candidate for interaction with ORF280 is the NtrBC-P_{II}-P_Z regulatory cascade because it links N-metabolism and N_2-fixation and has a mode of action based on protein phosphorylation (Revers et al. 2000).

Effect on plant growth and yield

Contrary to greenhouse studies on the effect of *Azospirillum* spp. on plant growth and yield that dominated older literature (Bashan and Holguin 1997*a*), most contemporary reports involved field studies as indicators of maturity of the field and its transformation to commercialization. Three notable phenomena reported are (*i*) inoculation of plant species other than cereals. There are a significant number of those, demonstrating that over 100 species are capable of inoculation by this bacteria. Although *Azospirillum* was isolated initially from cereals and most of the initial inoculation has been done on the main cereal crops (Bashan and Levanony 1990), there are more non-cereal species successfully inoculated with *Azospirillum* than cereals (Bashan and Holguin 1997*a*; Tables A1 and B1). That review proposed that *Azospirillum* should be considered a general plant-growth-promoting bacterium and not a cereal growth promoter. (*ii*) Apparently, in numerous cases, inoculation reduced the use of chemical fertilizers, especially nitrogen by 20%–50%, and provided superior results when organic fertilizers were incorporated. (*iii*) In many developing countries, inoculation increased the cost-benefit ratio. Brief highlights of recent studies, and their respective references, are given in Table A1.

Effects of co-inoculation with other microorganisms on plant growth and yield

The most notable phenomenon in *Azospirillum* inoculation, as in the early 1990s (Bashan and Holguin 1997*a*, *b*), is that inoculation is more successful and more profitable when other microorganisms are co-inoculated with *Azospirillum*. Inoculation consortia apparently work better when phosphate-solubilizing bacteria, *Azotobacter*, rhizobia, bacilli, and VAM fungi are incorporated, perhaps aiding the growth of each other by synergistically providing nutrients, removing inhibitory products, and in the process, enhancing plants' ability to grow better. Although most mechanisms by which co-inoculation affects plant growth are as yet unknown, apparently co-inoculation allows plants to achieve a more balanced nutrition and (or) absorption of nutrients is improved. The most notable phenomena reported are increased mineral uptake, reduction in the use of N and P fertilizers by 25%–50%, increases in available NPK from soil, enhanced quality characteristics of the yield, higher net return, and better cost-benefit ratio. Highlights of the recent studies, and their respective references, are given in Table B1. In many inoculation tests, especially in developing countries, it appeared that co-inoculation was the method of choice in the last decade. With better characterization of the strains and better inoculant carriers (see below), it might be the preferred future mode of application for *Azospirillum* at the field level.

Biological control

Azospirillum spp. are not known as typical biological control agents because they lack direct suppressive chemicals

likely to affect most plant pathogens, although some strains may produce cyanide (HCN) in vitro (Gonçalves and de Oliveira 1998), but this feature is uncommon. Most biological control effects reported earlier (Bashan and Holguin 1997*a*) and in this essay are indirect. Inoculation with *Azospirillum* spp. indirectly suppressed some pathogens, suggesting potential for *Azospirillum* inoculation in prevention programs. The possible mechanisms used by *Azospirillum* to reduce damage inflicted by pathogens that have been demonstrated so far are related to environmental competition and displacement of pathogens, inhibition of seed germination of parasitic weeds, general enhancement of plants to resist pathogen infection, and possible inhibition of fungal growth via the production, at least in vitro, of microbial toxic substances.

There were several observations demonstrating that some pathogen populations were reduced by *Azospirillum* inoculation. Inoculation of okra (*Abelmoschus esculentus*) with *A. brasilense* enhanced several plant characteristics and pod yield. At the same time, a significant reduction in adult female root-knot nematodes (*Meloidogyne incognita*) egg masses, eggs per egg mass, and total soil nematode population were recorded (Ramakrishnan et al. 1997). Similar results in sunflower (*Helianthus annuus*) were obtained with a commercial inoculant of *A. brasilense* (Ismail and Hasabo 2000). Maize (*Zea mays*) inoculated with a combination of mycorrhizal fungi, *Glomus fasciculatum*, *Azospirillum* sp., and phosphate-solubilizing bacteria reduced the population of the *Pratylenchus zeae* nematode and simultaneously induced the highest cob yield (Babu et al. 1998). *A. lipoferum* inoculated onto wheat plants reduced a *Heterodera avenae* nematode infection (Bansal et al. 1999). Inoculation of sorghum with *A. brasilense* to control the *Atherigona soccata* shoot fly that causes dead-heart in sorghum resulted in a 10-fold reduction of the disease and increased grain yield (Kishore 1998*a*, *b*). *A. brasilense* was applied as a foliar spray against foliar fungal and bacterial diseases of mulberry, such as powdery mildew caused by *Phyllactinia corylea*, black leaf spot caused by *Pseudocercospora mori*, black leaf rust caused by *Cerotelium fici*, and bacterial leaf blight caused by *Pseudomonas mori*. Inoculation reduced fungal pathogens and excelled as a treatment against bacterial blight (Sudhakar et al. 2000*a*, *b*). The addition of *Rhizobium*, *Azospirillum*, or *Azotobacter* inocula as a combined seed and soil treatment in cultivation of pearl millet (*Pennisetum glaucum*) reduced downy mildew (*Sclerospora graminicola*) disease in the leaves (Gupta and Singh 1999). Inoculation with arbuscular mycorrhizal (AM) fungi and *Azospirillum* sp. suppressed damping-off in chili (*Capsicum* sp.) caused by *Pythium aphanidermatum* (Kavitha et al. 2003). Combinations of several ineffective management tactics (spraying Cu and streptomycin combined with *Azospirillum* seed inoculation and seed disinfections, each singly ineffective against *Pseudomonas syringae* pv. *tomato* (PST, bacterial speck of tomato)), significantly reduced the occurrence and severity caused by PST and also improved plant growth. Additionally, this combined treatment significantly reduced the amount of chemical pesticides required to protect tomato plants from PST (Bashan and de-Bashan 2002*b*). The mechanisms by which all this happens remain unknown. So far, *Azospirillum* is not known to induce systemic resistance in plants (Bashan and de-Bashan 2002*b*), and therefore, one may suspect that the indirect effect of the inoculant is that the plant was healthier and had greater resistance to infection.

To evaluate displacement of pathogens by *Azospirillum* inoculation, PST and *A. brasilense* were inoculated onto tomato plants, as a mixed culture or consecutively. Inoculation of seeds with a mixed culture resulted in reduction of the pathogen population in the rhizosphere, increased the *A. brasilense* population, prevented PST development, and improved plant growth. PST did not survive in the rhizosphere in the presence of *A. brasilense*. Inoculation of leaves with the mixed bacterial culture under mist conditions significantly reduced the PST population and significantly decreased the severity of the disease. Challenge with PST after *A. brasilense* was established in the leaves further reduced the PST population and severity of the disease and significantly enhanced plant development. Selective enhancement of the *A. brasilense* population on leaves occurred by applications of malic acid (favorable for *A. brasilense*, but not for PST), decreased the PST population to almost undetectable levels, almost eliminated disease development, and improved plant growth to the level of uninoculated healthy controls (Bashan and de-Bashan 2002*a*). Seeds inoculated with *A. brasilense* Sp7 and later challenged by 2 foliar bacterial pathogens of tomato (*Clavibacter michiganensis* subsp. *michiganensis* [bacterial canker] and *Xanthomonas campestris* pv. *vesicatoria* [XCV, bacterial spot]) delayed leaf- and plant-death compared with untreated controls, but canker severity was not affected. Unfortunately, inoculation with *Azospirillum* increased the severity of XCV on cherry tomatos (Romero et al. 2003).

Sometimes, a hint of the possible mechanism of biocontrol was suggested. Inoculation of mung bean (*Vigna radiata*) infected with root-knot nematode (*Meloidogyne incognita*) and *A. lipoferum* led to a decrease in the number of root galls and egg masses/root system. Plant growth and biomass of plants inoculated with this nematode significantly increased after *A. lipoferum* inoculation, probably because of an increase in the number of functional nodules on nematode-infected roots by the inoculant (Khan and Kounsar 2000). *Azospirillum* spp. inhibited seed germination of the parasitic weed Striga (*Striga hermonthica*) infesting fields of tropical sorghum, and thus, promoted sorghum growth (Bouillant et al. 1997). *Azospirillum* cells suspended in a synthetic germination stimulant did not inhibit germination of striga seeds, but blocked radicle elongation. The affected radicles had an abnormal morphology and contained no vacuolated cells in the root elongation zone. Lipophilic compounds extracted from the medium of bacteria prevented germination of striga seeds (Miché et al. 2000). Several isolated bacterial strains showed antagonism towards the fungus *Aspergillus flavus* that produces aflatoxin (the most potent carcinogenic mycotoxin produced by some fungi), and were capable of degrading the toxin in vitro. Since identification of the microorganism was based on morphological characteristics, it is uncertain whether the tentative identification of the strains as *Azospirillum* is valid (Cho et al. 2000). An *A. brasilense* strain with an increased N_2-fixation capacity was tested in vitro against the soil-borne plant pathogens, *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia*

solani, and *Pythium* sp., all infecting cucumbers. The bacteria reduced the dry wt of *Fusarium* mycelium by 90%–96%, of *Rhizoctonia* by 72%–94%, of *Pythium* by 71%–95%, and completely eliminated *Sclerotinia* mycelium (Hassouna et al. 1998). In vitro, many Brazilian *Azospirillum* strains were capable of producing HCN similar to several known biological control agents. However, no biological control tests were conducted (Gonçalves and de Oliveira 1998). At this time, all of these reports indicate that there is no conclusive evidence that *Azospirillum* is a true biological control agent.

Environmental applications

Apart from agriculture and forestry, a subfield of research has emerged with the use of inoculation of environmentally important plants (terrestrial and aquatic) to solve environmental problems.

Prevention of soil erosion and dust pollution in arid zones

A major research priority in semi-arid and arid zones is soil erosion and dust pollution, the latter affecting the health of inhabitants. The obvious solution, re-vegetation of these areas with native plants, is difficult to achieve because most desert plants grow slowly and have difficulties of establishment and survival in the first years of growth in the absence of regular irrigation.

Survival and development of cactus transplants in urban and disturbed areas of 1 desert region in Mexico were monitored. During a 3.5-year period after transplantation in an eroded area, young plants of 3 tree-shaped cacti (*Pachycereus pringlei*, *Stenocereus thurberi*, and *Lophocereus schottii*), had a high survival rate and developed more rapidly when inoculated with *A. brasilense*, compared with uninoculated control plants. Soil erosion diminished in the experimental area covered with inoculated plants. Small, but significant amounts of soil particles accumulated in the experimental area where cactus roots grew into the wind-deposited dust. One demonstrated mechanism for stabilizing the dust was by enhanced growth of small roots during the rainy season. *A. brasilense* survived well in the rhizosphere of these cacti for 2 y, but not in root-free soil (Bashan et al. 1999). Inoculation of cardon (*P. pringlei*) seeds with *A. brasilense* did not improve survival of the seedlings, but resulted in significantly better root and shoot growth, and this effect increased linearly as soil nutrients declined, in comparison with untreated seeds. In rich desert soil (more nutrients and better texture and water-holding capacity), *A. brasilense* had no effect on cardon growth, but in poor soil, dry mass of shoots was almost 60% and root length over 100% greater as a result of inoculation (Carrillo-Garcia et al. 2000). Growth promotion of cacti seedlings might occur because inoculation enhanced proton efflux and rhizosphere acidification. These phenomena are responsible for lowering soil pH, resulting in higher availability of P and other poorly soluble minerals to seedlings (Carrillo et al. 2002). Enhanced proton efflux of roots by *Azospirillum* inoculation is a known phenomenon in crops (Bashan and Holguin 1997*a*) and was recently confirmed by Amooaghaie et al. (2002). Inoculation of desert plants with PGPBs associated with roots, including *Azospirillum* sp., may accelerate plant and soil restoration projects in disturbed areas since *Azospirillum* is known to promote growth of cacti.

Bioremediation of wastewater

Tertiary wastewater treatment (nutrient removal) is an essential step, even if it is not always practised, to avoid eutrofication of water bodies receiving treated wastewater and as a means of reducing the nutrient load in wastewater used for irrigation. Use of microalgae to that end is known, albeit not commonplace. The main limitations of microalgal technology are that there is a limit to the amount of nutrients single cell cultures can absorb and appropriate disposal of the large microalgal population generated by treatment. Inoculation with *Azospirillum* was shown to assist microalgal growth, and immobilization of both microorganisms in polymeric spheres significantly reduced the removal problem (de-Bashan et al. 2004).

When freshwater microalgae *Chlorella vulgaris* or *C. sorokiniana*, often used in wastewater treatment, were co-immobilized and co-incubated with *A. brasilense* in alginate beads designed for advanced wastewater treatment, significant increases in growth of the microalgae and significant changes in their metabolism were observed. Dry and fresh wt, total number of cells, size of microalgal colonies within the beads, number of microalgae per colony, cell cytology, amount of lipids and number of fatty acids, and the level of pigments all significantly increased (Gonzalez and Bashan 2000; de-Bashan et al. 2002*a*). Apparently, this association is controlled by the bacterial species applied to this artificial association. Electron microscopy comparisons at the cellular level were performed when *C. vulgaris* was co-immobilized and co-incubated with *A. brasilense* or its natural associative bacterium, *Phyllobacterium myrsinacearum*. Initially, most of the small cavities within the beads were colonized by micro-colonies of only 1 microorganism. Subsequently, the bacterial and microalgal micro-colonies merged to form larger, mixed colonies within the cavities. At this stage, the effect of bacterial association with the microalgae differed, depending on the bacterium present. Though the microalgae entered a senescent phase and eventually died in the presence of *P. myrsinacearum*, they remained in a growth phase in the presence of *A. brasilense* (Lebsky et al. 2001). Enhancement of microalgal growth by *Azospirillum* significantly increased removal of ammonium and soluble phosphorus ions from wastewater compared with immobilization of the microalgae alone, whether synthetic wastewater (de-Bashan et al. 2002*b*) or natural domestic wastewater from a municipal wastewater plant (de-Bashan et al. 2004) was used. These studies support artificial co-immobilization of microalgae and *Azospirillum* as an effective means to increase microalgal population within confined environments and removing nutrients from wastewater.

Restoration of arid zone marine mangrove ecosystems

Mangrove ecosystems are an essential component of the coastal tropics, serving as refuge and feeding grounds for juveniles of almost all tropical marine fish, shellfish, and crabs (all highly valuable and important species) (Holguin et al. 2001). Yet for many reasons, principally shrimp aquaculture,

mangrove ecosystems are being damaged or destroyed and now require reconstruction that may employ PGPBs as inoculants (Bashan and Holguin 2002).

Inoculation of axenic black mangrove seedlings in seawater with either the halotolerant *A. halopraeferens* or *A. brasilense* produced heavy colonization of the root surface. The colonization pattern was similar to colonization observed on terrestrial plants and bacteria were embedded in a thick sheath or in micro-aggregates. *A. brasilense* cells were anchored to the surface of the roots and to themselves by a network of fibrillar material. Both bacterial strains survived in seawater for more than 30 d, colonizing mangrove roots at a very high density. *A. halopraeferens* was a better root surface colonizer (Puente et al. 1999). Inoculation of pickleweed *Salicornia bigelovii*, a common annual halophyte present in and surrounding mangrove ecosystems, with *A. halopraeferens* significantly enhanced many growth and quality parameters of the plant (Bashan et al. 2000; Rueda-Puente et al. 2003). All of these are first attempts to incorporate *Azospirillum* into mangrove reforestation programs.

Survival and persistence in soil

Azospirillum sp. is a common rhizosphere bacterium. Most species and strains prefer rhizosphere and root habitats and not the bulk soil, where they survive poorly. Yet, a fraction of the population persists in bulk soil (for review, see Bashan 1999) and can be isolated directly from the soil (Egorenkova et al. 2000).

Soil observations

Effects of 3- and 19-y natural fallow treatment of a tropical sandy topsoil on soil structure and *Azospirillum* diversity was assessed by aggregate size fractionation. Long-term fallow (19 y) under the grass *Pennisetum*, stimulated aggregation, whereas all clay particles were easily dispersed from the soil left fallow for 3 y. Coarse soil fractions supported potential sites for N_2-fixation, suggesting that these microhabitats were favorable for active N_2-fixers. Yet, more than 70% of the N_2-fixing microorganisms and 90% of the recovered *Azospirillum* were isolated from the dispersed fine clay fraction. Use of specific genetic probes on colonies within each soil fraction indicated an abundance of *A. irakense* only in the 3-y fallow soil fractions, and a selective effect of fallow on *A. brasilense*/*A. amazonense* genomic species in a 19-y fallow soil (Chotte et al. 2002). Edaphic factors affecting survival of 5 species of *Azospirillum* in bulk soil (*A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. halopraeferens*, and *A. irakense*) were evaluated after the bacteria were inoculated into 2 natural, semi-arid soils (terra rosa and sandy loess), and 2 artificial soils constructed to simulate the native soils. Within 60 d, the populations of all 5 *Azospirillum* spp. declined significantly in a linear pattern in the native and analogous artificial soils. Higher levels of $CaCO_3$ and fine and rough sand had significant detrimental effects on the survival of the 5 species, whereas increased organic matter content improved survival. In contrast, when the bacterial strains were incubated in the rhizosphere of tomato seedlings grown in the artificial soils, manipulation of these soil variables had only a marginal effect on bacterial survival; all *Azospirillum* spp. survived well in the tomato rhizosphere under conditions that were otherwise detrimental. This indicated that most cells of the 5 Azospirilla strains died in a linear pattern in 2 semi-arid soils, and that only major soil components affected *Azospirillum* survival in soil (Bashan and Vasquez 2000).

Spreading pig waste slurry as organic fertilizer seems to be a good management practice in corn-cultivated agrosystems, as it does not affect the presence of *A. brasilense* in soil. *A. brasilense* was found only in unfertilized and organically amended plots, while fertilization with urea reduced its presence below the detection threshold of a molecular probe (Ceccherini et al. 2001).

All these studies enhance the notion that there is an effect on the community structure of azospirilla in bulk soil. Environmental factors affecting survival are organic matter content and clay fraction, as found in earlier studies. Yet, this does not provide sufficient evidence that azospirilla can pass from one season to the other as a saprophyte and inoculate crops in the next season or unintentionally promote weed growth in subsequent plantings, all fundamental and practical questions for the inoculant industry.

Storage material, poly-β-hydroxyalkanoates (PHA) (aka poly-β-hydroxybutyrate; PHB) and lipid formation and metabolism in relation to survival of the bacterium

Like many prokaryotes, *Azospirillum* spp. produce high levels of intracellular storage substances, such as polyhydroxyalkanoates (previously described as PHB) under nutritional limitation. The C/N ratio in the bacterial growth medium and oxygen partial pressure, are the governing factors for concentrations of PHA in the cell. Under N_2-fixation and without mineral N, the bacteria can accumulate up to 75% of the cell dry wt as PHA. This energy and carbon storage compound is then used under stress conditions, such as limitations of carbon and energy, a capacity that might enhance the competence of *Azospirillum* in the rhizosphere, which is desirable in commercial agricultural inocula (for review, see Bashan and Holguin 1997*a*; Bashan 1998). Not all *Azospirillum* strains produced PHA. Out of 49 isolates of *Azospirillum* from West Bengal, only 13 were PHA producers. The majority of these belonged to *A. brasilense* and a few belonged to *A. amazonense* and *A. lipoferum*. When grown in a medium with nitrogen, the PHA content of these strains ranged from 1% to 14% of dry cell mass (Manna et al. 1997). Three enzymes and their genes that are essential in the PHA biosynthetic and degradation pathways were identified in *A. brasilense* Sp7. A PHA^- mutant of *A. brasilense* decreased its starvation resistance compared with the parent strain, but otherwise, motility, cell aggregation, root attachment, and EPS and capsular polysaccharide production were superior to the wild-type (Kadouri et al. 2002; Edelshtein et al. 2003).

In normally grown *A. brasilense* Cd cells, 2 unsaturated fatty acids, octadecanoate (18:1 *cis*-9) and hexadecanoate (16:1 *cis*-9), accounted for approximately 80% of the fatty acid content in *A. brasilense* Cd. The major lipids were phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine. Flocculation (creation of massive cell aggregates) resulted in the de novo synthesis of a glycolipid

and cardiolipin. Flocculation also resulted in a decrease in cellular proteins and lipids and a proportional increase in cellular PHA and carbohydrates. PHA in flocculated cells reached 60%–65% of dry cell wt (Olubayi et al. 1998).

Genes involved in poly-β-hydroxybutyrate (PHB) synthesis and degradation

With oxygen limitation or high C/N ratio, *Azospirillum*'s vibrioid cells can develop into round encapsulated forms (called cyst-like or C-forms) that are rich in PHB (Itzigsohn et al. 1995). Recent findings support the suggestion that *Azospirillum*'s C-forms might prevail over cells without PHB in water and nutrient deficient soils (for review, Bashan and Holguin 1997*a*). Kadouri et al. (2002) cloned and sequenced the 3 essential genes in the PHB biosynthetic pathway: *phbA* (β-ketothiolase), *phbB* (acetoacetyl coenzyme A reductase), and *phbC* in *A. brasilense* Sp7. Construction of an *A. brasilense phbC* mutant obtained by insertion of a kanamycin-resistance cassette within the *phbC* gene (encoding for PHB synthase) revealed that the wild-type endured starvation, ultraviolet irradiation, heat, osmotic pressure, osmotic shock, desiccation, and growth in hydrogen peroxide better than the mutant. However, the mutant showed increased motility, cell aggregation, root adhesion, and EPS and capsular polysaccharide production, compared with the wild-type. The wild-type exhibited greater chemotactic response towards attractants. Synthesis and utilization of PHB as the carbon and energy source under stress might favor establishment and survival of *A. brasilense* in competitive environments (Kadouri et al. 2002, 2003), but so far shows no advantage in root colonization (Kadouri et al. 2003).

A. brasilense Sp7 *ntrBC* double mutants and *ntrC* mutants, produced by Tn*5* insertion, grew and accumulated PHB simultaneously in medium with high ammonia concentration. Since ammonia is critical to decoupling cell growth and PHB production, these results suggest that *ntrB* and *ntrC* genes are involved in synthesis regulation of PHB by ammonia in *A. brasilense* Sp7 (Sun et al. 2000). They found that the *A. brasilense* Sp7 *glnBglnZ* double mutant produced more PHB than the wild strain during active growth with excess nitrogen. A *glnD* mutant accumulated even more PHB during the growth phase than the *glnBglnZ* double mutant. These results suggest that the P_{II}-P_Z system is involved in nitrogen-dependent regulation of PHB synthesis in *A. brasilense* Sp7 (Sun et al. 2002).

Ecology of azospirilla and its interactions with soil microorganisms

It is well documented that azospirilla are mainly rhizosphere bacteria, and with the exception of some strains capable of living in bulk soil or as endophytes, most survive poorly in bulk soil (Bashan 1999). Yet, environmental factors and other native soil microorganisms influence and interact with the inoculant.

Tn5-lacZ-tagged *A. brasilense*, capable of growing at suboptimal temperatures, was compared with the parental strains. While the mutants fixed nitrogen, produced IAA and siderophores, and could colonize and promote wheat growth similar to the parental strains on their own, co-inoculation of the mutants with their respective parental strains showed that their colonization potential was very negatively affected (Rajeev et al. 2000). However, 2 *A. brasilense* strains, capable of both growth promotion and growth at a suboptimal temperature of 22 °C, were better inoculants for wheat than the regular, medium-high temperature strains (Kaushik et al. 2002). This indicates that strains capable of growing at low temperatures and producing plant hormones are perhaps a better choice for wheat cultivation.

The positive relationship between nitrogenase activity and N_2-fixing populations in the top layers of 4 flooded tropical rice soils was demonstrated. Application of organic sources (cellulose and rice straw) to the topsoil (1–2 cm) produced higher nitrogenase activity associated with larger populations of *Azospirillum* sp., irrespective of soil type (Kanungo et al. 1997). Microbiological analysis of field-grown rice indicated that cultivars with high nitrogen absorption efficiency harbored higher populations of N_2-fixing *Azospirillum* sp., *Azotobacter* sp., and anaerobic bacteria, compared with low nitrogen absorbing cultivars. Notwithstanding, the effect of nitrogen fertilizer management on N_2-fixation, nitrogen absorption efficiency of rice and the rhizosphere-associated nitrogenase activity and N_2-fixing bacteria were apparently interrelated (Kanungo et al. 1998). N_2-fixation capacity of 285 strains of *Azospirillum* isolated from soils from 7 geographic regions in Australia varied from zero or negligible nitrogenase activity compared with the normal activity of *Azospirillum*. Most of the isolates, when associated with wheat roots, reduced between 1–5 nmol C_2H_4·(mg dry root)$^{-1}$·d and some strains showed considerably higher activity. The majority of these strains were *A. lipoferum*. N_2-fixation on wheat roots did not correlate well with N_2-fixation in pure culture. Some strains that fixed nitrogen vigorously in pure culture had low rates of N_2-fixation on roots, and vice versa, where isolates of *A. lipoferum* had a higher average nitrogenase activity than *A. brasilense* in nitrogen-free medium and in association with wheat roots (Han and New 1998*a*, *b*).

Azospirillum spp. were found associated with natural arid vegetation of the North Sinai (Egypt). *Azospirillum* spp., and *Pseudomonas* spp., presumably dominating the diazotrophic community, were identified according to morphological, cultural, and physiological characteristics of the isolates. Acetylene-reducing activities of these isolates were highest with *A. brasilense* (Sedik 1997). Similarly, *Azospirillum* was found to share with *Herbaspirillum* and *Acetobacter diazotrophicus* the rhizosphere, and the interior of sugarcane *Saccharum officinarum* stems (Indira and Bagyaraj 1997).

Azospirillum is usually inoculated into natural soils containing a multitude of microorganisms with a wide range of characteristics and microfauna. Interactions with some of them are inevitable. Effects of a mixed inoculant of *Azospirillum* spp. and several AM fungi (*Glomus mosseae*, *G. deserticola*, and natural AM fungi from the test soil) upon mycorrhizal colonization in maize plants were evaluated for enzymatic activities (esterase, phosphatase, trehalase, and chitinase). The enzymes were indicators for detecting changes in the microbial functional groups in the soil. *Azospirillum* mixed with the AM fungi showed no neg-

ative effects on AM fungal establishment. As a result of mycorrhizal colonization and microbial inoculation, modifications of the microbial community structure and ecology were induced (Vázquez et al. 2000).

Anchoring of PGPB to AM fungal structures may have special ecological and biotechnological significance because it may facilitate colonization of new rhizosphere by the bacteria traveling on or moving with fungal hyphae, and may be an essential trait for development of mixed inocula. Mutants of *A. brasilense* deficient in EPS were shown to be strongly impaired in their capacity to attach to AM roots and AM fungal structures (Bianciotto et al. 2001). When barley was inoculated with the AM *Glomus intraradices*, addition of *A. lipoferum* 137 and the phosphate-solubilizing bacteria, *Agrobacterium radiobacter* and *Flavobacterium* sp., did not affect the intensity of development of mycorrhizal structures in the roots. However, while mycorrhization of the plants was conducive for better establishment of *A. lipoferum* 137 in the rhizoplane, it significantly decreased root colonization of *Flavobacterium* sp. (Belimov et al. 1999). Addition of *A. brasilense* to the AM fungi (*Hebeloma crustuliniforme* and *Pisolithus tinctorius*) used as a biocontrol inoculant for pine seedlings against the pine pathogens, *Rhizoctonia solani* and *Fusarium oxysporum*, increased fungal biomass and the shoot/root ratio of the seedlings (Dahm et al. 1998). Synergistic and antagonistic effects on AM fungi (*G. fasciculatum*) co-inoculated with *A. brasilense* or *Rhizobium meliloti* on the photosynthesis in alfalfa plants might serve as a parameter to measure the effect of the microbial activity and their possible beneficial effects in the field. Beneficial effects of AM on photosynthesis were clearly revealed as enhancement of the electron transport activity per leaf area. When AM was co-inoculated with one of the PGPBs, the antagonistic effect of *A. brasilense* was higher than that of *R. meliloti*, though not strong enough to fully counterbalance the beneficial effect of AM. However, in the case of triple inoculation with both PGPB and AM, electron transport was found to be only slightly lower than in the case of single inoculation by AM, indicating that, in the presence of each other, PGPBs are no longer antagonistic to AM (Tsimilli-Michael et al. 2000). Two naturally occurring bacterial endophytes of rice seeds, *Corynebacterium flavescens* and *Bacillus pumilus*, competed with inoculated *A. brasilense* and excluded it from the rhizoplane (Bacilio-Jiménez et al. 2001). Mixed cultures of *A. brasilense* Sp7 and *Penicillium corylophillum* allowed the efficient utilization of pectin by the fungus (93% pectin degradation within 9 d). The degraded components served as a carbon source for N_2-fixation by *A. brasilense*, which is incapable of degrading pectin (El-Katatny et al. 1997).

Azospirillum in the soil could indirectly fall prey to soil macrofauna. *A. brasilense* was introduced into oak leaf litter, or soil containing litter-dwelling diplopods and isopods or earthworms. In the presence of these animals, the number of bacteria in excrement was always 2–10 times lower than in food. Digestive fluid taken from the middle part of the gut of the diplopod *Pachyiulus flavipes* showed a strong antibacterial activity, indicating that *A. brasilense* appeared to be sensitive to digestion by the midgut fluid (Byzov et al. 1997).

All these studies add evidence for the rhizocompetence of *Azospirillum* as rhizosphere bacteria. Its capacity to accumulate large amounts of PHA, massively aggregate and flocculate, and produce cyst-like forms under stress conditions show that *Azospirillum* has a potential as a formidable competitor in its ecological niche, the rhizosphere. Yet, its interaction with other soil microorganisms is unclear, and the available information allows only speculations, at best.

Stresses

Salt stress

Numerous cultivated soils worldwide are becoming more saline mainly from the use of marginal irrigation water, from excess fertilization, and various desertification processes. Inoculation with *Azospirillum* sp. under saline stress conditions is therefore commonplace. Prior findings showed that common agricultural *Azospirillum* strains tolerated high salinity (≤2%). Salt resistance among species increased from *A. amazonense* (lowest) to *A. halopraeferens* (highest), the latter tolerating over 3% NaCl (seawater salinity). The common cellular mechanism of osmotic stress adaptation is intracellular accumulation of organic solutes (osmolytes). In sorghum, inoculation with *A. brasilense* diminished the adverse effects caused by osmotic stress (for review, see Bashan and Holguin 1997*a*).

More recent and detailed confirmatory in vitro studies demonstrated that *A. brasilense* Cd can tolerate up to 200 mmol/L NaCl in the medium without appreciable decline in growth. Higher concentrations of salt caused inhibition of bacterial growth. At 300 mmol/L NaCl, growth decreased 66% after 24 h. After 48 h at this concentration, bacteria reached maximum optical density, comparable to the optical density of control cultures after 12 h. *A. brasilense* responded to saline stress by elevating intracellular concentrations of glutamate at 24 h and K^+ at 48 h. Although several cellular functions are affected by saline stress, it seems that *A. brasilense* Cd has remarkable tolerance for saline conditions (Rivarola et al. 1998). As already known, *Azospirillum* spp. can accumulate compatible solutes, such as glycine betaine, glutamate, proline, and trehalose, to allow adaptation to fluctuations in soil salinity/osmolarity. Addition of these osmo-protectants to bacterial cultures under saline stress usually increased cellular growth and nitrogenase activity (Choi and Gal 1998; Tripathi and Mishra 1998*a*). As inhibition of *A. lipoferum* growth by NaCl was relieved by exogenous glycine betaine, this suggested that *A. lipoferum* has a salinity-induced glycine betaine transport system (Tripathi and Mishra 1998*b*). Additionally, proline seems to play a major role in osmoadaptation. With increases in osmotic stress, the dominant osmolyte in *A. brasilense* shifted from glutamate to proline. Accumulation of proline occurs by uptake and synthesis. At higher osmolarity, *A. brasilense* Sp7 accumulated a high intracellular concentration of glycine betaine that was taken up via the glycine betaine transport system, probably similar to that of *A. lipoferum*. Except for *A. halopraeferens*, all other species of *Azospirillum* lacked the ability to convert choline to glycine betaine. Mobilization of the *betABT* genes

of *E. coli* in *A. brasilense* enabled it to use choline for osmo-protection (Tripathi et al. 1998).

Saline stress alters *A. brasilense* Cd maize and wheat interactions, normal colonization, and N_2-fixation. Saline stress altered the early stages of plant development, which led to inadequate colonization and expression of *A. brasilense nif* gene promoters. While *nifA* expression increased in stressed bacteria, *nifH* transcription was diminished (Jofré et al. 1998*a*, *b*). Yet, *A. halopraeferens* and *A. brasilense* were capable of colonizing roots of mangrove trees in seawater (Puente et al. 1999). Attachment of *A. brasilense* Cd to maize and wheat roots was altered when the bacteria were grown under saline stress. Alteration in the adsorption phase of attachment appeared to be related to the disappearance of a 100 kD external membrane protein in the bacterium. Gene expression in *A. brasilense* was influenced by the presence of plant root exudates; wheat root exudates induced the reappearance of the 100 kD protein (Fischer et al. 1999). Unstressed bacteria grown under standard conditions were distributed over the entire root system of wheat, except the elongation zone. Bacteria subjected to saline stress were mainly found at the root tips and the lateral roots. Interestingly, salt treatment reduced surface colonization, but not colonization inside the root (Fischer et al. 2000). *Azospirillum* inoculation at NaCl concentrations up to −1.2 MPa significantly increased chlorophyll, K, Ca, soluble saccharides, and protein contents as compared with control plants growing without NaCl (Hamdia and El-Komy 1997), similar to alleviating water stress on wheat plants grown under drought conditions (El-Komy et al. 2003). Inoculating *A. brasilense* on wheat seedlings exposed to severe salt (NaCl) or osmotic (polyethylene glycol) stresses significantly reversed part of the negative effects; both stresses reduced relative elongation rate of shoots. Fresh wt, fresh wt/dry wt, water content, and relative water content were higher in shoots from inoculated plants than in stressed controls (Creus et al. 1997). Turgor pressure at low water potential was higher in inoculated seedlings in 2 wheat cultivars under osmotic stress. This could result from better water uptake as a response to inoculation that, in turn, is reflected by faster shoot growth in inoculated seedlings exposed to these stresses. These results are similar to what was found in water-stressed wheat inoculated with *Azospirillum*. They showed better water status and effects on cell wall elasticity and (or) apoplastic water (Creus et al. 1998).

Soil salinity plays a major role in diversity of indigenous azospirilla associated with rice along the coast of India. All the strains detected were *A. brasilense* or *A. lipoferum*. Decreasing diversity of *Azospirillum*, probably from higher salinity, is apparently more important than the clay fraction of the soil, a factor demonstrated as having a major role in several studies (see above). If true, this association between soil salinity and range of *Azospirillum* should be considered when developing inoculants, especially for coastal salt-affected agricultural areas (Saleena et al. 2002).

The effects of salt on *Azospirillum*-plant interactions is a major factor to be considered in applied studies. In spite of its importance, meagre molecular studies have been conducted on the bacterium per se, and almost none as to interactions with plants. The most fundamental omission in current knowledge is uncertainty on whether improved salt tolerance of the bacterium is needed to enhance the bacterium's effect on plants or if existing salt tolerance is adequate to ensure positive growth-promotion by inoculation.

Pesticides

In view of the high input of agrochemicals in contemporary crop production and the likelihood that *Azospirillum* inoculation is and will be used in regular crop production, not solely in organic agriculture, studies on the interactions of *Azospirillum* inoculants with common pesticides are essential. Apparently, as in the past (for review, Bashan and Holguin 1997*a*), this is an unattractive subject of *Azospirillum* field research. Few tests, mostly at a laboratory scale, have been conducted.

Insecticides

The effects of 4 insecticides, bromopropylate, diazinon, methidathion, and profenofos on the cells of *A. brasilense* were evaluated in vitro in chemically defined media. While bromopropylate and diazinon were completely harmless, methidathion at high doses significantly reduced N_2-fixation, intracellular levels of ATP, and growth in cells. However, these negative effects were not significant in cells grown in dialyzed soil media, indicating that *A. brasilense* can potentially tolerate high concentrations (300 µg/mL) of methidathion (Gomez et al. 1998). Similarly, profenofos produced the same negative effect as methidathion, including inhibition of production of several vitamins. The effect was significantly greater in dialyzed soil media (Gomez et al. 1999). The insecticide carbofuran affects nitrogenase activity in vitro, where low levels (2 and 5 µg/mL) significantly stimulated nitrogenase activity, while higher levels (>5 µg/mL) caused significant inhibition. Interestingly, the usual inhibitory effect of combined high nitrogen levels on nitrogenase diminished gradually in the presence of carbofuran over a 10-day incubation period (Kanungo et al. 1998).

Of 4 insecticides (carbofuran, chlormephos, terbufos, and benfuracarb) currently used on maize at the time of sowing, only terbufos had a slight effect on growth of *A. lipoferum* in solid medium cultures. Physical contact of the insecticides with the commercial inoculant (Azogreen-m), bacteria inoculated directly on insecticide granules, or inoculant mixed with an insecticide all decreased survival of *A. lipoferum* (Revellin et al. 2001).

Acaricides

A. lipoferum survived high concentrations of the organochlorine acaricide dicofol (1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethanol). The bacteria accumulated the acaricide in the cell envelope connected to neutral lipids and phospholipids of membranes (Mano and Langenbach 1998).

Fungicides

Three fungicides (carbendazim, thiram, and Bordeaux mixture) and 2 insecticides (carbofuran and phorate) were evaluated for their effect on *Azospirillum* sp. in cowpea in vitro and in the field. Only thiram caused growth reduction of the bacterium in vitro. In the field, the rhizosphere bacterial population was reduced in thiram-treated plants, but not in plants treated with the other chemicals. A mix of thiram and insecticides reduced the *Azospirillum* population, but a

gradual bacterial build-up was observed in the rhizosphere following the treatment (Raji and Pillai 2000).

Herbicides

Cotton plants could be protected from harmful effects of 2,4-D by inoculation with *A. brasilense*. The 2,4-D-degrading plasmid pJP4 was transferred into *A. brasilense* Sp7. Transconjugants degraded 2,4-D in pure culture via co-metabolism at concentrations up to 50 µg/mL. However, when the transconjugants were inoculated on cotton seeds, the plants were resistant only to low levels of the herbicide (0.5 µg/mL), which is not sufficient for protection of the cotton plant. Plants growing in soils with this concentration of herbicide, and inoculated with wild-type strains, died (Feng and Kennedy 1997).

Toxic metals and antibiotics

Five strains of *Azospirillum* spp. were able to tolerate chromium to levels of 1000–1400 µg/mL in vitro (Govindarajan 1999). *Azospirillum* spp. are highly sensitive to heavy metals; the order of toxicity is Hg > Cd > Pb. A mixture of these heavy metals (at 5 µg/mL each) was more toxic to *Azospirillum* cultures than the individual metals (Subramaniane and Govindarajan 1998). The effect of heavy metals on 3 strains of *Azospirillum* was evaluated. At concentrations of 10 and 100 mg/L, Cd^{+2}, Zn^{+2}, and Cu^{+2} inhibited growth and biomass of *Azospirillum* in media containing these metals. At a concentration of 500 mg/L, growth was completely inhibited (Strzelczyk et al. 1997). In the presence of 50 µmol/L $CdCl_2$, inoculation with *A. lipoferum* of barley seedlings partly decreased Cd toxicity, possibly through the improvement of mineral uptake. Cadmium causes severe inhibition of growth and nutrient uptake in barley plants. Additionally, inoculation with the bacteria slightly enhanced root length and biomass of the seedling treated with Cd, and the amount of nutrients absorbed by the inoculated plants increased significantly. There was only some protection against Cd toxicity, but no uptake of Cd since Cd content in the inoculated plants was unchanged (Belimov and Dietz 2000). *A. lipoferum* was found to be naturally resistant to antibiotics of the penicillin and cephalosporin families (Boggio and Roveri 2003).

In summary, the data available from the onset of research on *Azospirillum* preclude a firm conclusion about the interactions of this bacterium with pesticides.

Compost

Some composts may be toxic to plant growth because of elevated humic acids or inappropriate preparation. Inoculation of wheat seeds with *A. brasilense* or *A. lipoferum* prior to sowing in soil amended with 2 types of composts improved seed germination and development of plants. The bacteria possibly changed the humic acids since either bacteria could survive and grow in high humic acid solution as the sole source of carbon, modifying the composition during in vitro tests (Bacilio et al. 2003).

Inoculants and carriers

An *Azospirillum* inoculant is a formulation containing 1 or more bacterial strains or species in an easy-to-use and economical carrier material, either an organic material, or a synthesis of defined molecules. The inoculant is the means of bacterial transport from the factory/laboratory to the living plant. The inoculant formulation has a crucial effect on the inoculation process because the chosen formulation determines potential success of the inoculant (Bashan 1998). Limited development of inoculants involving *Azospirillum* has occurred in recent years.

Organic undefined carriers

Traditional turf/peat carriers (Bashan 1998) continue to be evaluated. Survival time of *A. brasilense* in solid turf used as a carrier for seeds of different grasses revealed that bacterial concentrations in the carrier during the first 30 d was 10^8 CFU/g inoculant and decreased after 60 d to 10^7 CFU/g, staying at this level up to 4 months. This level is sufficient, albeit marginal, for successful plant inoculation (Garcia and Sarmiento 2000).

Sugar industry byproducts (pressed-mud and surplus bagasse) were composted, using vermicomposting techniques and then used as a carrier for *Azospirillum* spp. The vermicompost sustained higher bacterial populations than lignite, which is more commonly used in India as a carrier, and had superior physicochemical properties (greater density, porosity, water-holding capacity, pH, and a lower C to N ratio (Muthukumarasamy et al. 1997). Cultivation of *Azotobacter chroococcum*, *A. lipoferum*, and the phosphate-solubilizing *Pseudomonas striata*, together with rock phosphate in vermicompost, produced a superior final product, with increased levels of N and available P. However, it is not known whether this product is a better inoculant than peat carriers (Kumar and Singh 2001). Sawdust composted by the cellulose-decomposing fungus (*Cephalosporium* sp.) and *A. brasilense* was evaluated as a possible carrier for *Bradyrhizobium*, *Rhizobium*, and *Azospirillum*. The carrier supported good growth and survival of 3 PGPB species. Yield increases following crop inoculation, with the carrier containing a mixture of 3 PGPBs, were observed on soybean, groundnuts, lucerne, and a grass mixture of bird's foot trefoil and ryegrass (Kostov and Lynch 1998).

Organic defined carriers

A method of inoculating seeds with *A. brasilense* immobilized in wet and dried alginate microbeads (100–200 µm diameters) was developed. Although the process of entrapping the bacteria killed part of the population, bacteria residing in the microbeads were sufficient (>10^{11} CFU/g inoculant) for seed inoculation. Microbeads could be used either wet or dry. Dry inoculant was produced using dry air at 38 °C, which created a powdery substance loaded with >10^9 CFU/g beads. Alternatively, dry microbeads were produced using a standard freeze-drying procedure. This dry preparation was easily attached to the surface of dry seeds with the addition of organic adhesives. Bacteria were slowly released from the microbeads in amounts ranging from 10^4 to 10^6 CFU/g, depending on the type (wet or dry, with or without skimmed milk) of carrier and duration of incubation (smaller amounts of bacteria were released over time). Wet and dry inoculants enhanced development of wheat and tomato seedlings growing in infertile soil, and biodegraded within 15 d in moist soil in greenhouse pots (Bashan et al. 2002). Survival of *Azospirillum* spp. in alginate beads can last for very long pe-

riods. An *A. brasilense* Cd inoculant, produced in 1983 and dried and stored at ambient temperature, was recovered in 1997. The population had decreased, yet significant numbers survived (10^5–10^6 CFU/g beads). This *A. brasilense* Cd retained several of its original physiological features. When inoculated on wheat plants, it colonized and produced plant growth effects equal to those of the contemporary strain (Bashan and Gonzalez 1999).

Non-organic carriers

An attempt to substitute the peat-based inoculant with gamma-irradiated vermiculite as a carrier for *Azospirillum* apparently failed. Survival and shelf life quality control tests revealed that the number of viable *Azospirillum* cells in vermiculite decreased gradually to reach 1.3×10^7 viable cells·(g dry wt)$^{-1}$ carrier at the end of 44 weeks of storage. Shelf life of an *Azospirillum* vermiculite inoculant was only 20 weeks at ambient temperature, in comparison to 44 weeks for a peat-based inoculant (Saleh et al. 2001). A granular clay inoculant containing AM fungus (*Glomus fasciculatum*), *Azospirillum*, and phosphate solubilizing bacteria (PSB) for easy application in nurseries had a shelf life of up to 60 d (Lilly and Santhanakrishnan 1999).

As not much attention has been placed on superior formulations of azospirilla, this domain has been left to the inoculant industry, which has published few results concerning inoculant formulation since the onset of *Azospirillum* research. As such, and because there are commercial inoculants of *Azospirillum*, albeit to a limited extent, it seems that this technological section of *Azospirillum* is not, and probably will not be, in the public domain.

Concluding remarks and possible lines of research in the future

A critical analysis of the state-of-the-art of *Azospirillum* applications reveal the following general conclusions: the major problems of inconsistent yield response by single inoculations and a suitable and practical delivery system are not solved, as yet. At the same time, there is a proliferation of studies on the role of *Azospirillum* inoculants mixed with other microorganisms as an inoculant consortium. In view of the numerous field successes reported in the last decade (Bashan and Holguin 1997*a*, *b*, Table B1), the short-term goal of *Azospirillum* applications in agriculture should focus on this issue.

Azospirillum was discovered as a growth promoter of cereals. Because much *Azospirillum* research is still conducted on cereal, the perception of *Azospirillum* as a PGPB for cereals is still widespread. This ignores the multitude of studies in which *Azospirillum* affected and promoted the growth of numerous other species, including trees, cacti, microalgae, vegetables, fruit, flowers, medicinal plants, and spices. Over 100 crops and environmentally important species are reported to respond to inoculation with *Azospirillum*. In view of the amount of information on other plant species, we propose that *Azospirillum* be considered a non-specific plant-growth-promoting bacterium.

Azospirillum inoculants today are used mainly for cereals, mostly in developing countries. By default, this market dictates that the inoculant must be as cheap as possible. The cost of developing novel inoculant materials quickly moves the price out of practical range for agriculture, especially in developing countries. However, there are several high-value specialty crops, such as flowers, fresh vegetables, fruit, and general organic farming, where chemicals are undesirable and *Azospirillum* is known to have an effect, even though much of the results are experimental. Success in these markets might sustain *Azospirillum* inoculation technology until a cheap solution for *Azospirillum* inoculation of major crops (wheat, rice, corn, and barley) can be found. High input greenhouse crops are an additional candidate for commercial *Azospirillum* inoculants. The cost of inoculation should not unduly increase the economic burden on the grower. These markets are an opportunity for novel *Azospirillum* inoculants.

In agricultural practices, contemporary synthetic inoculants are frequently too expensive, and therefore, there is reluctance by agro-industry to develop them. However, the bioremediation industry is continuously supporting development of advanced inoculants. In emergencies, novel synthetic, efficient inoculants will undoubtedly be used for bioremediation processes, regardless of the cost. This trend may provide *Azospirillum* inoculation technology a strong foothold for new inoculant materials and formulations. A wider use of encapsulated microorganisms in nonagricultural applications may help these materials become cost competitive for *Azospirillum*, which is currerntly not the case.

Of all bacterial genera associated with plant roots, *Azospirillum* is the most studied. The bacteria's plant growth promotion mechanisms are only partially understood. The main handicap is lack of a clear plant phenotype that appears after inoculation, like nodules in rhizobia. Consequently, screening of large numbers of isolates or mutants is necessary.

There has been a proliferation of studies on the physiology and molecular biology of *Azospirillum* in an effort to circumvent the absence of a clear plant phenotype. Many genes involved in PGPB interactions such as N_2-fixation, hormone and polysaccharide production, chemotaxis, and motility genes, were identified, cloned, and studied intensely, irrespective of direct effects on plants after *Azospirillum* inoculation, a fact that so far contributes only marginally to producing a better inoculant. Most studies on *Azospirillum* genetics have focused on nitrogen metabolism. However, a genetically manipulated "super bacterium" that provides plenty of nitrogen to the plant by fixing more N_2 or excreting ammonium without compromising its fitness, is still far from development.

Most genetic and molecular tools function well with *Azospirillum*. For example, reporter genes like *gfp*, *gusA*, and *lacZ*, an assortment of immunological methods combined with laser microscopy, and specific immuno-gold labeling in electron microscopy identify and track *Azospirillum* on roots. These powerful tools trace even small numbers of bacteria, and differentiate between strains of the same species. One doubts if there is need to develop more detection methods.

As molecular knowledge of *Azospirillum* significantly increases, researchers are tempted to create transgenic plants expressing bacterial genes, as has already been done for several plants. Transfer of the nitrogenase enzyme to plants seems impractical for the foreseeable future because of the complexity of nitrogenase biosynthesis and the necessity of

providing appropriate physiological environments for enzymatic activity. However, for *Azospirillum* spp. affecting plants through other mechanisms, there is a theoretical option of engineering plant species (containing other bacterial genes), as has been done for a few other bacterial genes introduced into plants. Despite this, the major question is whether it is practical to consider the introduction of *Azospirillum* genes into the plant. Based on our current knowledge of this bacterium's physiology, the answer is probably, "not yet". The success achieved so far in developing transgenic plants tends to indicate that obstacles could eventually be solved if the desired genetic trait is expressed by 1 gene or a small cluster of genes. If the chosen genetic trait involves several clusters of genes (as in the case of N_2-fixation genes), and these are subjected to elaborate controls, the probability of success is difficult to predict. Furthermore, the Additive Hypothesis (see earlier commentary), suggesting involvement of multiple mechanisms employed by the bacteria to affect plant growth, implies it would be pointless to introduce individual genes responsible for only 1 of the several mechanisms involved in a growth process. In conclusion, currently, our opinion is that the best approach is to try to understand mechanisms by which *Azospirillum* promotes plant growth. Once this happens, the transfer of *Azospirillum* genes into plants might be considered.

The following areas deserve attention for future research. These issues are raised to stimulate thought and research on *Azospirillum*.

(*i*) Establish biological markers for the interaction between *Azospirillum* and other microorganisms (especially AM fungi and PSB) to select the most compatible combinations for plant inoculation.

(*ii*) Explore the use of *Azospirillum* for solving environmental problems not related to agriculture, such as purifying toxic effluents and urban wastewater and preventing soil erosion.

(*iii*) Explore the possibility of gaining nitrogen from N_2-fixation, although many studies have shown negligible incorporation of nitrogen into plants. This could be done by further testing of the nutritional requirements of *Azospirillum* and the artificial system of paranodules where nitrogen is transferred to the plant. Construct strains that provide more nitrogen to plants, through N_2-fixation or excretion of ammonium, without compromising the fitness of the strains. Determine if N_2-fixation becomes crucial to the survival and growth of a plant under certain environmental conditions, such as nitrogen deficiency.

(*iv*) Use accumulating knowledge on *Azospirillum* physiology to produce industrial products based on its physiological features, but not related to agriculture, such as PHA, enzymes and vitamins, and the breakdown of polymers like cellulose in combination with cellulolytic bacteria.

(*v*) Develop cheap synthetic carriers of inoculants as substitutes for organic and mineral carriers that have proved less efficient for *Azospirillum*. Without a practical means of delivery, no PGPB (including *Azospirillum*) will be commercially successful.

(*vi*) Intensify study of the effects of stress (pesticides, salt, water shortage, toxic materials), which are currently in the backwater of *Azospirillum* research. Successful applications and research in these areas are progressing slowly, and most studies are conducted under in vitro conditions. Research in these areas is definitely needed, as *Azospirillum* is tested under stressed environmental conditions.

(*vii*) Determine which physiological features of the bacterium have a role in microbe-plant association that are for self-functioning only and that are involved in both.

(*viii*) Study the role of each IAA pathway in the plant–bacterium interaction. Determine the environmental conditions and processes that regulate known IAA pathways. Determine conditions that regulate *ipdC* gene expression.

(*ix*) Determine the role of gibberellins in plant-*Azospirillum* interaction.

(*x*) Use microarrays and proteomics to elucidate the "cross talk" among *Azospirillum*, plants, and other rhizosphere bacteria.

(*xi*) Investigate how root exudates trigger or silence bacterial genes involved in plant–bacteria interaction.

(*xii*) Study field survival and plant growth promotion of *Azospirillum* ACC deaminase transformants.

(*xiii*) Investigate the role of polysaccharides and lectins in *Azospirillum* root attachment.

(*xiv*) Understand the role of *Azospirillum* aggregation in its life cycle and in the plant–bacteria interaction.

(*xv*) Clarify whether inoculated bacteria have the capacity to survive from one growing season to the other.

(*xvi*) Focus attention on the plant part of the interaction. While the bacterial part is extensively studied, plants are not, partly because many plants species respond positively to inoculation; hence, diluting research efforts. There are very few molecular studies involving plant genes participating in the interaction.

In summary, this review emphasized the central issues of *Azospirillum* research today, such as physiological and molecular studies as a general model for rhizosphere bacteria, co-inoculation with other microorganisms, hormonal studies and re-consideration of nitrogen contribution by the bacteria under specific environmental conditions, proposed *Azospirillum* as a non-specific plant-growth-promoting bacterium, re-introduced the Additive Hypothesis suggesting involvement of multiple mechanisms employed by the bacteria to affect plant growth, showed the less researched areas such as inoculant and pesticide research, and proposes possible avenues for the exploitation of this bacterium in environmental areas other than agriculture. Given the current knowledge of *Azospirillum*, the most studied plant-growth-promoting bacterium lacking major biocontrol activity, the prospects of using it for the benefit of human societies are greater than for other PGPBs. Some important agronomic applications will likely become available in the immediate future.

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Appendix A and B:

Appendixes appear on the following page.