Recent advances in nitrogen-fixing acetic acid bacteria

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Abstract

Nitrogen is an essential plant nutrient, widely applied as N-fertilizer to improve yield of agriculturally important crops. An interesting alternative to avoid or reduce the use of N-fertilizers could be the exploitation of plant growth-promoting bacteria (PGPB), capable of enhancing growth and yield of many plant species, several of agronomic and ecological significance.

PGPB belong to diverse genera, including *Azospirillum*, *Azotobacter*, *Herbaspirillum*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, and *Gluconacetobacter*, among others. They are capable of promoting plant growth through different mechanisms including (in some cases), the biological nitrogen fixation (BNF), the enzymatic reduction of the atmospheric dinitrogen (N_2) to ammonia, catalyzed by nitrogenase.

Aerobic bacteria able to oxidize ethanol to acetic acid in neutral or acid media are candidates of belonging to the family *Acetobacteraceae*. At present, this family has been divided into ten genera: *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Saccharibacter*, *Swaminathania*, *Neoasaia*, and *Granulibacter*. Among them, only three genera include N_2-fixing species: *Gluconacetobacter*, *Swaminathania* and *Acetobacter*.

The first N_2-fixing acetic acid bacterium (AAB) was described in Brazil. It was found inside tissues of the sugarcane plant, and first named as *Acetobacter diazotrophicus*, but then renamed as *Gluconacetobacter diazotrophicus*. Later, two new species within the genus *Gluconacetobacter*, associated to coffee plants, were described in Mexico: *G. johannae* and *G. azotocaptans*. A salt-tolerant bacterium named *Swaminathania salitolerans* was found associated to wild rice plants. Recently, N_2-fixing *Acetobacter peroxydans* and *Acetobacter nitrogenifigens*, associated with rice plants and Kombucha tea, respectively, were described in India.

In this paper, recent advances involving nitrogen-fixing AAB are presented. Their natural habitats, physiological and genetic aspects, as well as their association with different plants and contribution through BNF are described as an overview.
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Keywords: Acetobacteraceae; Nitrogen fixation; *Gluconacetobacter diazotrophicus*; *G. johannae*; *G. azotocaptans*; *Swaminathania salitolerans*; *Acetobacter peroxidans*; *A. nitrogenifigens*

1. Introduction

Nitrogen is an essential plant nutrient and, in agriculture, fertilization with nitrogen products is widely and increasingly practiced to increase the production yield of food (Reinhold-Hurek and Hurek, 2003). However, the use of elevated doses of fertilizers, as well as pesticides, may have negative and unpredictable effects on the environment, and contribute to the contamination of soil, water and natural areas. Such impacts pose a serious threat to human and animal health. In addition, developing countries have to face the demand of high costs for such technology and chemical utilization. An interesting option for decreasing the use of chemical fertilizers, could be the exploitation of plant growth-promoting bacteria (PGPB). These bacteria may provide a natural and harmless means to improve the growth and yield of crops ,thereby minimizing the use of agrichemicals.

PGPB are defined as free-living soil, rhizosphere, rhizoplane, endophytic, and phylosphere bacteria that, under certain conditions, are beneficial for plants (Bashan and de-Bashan, 2005). They are capable of promoting plant growth through different mechanisms, such as biological nitrogen fixation (BNF), phytohormone production, phosphate solubilization and siderophore production.

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The biological reaction that counterbalances the loss of nitrogen from soils or agroecosystems is the BNF, which is the enzymatic reduction of the atmospheric dinitrogen (N_2) to ammonia, catalyzed by nitrogenase. The global contribution of BNF is estimated between 200 and 300 million tons of fixed N per year, including terrestrial and marine systems (Galloway et al., 1995; Karl et al., 2002). This process is unique to *Bacteria* and *Archaea*, and the microorganisms that fix nitrogen are named diazotrophs. Those microbes that fix nitrogen independent of other organisms are named free-living. The symbiotic relationship between the diazotrophic rhizobia and legumes (e.g. soybean, clover, common bean) can provide large amounts of N to the plant and has a significant impact on agriculture. Associative N_2-fixing microorganisms are those diazotrophs that live in close proximity to plant roots, in the rhizosphere or within the plants (endophytes), and can obtain energy and materials from the plants, giving in turn ammonia from the reduced atmospheric dinitrogen.

PGPB belong to diverse genera including *Azospirillum*, *Azotobacter*, *Herbaspirillum*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, and *Gluconacetobacter*, among others. However, this article, will focus on the nitrogen-fixing acetic acid bacteria (AAB).

2. Nitrogen-fixing bacteria within the family Acetobacteraceae

Historically, aerobic bacteria able to oxidize ethanol to acetic acid in neutral or acid media are candidates for the family Acetobacteraceae (Swings, 1992). At present, this family has been divided into ten genera: *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Acidomonas* (Yamada et al., 1997), *Asaia* (Yamada et al., 2000), *Kozakia* (Lisdiyanti et al., 2002), *Saccharibacter* (Jojima et al., 2004), *Swaminathania* (Loganathan and Nair, 2004), *Neoasaia* (Yukphan et al., 2005) and *Granulibacter* (Greenberg et al., 2007). Among them, only three genera include N_2-fixing species: *Gluconacetobacter*, *Acetobacter* and *Swaminathania*.

The first N_2-fixing AAB was described in Brazil by Dr. Johanna Döbereiner and her research group. It was found inside sugarcane plant tissues and was first named *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988; Gillis et al., 1989), then renamed as *Gluconacetobacter diazotrophicus* (Yamada et al., 1997). Later, two new species within the genus *Gluconacetobacter*, associated with coffee plants, were described in Mexico: *G. johannae* and *G. azotocaptans* (Fuentes-Ramírez et al., 2001).

In 2004, *Swaminathania salitolerans* was described (Loganathan and Nair, 2004). Recently, *Acetobacter peroxydans* and *Acetobacter nitrogenifigens*, associated with rice plants and Kombucha tea, respectively, were described as N_2-fixing AAB in India (Dutta and Gachhui, 2006; Muthukumarasamy et al., 2005).

3. Natural habitat

G. diazotrophicus was first isolated from sugarcane, colonizing inner tissues of roots, stems and leaves (Cavalcante and Döbereiner, 1988). Although *G. diazotrophicus* was originally described as an endophytic species, its natural occurrence in the rhizosphere of different plants has been documented in old and recent articles (Jiménez-Salgado et al., 1997; Loganathan et al., 1999; Muthukumarasamy et al., 2005; Santos et al., 2006). Although *G. diazotrophicus* was described as a species associated with sugar rich plants, it has been found naturally associated with other types of plants, and can be recovered from inoculated, non-sugar rich plants (Jiménez-Salgado et al., 1997; Muthukumarasamy et al., 2002a, 2005; Sevilla and Kennedy, 2000).

Since its first report (Cavalcante and Döbereiner, 1988), *G. diazotrophicus* has been isolated from sweet potato (Paula et al., 1991), *Pennisetum purpureum* (Reis et al., 1994), coffee plants in Mexico (Jiménez-Salgado et al., 1997), field-grown pineapples in Mexico (Tapia-Hernández et al., 2000), a grass called "Finger millet" (*Eleusine coracana* L. Gaertn) (Loganathan et al., 1999), tea plants (roots), mango (fruits), the rhizosphere of banana plants, (Muthukumarasamy et al., 2002a) and from wetland rice (Muthukumarasamy et al., 2005). *G. diazotrophicus* was found also in trash of sugarcane (e. g. senescent leaves left on the ground from the last cut) (Reis et al., 1994) and it was also isolated from a mealy bug (*Saccharococcus sacchari*) associated with sugarcane plants (Ashbolt and Inkerman, 1990). Caballero-Mellado et al. (1995) also found *G. diazotrophicus* associated with mealy bugs. This information suggests that this diazotroph is sucked from sugarcane plants by mealy bugs. While *G. diazotrophicus* is preferably suited to survival and growth inside cane tissues, it does not infect intact sugarcane plants, and barely survives in soil (Baldani et al., 1997).

In contrast to *G. diazotrophicus*, which inhabits inner plant tissues as an endophyte, *G. johannae* and *G. azotocaptans* were only found colonizing the rhizosphere of coffee plants (Fuentes-Ramírez et al., 2001). Very recently, *G. azotocaptans* was isolated from the rhizosphere of corn (Mehnaz et al., 2006).

S. salitolerans, a salt-tolerant, N_2-fixing and phosphate-solubilizing bacterium was isolated from wild rice plants (Loganathan and Nair, 2004). *A. peroxydans* constitutes the first report of a N_2-fixer in the genus *Acetobacter*, and was associated with cultivated wetland rice varieties in India (Muthukumarasamy et al., 2005). Recently, *A. nitrogenifigens*, isolated from Kombucha tea in India, was described as a novel N_2-fixing acetic acid bacterium (Dutta and Gachhui, 2006). Examples of N_2-fixing AAB and the source of isolation are presented in Table 1. Because of its first discovery, most of the literature about N_2-fixing AAB is related to *G. diazotrophicus* (Cavalcante and Döbereiner, 1988); for that reason, most of the information in this overview concerns this species.

4. *G. diazotrophicus*: physiological aspects

The most common physiological characteristics of *G. diazotrophicus* are its high sucrose tolerance (10%), growth and nitrogen fixation at low pH (5.0 or less), chocolate colonies on potato agar medium with 10% sucrose, absence of nitrate reductase, nitrogen fixation not affected by high concentrations of NO_3^- (25 mM) and partial inhibition by NH_4^+, especially at

Table 1
Source of isolation of different genera and species of nitrogen-fixing AAB

Genera and species	Source of isolation	Reference
Gluconacetobacter diazotrophicus	Sugarcane	Cavalcante and Döbereiner, 1988; Li and MacRae, 1991; Fuentes-Ramírez et al., 1993; Caballero-Mellado et al., 1995
	Cameroon grass	Reis et al., 1994
	Sweet potato	Paula et al., 1991
	Coffee	Jiménez-Salgado et al., 1997
	Pineapple	Tapia-Hernández et al., 2000
	Eleusine coracana	Loganathan et al., 1999
	Tea	Muthukumarasamy et al., 2002a
	Mango	Muthukumarasamy et al., 2002a
	Banana	Muthukumarasamy et al., 2002a
	Mealy bugs	Ashbolt and Inkerman, 1990; Caballero-Mellado et al., 1995
	Rice	Muthukumarasamy et al., 2005
Gluconacetobacter johannae	Coffee	Fuentes-Ramírez et al., 2001
Gluconacetobacter azotocaptans	Coffee	Fuentes-Ramírez et al., 2001
	Maize (corn)	Mehnaz et al., 2006
Swaminathania salitolerans	Wild rice	Loganathan and Nair, 2004
Acetobacter peroxydans	Wetland rice	Muthukumarasamy et al., 2005
Acetobacter nitrogenifigens	Kombucha tea	Dutta and Gachhui, 2006

high sucrose concentrations (Boddey et al., 1991; Stephan et al., 1991). It was observed that most nitrogen fixers cease growth when the pH is as low as 5, but *G. diazotrophicus* grows at pH 3 (Burris, 1994). Acid production by *G. diazotrophicus* has an additional value as it can solubilize insoluble P and Zn compounds (Muthukumarasamy et al., 2002a; Madhaiyan et al., 2004), although this feature is reduced when the organism is in contact with pesticides, as reported by Madhaiyan et al. (2006).

Another unique characteristic of this bacterium is its ability to excrete part of the fixed nitrogen into the medium as demonstrated by Cojho et al. (1993) who used an amylolytic yeast (*Lipomyces kononenkoae*), grown in a N-deficient medium, to mimic the plant needs. They observed that this bacterium could supply more than 40% of the fixed nitrogen required by the yeast. This observation suggests that, within sugarcane stems, where *G. diazotrophicus* occurs in numbers up to 10_7/g of plant tissue (Cavalcante and Döbereiner, 1988), substantial amounts of nitrogen could be made available to the plant, hence providing a basis for the very high estimates of N_2-fixation (up to 220 kg N/ha) reported in certain sugarcane genotypes under field conditions in Brazil (Boddey et al., 1991; Urquiaga et al., 1992).

These characteristics, in addition to a lack of a nitrate reductase (Cavalcante and Döbereiner, 1988) and only partial inhibition of nitrogenase activity by NH_{4+}, enable *G. diazotrophicus* to fix N_2 in the presence of soil nitrogen, thus making it an even more interesting plant-associated diazotroph.

An additional aspect reported by Cojho et al. (1993) was the commensalism observed between *G. diazotrophicus* and the

yeast *L. kononenkoae* when grown on starch, suggesting the possibility for single cell protein production from starchy plant residues without the need for nitrogen supplements.

The respiratory system and diazotrophic activity of *G. diazotrophicus* PAL5 (the most studied strain) was investigated by Flores-Encarnación et al. (1999), using increasing aeration (from 0.5 to 4.0 liters of air/min/liter of medium). Aeration showed a strong, positive effect on the growth and diazotrophic activity of *G. diazotrophicus*. Spectral and high-pressure liquid chromatography analysis of membranes revealed the presence of cytochrome *ba* as a putative oxidase in cells obtained from diazotrophically active cultures, concluding that glucose dehydrogenase and cytochrome *ba* are key components of the respiratory system of *G. diazotrophicus* during aerobic diazotrophy.

Levansucrase (LsdA, EC 2.4.1.10) was identified as a constitutive exoenzyme in 14 *G. diazotrophicus* strains recovered from different host plants cultivated in diverse geographical regions (Hernández et al., 2000). The enzyme, consisting of a single 60 kDa polypeptide, hydrolyzed sucrose to synthesize oligofructans and levan. They observed that targeted disruption of the *lsd*A gene in four representative strains abolished their ability to grow on sucrose, indicating that the endophytic species *G. diazotrophicus* utilizes plant sucrose via levansucrase. Arrieta et al. (2004) reported that LsdA, unlike other extracellular levansucrases from Gram-negative bacteria, is transported to the periplasm by a signal peptide-dependent pathway. They identified an unusually organized gene cluster encoding at least the components LsdG, -O, -E, -F, -H, -I, -J, -L, -M, -N, and -D of a type II secretory system required for LsdA translocation across the outer membrane, being the first description of a type II pathway for protein secretion in the Acetobacteraceae (Arrieta et al., 2004).

It has been reported that extracellular glucose oxidation is considered the main route for glucose catabolism in *G. diazotrophicus* (Alvarez and Martínez-Drets, 1995; Attwood et al., 1991). However, low but significant hexokinase activities have been reported in this organism; moreover, a nicotinamide adenine dinucleotide-linked glucose dehydrogenase (NAD-GDH) was found to be actively synthesized in glucose-containing cultures (Alvarez and Martínez-Drets, 1995; Attwood et al., 1991). Therefore, two oxidative routes seem to be simultaneously expressed in *G. diazotrophicus*, one being intracellular by way of a NAD-GDH, and the other being periplasmic by way of a pyrrolo-quinoline-quinone-linked glucose dehydrogenase (PQQ-GDH). It was observed also that a PQQ-GDH was primarily responsible for the high rates of gluconic acid formation exhibited by *G. diazotrophicus* (Attwood et al., 1991). Studying the regulation of both enzymes, Luna et al. (2006) observed that *G. diazotrophicus* metabolizes glucose mainly by way of a PQQ-GDH, particularly under BNF and/or limited conditions. However, under glucose excess, a NAD-GDH is simultaneously expressed, and this enzyme would likely participate in glucose oxidation. Studies conducted in batch and continuous cultures by Luna et al. (2006) showed that glutamate is the central molecule of C-metabolism in *G. diazotrophicus*, and that metabolic flux proceeds mainly by the

pentose–phosphate pathway, as has already been reported for other AAB (Matsushita et al., 1994).

4.1. Nitrogenase activity

Biological fixation of atmospheric dinitrogen by its reduction and protonation to ammonia is achieved in diazotrophic bacteria by the enzyme complex known as nitrogenase. *G. diazotrophicus* has the capacity to fix N_2 at environmental atmospheric partial O_2 pressures (pO_2) (e. g. approximately 20 kPa of O_2) when grown in semisolid medium (Cavalcante and Döbereiner, 1988) and as colonies on solid medium (Dong et al., 1995). Growing *G. diazotrophicus* on solid medium at different pO_2, Pan and Vessey (2001) demonstrated that colonies of *G. diazotrophicus* can fix N_2 at a wide range of atmospheric pO_2, and can adapt to maintain nitrogenase activity in response to both long-term and short-term changes in atmospheric pO_2, and that the bacterium has a switch-off/switch-on mechanism for protecting the nitrogenase from rapid changes in atmospheric pO_2. This is relevant, given that *G. diazotrophicus* exists *in situ* as microcolonies adhered to plant cell walls (Dong et al., 1994). Studying the positions of *G. diazotrophicus* in the mucilaginous matrix of the colony, Dong et al. (2002) observed that bacteria utilize the path-length of colony mucilage between the atmosphere and the biomass to achieve a flux of O_2 that maintains aerobic respiration, while not inhibiting nitrogenase activity. Likewise, evidence for conformational protection of nitrogenase against O_2 in *G. diazotrophicus* was presented by Ureta and Nordlund (2002), in which a putative FeSII Shethna protein is involved.

The influence of high sugar content on nitrogenase activity of *G. diazotrophicus* in the presence of O_2 and of N added in the form of ammonium and amino acids was studied by Reis and Döbereiner (1998). They observed that 10% sucrose protected nitrogenase against inhibition by oxygen, ammonium, some amino acids, and also to some extent by salt stress, indicating the presence of different osmotolerance mechanisms for sucrose and salt (Reis and Döbereiner, 1998). When evaluating the effect of the addition of increasing amounts of two sources of mineral nitrogen (ammonium sulphate and calcium nitrate) on the nitrogenase (acetylene reduction) activity of *G. diazotrophicus* in two sugarcane hybrids, SP70-1143 and SP79-2312, Medeiros et al. (2006) determined that it was inhibited by both sources, especially in variety SP79-2312. Likewise, Madhaiyan et al. (2006) noticed that addition of pesticides to the growth media substantially reduced the nitrogenase activity of pure cultures of *G. diazotrophicus*.

4.2. Production of phytohormonal substances

Phytohormones play an important role as signals and regulators of growth and development in plants. The capacity to produce them is often considered as a trait of the plant kingdom. However, production of phytohormones is also widespread among soil and plant-associated prokaryotes (Costacurta and Vanderleyden, 1995). The production of phytohormonal substances such as auxins and gibberellins by

different PGPB has been proposed as one of the mechanisms, besides N_2-fixation, to explain plant growth-promotion.

Indole-3-acetic acid (IAA) is a naturally occurring auxin with broad physiological effects. It can be implicated in plant pathogenesis or stimulates plant growth (Patten and Glick, 1996). The IAA production by *G. diazotrophicus* was first reported by Fuentes-Ramírez et al. (1993). Further evidence of IAA production by *G. diazotrophicus* was also observed by Bastián et al. (1998), Lee et al. (2004), Pedraza et al. (2004), and Madhaiyan et al. (2006). Lee et al. (2004) also reported that IAA biosynthesis is deficient in *G. diazotrophicus* strains with mutations in cytochrome *c* biogenesis genes.

The presence of phytohormones seems to play an important role in *G. diazotrophicus*-plant interactions. Sevilla et al. (2001) observed that a *nif*H_ mutant of *G. diazotrophicus* PAL5 (altered in its N_2-fixing capacity) could still stimulate plant growth when inoculated to sugarcane plants, suggesting the implication of auxin production.

Aromatic amino acid aminotransferases (AATs) are ubiquitous enzymes that reversibly catalyze the conversion of amino acids to the corresponding α-ketoacids; they are involved in multiple metabolic pathways, such as the IAA synthesis (Patten and Glick, 1996). The detection of AATs activity in cell-free crude extracts from *G. diazotrophicus*, *G. johannae* and *G. azotocaptans*, as well as their IAA production, was reported by Pedraza et al. (2004). After non-denaturating PAGE, they observed only one isoform for each strain of *G. diazotrophicus*, *G. johannae* and *G. azotocaptans*.

The second phytohormonal substances detected in *G. diazotrophicus* are the gibberellins, A_1 and A_3. They were characterized by capillary gas chromatography–mass spectrometry from chemically-defined culture media containing 10% sucrose (Bastián et al., 1998). Recently, under laboratory conditions, it was observed that the addition of pesticides to the growth media of pure cultures of *G. diazotrophicus* reduced the production of IAA and gibberellin A_3 (Madhaiyan et al., 2006).

5. *G. diazotrophicus*: genetic aspects

It is known that some *G. diazotrophicus* strains carry plasmids of sizes varying from 50 to 110 MDa, and that the *nif* genes (involved in the N_2-fixing process) are not located in plasmids but in the chromosome (Caballero-Mellado et al., 1993; Teixeira et al., 1994). Caballero-Mellado and Martínez-Romero (1994) observed that not all *G. diazotrophicus* strains harbor plasmids, and that two plasmids (a 20–24 kb plasmid and a 170 kb plasmid) were highly conserved among the isolates examined. Although their functions are yet to be identified, the fact that some strains do not harbor plasmids may indicate that fundamental phenotypic characteristics of *G. diazotrophicus* (e.g., nitrogen fixation, IAA production, the use of different carbon substrates) are not plasmid-encoded. However, plasmids may confer some advantage on strains that harbor them, as most of the isolates contain highly conserved plasmids (Caballero-Mellado and Martínez-Romero, 1994).

It was also shown that this diazotroph has a narrow genetic diversity. Measurements of polymorphism in the electrophoretic mobilities of metabolic enzymes revealed that the mean genetic diversity per enzyme locus was 0.064, and that the genetic structure of *G. diazotrophicus* is clonal, with one largely predominant clone (Caballero-Mellado and Martínez-Romero, 1994). It has been exposed that one electrophoretic type (ET1) of *G. diazotrophicus* is distributed among different host species, including sugarcane, sweet potato, Cameroon grass, coffee and pineapple plants (Caballero-Mellado and Martínez-Romero, 1994; Caballero-Mellado et al., 1995; Jiménez-Salgado et al., 1997; Tapia-Hernández et al., 2000). According to Tapia-Hernández et al. (2000), the detection of one genotype (ET1) of *G. diazotrophicus* in taxonomically unrelated plants and the predominance of this genotype in some of those species, as well as the very low genetic diversity in populations of *G. diazotrophicus,* suggest two possibilities: *i*) only some genetically related groups in this species or its ancestor have acquired the aptitude of colonizing plants by themselves or with the support of vectors such as insects or fungi; or *ii*) *G. diazotrophicus* had been associated with a particular plant taxonomic group, maybe untested, and lately some selected genotypes have been able to extend their distribution to plants in other taxa.

In another study conducted in Brazil, Perin et al. (2004) evaluated the diversity of *G. diazotrophicus* strains from different sugarcane varieties and geographic origin; they also included strains isolated from coffee, pineapple and Cameroon grass. Cluster analysis of enzyme-linked immunosorbent assays indicated that variations were not correlated with the plant species, sugarcane variety, geographic origin, parts of plants or with sampling time. They suggest that high nitrogen doses lowered the diversity of *G. diazotrophicus* (Perin et al., 2004).

It was reported by Caballero-Mellado and Martínez-Romero (1994) that, regardless of the presence of plasmids, all of the *G. diazotrophicus* isolates analyzed shared a common pattern of *nif* structural gene organization on the chromosome. Different genes involved in the N_2-fixing process and its regulation such as *nif*HDK, *nif*A, *nif*B, *nif*V, *nif*E and *ntr*BC have been identified by Sevilla et al. (1997). Later, a major cluster (30.5 kb) of *nif* and associated genes of *G. diazotrophicus* was sequenced and analyzed by Lee et al. (2000). Northern blots and promoter sequence analysis showed that the genes are organized into eight transcriptional units (Lee et al., 2000). They also observed that the overall arrangement of genes is most likely that of the *nif-fix* cluster in *Azospirillum brasilense*, while the individual gene products are more similar to those in species of Rhizobiaceae or in *Rhodobacter capsulatus* (Lee et al., 2000).

In order to investigate the role played by sugarcane in the interaction between this plant and endophytic diazotrophs, Nogueira et al. (2001) studied gene expression profiles of sugarcane plants colonized by *G. diazotrophicus* and *Herbaspirillum rubrisubalbicans*. They produced an inventory of sugarcane genes, candidates for exclusive or preferential expression during the N_2-fixing association, and suggest that sugarcane plants might be actively involved in the establishment of the interaction with the diazotrophs assessed.

At present, a network comprised of eight sequencing laboratories and one bioinformatics laboratory has been assembled in Brazil, aimed at determining the complete genome sequence of *G. diazotrophicus*, strain PAL5 (Ferreira and RioGene Consortium, 2004), which could have a great impact on the research conducted with this diazotroph. They estimated the genome length as 4.3 Mb, and 98.89% of the *G. diazotrophicus* genome was assembled in 572 contigs with low overall redundancy. Using shotgun strategy, 73,906,554 bases were deposited and 102.813 reads were generated. They found that 92% of the estimated genome is in contigs longer than 3 Kb. A Bacterial Artificial Chromosome (BAC) library was constructed with the purpose to create a physical map and help to finish the sequence. The genome is currently comprised of around 6100 ORFs by Glimmer analysis. The annotation effort is already resulting in the identification of genes, metabolic pathways and regulatory circuits of *G. diazotrophicus* (Padua and RioGene Sequencing Consortium, 2004).

6. Associatiation between *G. diazotrophicus* and sugarcane plants

6.1. Endophytic nature

The endophytic nature of *G. diazotrophicus* was confirmed in Brazil by counts of this diazotroph in roots, stems, aerial parts and cane trash (Reis et al., 1994). Numbers in all plant parts were between 10^3 and 10^6 cells/g fresh weight. High numbers (10^6–10^7 cfu/g fresh weight) were found also in sugarcane plants in India (Muthukumarasamy et al., 1999). This diazotroph was also isolated from sugarcane trash and xylem sap, indicating latter translocation of the bacterium through the plant tissues in the xylem (Reis et al., 1994). The presence of *G. diazotrophicus* on and within sugarcane plant tissues was also confirmed by immunogold labeling by James et al. (1994). They observed that the loose cells of the root cap at root tips were a site of entry of the organism into root tissues. Both at lateral root junctions and root tips, bacteria were also seen in enlarged, apparently intact, epidermal cells. Likewise, bacteria were present in xylem vessels at the base of the stem, without pathogenic reaction to the bacteria within the xylem (James et al., 1994). Further evidence that the N_2-fixing endophytic bacterium from the intercellular spaces of sugarcane stems is *G. diazotrophicus* was reported by Dong et al. (1995). The occurrence of this diazotroph was directly demonstrated in tissues of micropropagated sugarcane plants using a species-specific oligonucleotide probe and PCR amplification (Kirchhof et al., 1998).

In commercial plantations, sugarcane is commonly propagated through stem cuttings (setts). The method used to treat setts before planting to eliminate the plant pathogen *Clavibacter xyli* subsp. Xili, which causes ratoon stunting disease, is heat treatment (50 °C for 30 min). Reis et al. (1994) observed that the number of *G. diazotrophicus* within the plant was not affected after heat treatment, confirming the endophytic habitat of this diazotroph and its propagation within the stem cuttings. It was also observed that when inoculating stem cuttings with *G.*

diazotrophicus, the bacterial colonization stimulated the production and development of root hairs (Bellone et al., 1997).

Although the colonization of sugarcane by *G. diazotrophicus* and its endophytic nature was reported in different works (Cavalcante and Döbereiner, 1988; Dong et al., 1995; James et al., 1994; Kirchhof et al., 1998; Reis et al., 1994), Walsh et al. (2006) observed in Australia that sugarcane-N_2-fixing *G. diazotrophicus* association is not easily achievable, being primarily limited by a lack of infection.

6.2. Nitrogen fertilization

In order to study the performance of *G. diazotrophicus* in the presence of N-fertilizers, different experiments were carried out. The early study by Fuentes-Ramírez et al. (1993) suggested a close relationship between N fertilization level added to sugarcane and isolation frequency of *G. diazotrophicus*; when sugarcane was fertilized with higher than 200 kg N/ha, isolation frequencies of 0–2% were found, while at levels lower than 120 kg of N/ha, frequencies increased up to 70%. Such a relationship on the isolation frequency of *G. diazotrophicus* from field-grown sugarcane in Australia was analyzed by Li and MacRae (1991),and later by Reis Junior et al. (2000) and Muthukumarasamy et al. (2002b) in Brazil and India, respectively.

Reis Junior et al. (2000) studied the effects of fertilization with high levels of N (300 kg of N/ha) on the occurrence and numbers of *Herbaspirillum* spp. and *G. diazotrophicus* in sugarcane plants in Brazil. They observed that in the sugarcane genotype SP79-2312, the N-fertilized plants generally showed higher concentrations of this nutrient and lower numbers of *G. diazotrophicus*, while the population of *Herbaspirillum* spp. was not affected by N application. These differences in the concentration of N and numbers of *G. diazotrophicus* due to N application were not observed in the variety SP70-1143. In the same study, it was found that the numbers of *G. diazotrophicus* detected were also influenced by the harvest time, becoming reduced in the harvests that coincided with dry periods of the year (Reis Junior et al., 2000). Muthukumarasamy et al. (2002b) also investigated in India whether *G. diazotrophicus* could be recovered from sugarcane plants either with low or no application of N-fertilizers. They observed that *G. diazotrophicus* numbers were at a minimum compared to other non-N_2-fixing bacteria in high N-fertilized samples; the colonization and acetylene reduction activity suffered a setback in high levels of ammonium chloride and ammonium nitrate, and that *G. diazotrophicus* formed long pleomorphic, immobile cells in the presence of high concentration of N-sources, especially NH_4^+. They also suggested that the morphological changes and the increased heterotrophic populations may play a role on the survival of *G. diazotrophicus* in high N-fertilized samples/ environments (Muthukumarasamy et al., 2002b).

Fertilizing with different levels of urea (0, 75, and 150 kg N/ha), Suman et al. (2005) observed an improvement in sugarcane growth and nutrient uptake by inoculating with *G. diazotrophicus*. Recently, Muthukumarasamy et al. (2006) noticed that inoculation of *G. diazotrophicus* and *Herbaspirillum* sp. in micropropagated sugarcane plants could mitigate N-fertilizer application considerably in sugarcane cultivation.

Medeiros et al. (2006) evaluated the effect of the addition of increasing amounts of ammonium sulphate and calcium nitrate on the population of *G. diazotrophicus*, nitrogenase activity and accumulation of N by two sugarcane hybrids: SP70-1143 and SP79-2312. The two varieties differed in the chemical nitrogen type they prefer to uptake from the soil: SP70-1143 preferred ammonium sulphate, whilst the variety SP79-2312 preferred N from calcium nitrate. In both varieties, the addition of increased doses of ammonium and nitrate inhibited the population of *G. diazotrophicus* but in variety SP70-1143, the inhibition was more pronounced in the presence of calcium nitrate (Medeiros et al., 2006).

Tejera et al. (2006) identified and quantified several nitrogen compounds in the apoplastic and symplastic sap of sugarcane stem, composed mainly of soluble sugars. Sap also contained nitrogen compounds, with amino acids (50–70% of N) and proteins (20–30% of N), being the main nitrogenous sub-stances, as well as inorganic forms as ammonium, nitrite and nitrate, in low concentrations (<20% of N). They found that the total amino acid content of apoplastic sap was six to nine times lower in non-nitrogen fertilized plants than in fertilized ones. Hence, a high concentration of amino acids in the apoplast of high N-fertilized sugarcane could inhibit the nitrogen fixation process in such conditions (Tejera et al., 2006). Besides, it has been reported by Fuentes-Ramírez et al. (1999), Muthukumarasamy et al. (2002b) and Tejera et al. (2004) that the population of *G. diazotrophicus* was sensitive to the application of N-fertilizers; thus it could be speculated that the number of N_2-fixing bacteria inside the apoplast might also be controlled by the content of some amino acids. The low concentration of amino acids in the apoplastic sap of inoculated plants, as compared with non-inoculated plants, also supports the hypothesis that these compounds should be involved in plant-endophyte associa-tions (Tejera et al., 2006).

Another aspect that could be involved in the *G. diazotro-phicus*-sugarcane association is the antagonism among strains. The antagonistic activity among 55 *G. diazotrophicus* strains in culture media was analyzed by Muñoz-Rojas et al. (2005). Antagonistic effects were variable among them and against closely related strains of *Gluconacetobacter* species, including *G. johannae*, *G. azotocaptans* and *G. liquefaciens* but not against other phylogenetically distant species. They found that the substance responsible for such antagonistic activity is a low molecular weight molecule (approximately 3400 Da), related to bacteriocin-like molecules. The antagonistic ability of some strains of *G. diazotrophicus* could be an advantage for the natural colonization of the sugarcane environment, as was observed in experiments with micropropagated sterile sugar-cane plantlets co-inoculated with a bacteriocin-producer strain and a bacteriocin-sensitive strain of *G. diazotrophicus* (Muñoz-Rojas et al., 2005). In these experiments, both in the rhizosphere as well as inside the roots, the bacteriocin-sensitive population decreased considerably. In addition, this study shows that inside the plants there may exist antagonistic interactions among

endophytic bacteria like those described among the rhizospheric population (Muñoz-Rojas et al., 2005).

Glycoproteins from sugarcane stalks have been isolated by size-exclusion chromatography from field-grown plants (Blanco et al., 2005). Some of these glycoproteins were able to bind to the cell wall of *G. diazotrophicus*, and largely removed after washing the bacterial cells with sucrose. This implies that sugarcane glycoproteins use β(1–2)-fructofurano-syl fructose domains in their glycosidic moiety to bind to specific receptors in the bacterial cell walls, acting as recognition factors (Blanco et al., 2005). Legaz et al. (2000) reported conclusive evidence about the ability of some sugarcane glycoproteins to bind to the cell wall of *G. diazotrophicus*. This could be the first step of biological discrimination of a compatible endophyte, thereby resembling a mechanism of specific tolerance such as found in the immune system of higher eukaryotics.

6.3. Studies on micropropagated sugarcane plants

Reis et al. (1999) reported a protocol to be used in micropropagated sugarcane plantlets as a means to introduce selected strains of endophytic diazotrophs.

Analysis of population dynamics of *G. diazotrophicus* strains in different sugarcane varieties (bacteria-free micro-propagated plantlets) showed that the bacterial populations decreased drastically in relation to plant age, regardless of the nitrogen fertilization level, bacterial genotype or sugarcane cultivars (Muñoz-Rojas and Caballero-Mellado, 2003). The inoculation of *G. diazotrophicus* may be beneficial for sugarcane plant growth, but this response is dependent both on the *G. diazotrophicus* genotype and the sugarcane variety (Muñoz-Rojas and Caballero-Mellado, 2003). In the same study, they observed that although the positive effect on sugarcane growth apparently occurred by mechanisms other than nitrogen fixation, their results showed the importance of the sugarcane variety for the persistence of the plant–bacteria interaction, which could explain the different rates of BNF estimated among sugarcane cultivars. However, according to Boddey et al. (2003), if attempts are to be made to select sugarcane varieties for high BNF, then the soils should be N-deficient, irrigated, and fertilized with molybdenum, (a key component of the enzyme nitrogenase responsible for the BNF), either to the soil or as foliar spray (especially in acid soils where the availability of this element is depressed).

Improved yield of micropropagated sugarcane plants following inoculation by *G. diazotrophicus* and *Burkholderia vietnamiensis* was also observed in Tamil Nadu, India (Govindarajan et al., 2006). In Brazil, Martinez de Oliveira et al. (2006) demonstrated the feasibility of inoculation technology using diazotrophic bacteria in micropropagated sugarcane varieties grown in soils with low, medium and high levels of natural fertility. They observed that the stem yield and BNF contribution in response to bacterial inoculation were influenced by the strain combinations in the inoculum, the plant genotype, and the soil type and nitrogen fertilization, thus confirming the genetic and environmental influence in PGPB interactions.

7. Other plant associations with *G. diazotrophicus* and *G. azotocaptans*

To test the qualities of *G. diazotrophicus* as a promising N$_2$-fixing and PGPB in other agriculturally important crops, different assays have been carried out. Bastian et al. (1999) inoculated sorghum seedlings with pure cultures of *G. diazotrophicus* strain PAL5. They observed that fructose and glucose levels determined on shoot were significantly augmented by *G. diazotrophicus* as compared to control and phytohormone treatments (gibberellin A$_3$ and IAA).

The potential of *G. diazotrophicus* to colonize and promote the growth of rice, maize and wheat was investigated by Sevilla and Kennedy (2000) using Nif$_+$ and Nif$_-$ strains tagged with the *uid*A gene (β-glucuronidase). They observed that *G. diazotrophicus* was able to colonize maize, rice and wheat, but the colonization was apparently restricted to root tissues. They found that inoculation with *G. diazotrophicus* did not enhance the growth of wheat seedlings. On the other hand, when nitrogen was not limiting, there were no differences between inoculated and uninoculated plants, indicating that, unlike in sugarcane, *G. diazotrophicus* may not benefit plant growth in the presence of sufficient nitrogen (Sevilla and Kennedy, 2000).

Working with common bean (*Phaseolus vulgaris* L.), Trujillo-López et al. (2006) tested the effect of UV light, as an inducer of secondary metabolite accumulation, on the association between common bean seedling roots and *G. diazotrophicus*. They observed that *i*) UV irradiation of seedlings 4 h prior to bacterial inoculation increased the number of bacterial cells associated with the roots by 5.65-fold with respect to a non-irradiated control ($p < 0.05$); *ii*) *G. diazotrophicus* associates with root hairs and root border cells; *iii*) aggregation of bacterial cells occurred in root structures from UV-induced seedlings; and that *iv*) secondary metabolites accumulated in roots from UV-irradiated seedlings.

In an assay of root inoculation of maize and sorghum with *G. diazotrophicus* and the arbuscular mycorrhizal fungus *Glomus intraradices*, Adriano-Anaya et al. (2006) observed that the two strains of *G. diazotrophicus* (PAL5, UAP5541) and *G. intraradices* increased both the shoot and root dry weight of sorghum, whereas they had no effect on the shoot and root dry weight of maize. Co-inoculation (using both microorganisms together) did not increase the shoot and root dry weight of either plant. They reported a synergistic effect of *G. diazotrophicus* on root colonization of maize by *G. intraradices*, whereas an antagonistic interaction was observed in the sorghum root where the number of *G. diazotrophicus* and the colonization by *G. intraradices* were reduced. Plant roots inoculated with *G. diazotrophicus* and *G. intraradices*, either separately or together, significantly increased root endoglucanase, endopo-lymethylgalacturonase and endoxyloglucanase activities. They suggest that hydrolytic enzyme activities increased as a result of inoculation with *G. diazotrophicus*, considering this to be one of the mechanisms by which these bacteria may increase root

colonization by arbuscular mycorrhizal fungi (Adriano-Anaya et al., 2006). The latter was already observed by Paula et al. (1991) when using *Glomus clarum* spores containing *G. diazotrophicus* to introduce this diazotroph into roots and aerial parts of sugarcane, sweet potato and sorghum seedlings.

Recently, Cocking et al. (2006) have investigated the interaction of *G. diazotrophicus* with *Arabidopsis thaliana* and the crop plants maize, rice, wheat, oilseed rape, tomato, and white clover. They have shown that inoculation with very low numbers of *G. diazotrophicus* results in extensive intracellular root colonization. Light microscopic examination of thin sections of resin-embedded root tips of *Arabidopsis* and these crop plants inoculated with β-glucuronidase (GUS)-labeled and with *nif*H promoter-GUS-labeled *G. diazotrophicus* showed blue-stained *G. diazotrophicus* within the cytoplasm of root cells, indicating that intracellular conditions were suitable for nitrogenase gene expression. Electron microscopy confirmed that these blue-stained intracellular *G. diazotrophicus* were within membrane-bounded vesicles. After these observations, they suggest whether these novel inoculations with *G. diazotrophicus* are likely to enable non-nodular endosymbiotic nitrogen fixation and whether these inoculations can also provide a plant system to investigate the endosymbiotic theory of the origin of eukaryotic organelles (Cocking et al., 2006).

Little information is available on other N_2-fixing AAB–plant interaction. In the case of *Gluconacetobacter azotocaptans*, Mehnaz and Lazarovits (2006) conducted an assay inoculating corn plants (under greenhouse conditions) with *G. azotocaptans*, *Pseudomonas putida* and *Azospirillum lipoferum*. Bacterial suspensions were applied to pre-germinated seeds of four corn varieties (39D82, 39H84, 39M27, 39T68) planted in sterilized sand and unsterilized soil. After 30 days from inoculation, they observed that some of the strains used (isolated from corn rhizosphere in Canada) provided significant plant growth promotion, expressed as increased root/shoot weight, when compared to uninoculated plants, in sand and/or soil, depending on the inoculated strain and corn variety combination (Mehnaz and Lazarovits, 2006).

8. Final remarks

In the last decades, it has been observed that plant-associated prokaryotes are valuable for agriculture as a tool for improving crop performance and environmental conditions, as they may reduce and avoid the use of chemical fertilizers.

Within the Acetobacteraceae family, *G. diazotrophicus*, *G. johannae*, *G. azotocaptans*, *S. salitolerans*, *A. peroxydans* and *A. nitrogenifigens* have been found to fix N_2. Little information is available on the last five cited species as they were recently described. Since its discovery, *G. diazotrophicus* has become a very interesting microorganism to study due to the characteristics overviewed in this article.

The *G. diazotrophicus*–sugarcane relationship represents a model system for monocot–diazotrophic association and, even though their interactions have not been fully understood, different reports indicate that *G. diazotrophicus* is able to promote plant growth and that the mechanisms attributed include nitrogen fixation and phytohormones production. It was observed that this bacterium has also the capacity to solubilize P and Zn compounds, to produce a bacteriocin that inhibits the growth of *Xanthomonas albilineas*, the causal agent of leaf scald disease in sugarcane (Piñón et al., 2002), as well as having resistance to different antibiotics and heavy metals (Ahmad et al., 2004).

Nevertheless, Boddey et al. (2003) consider it premature to use inoculants of N_2-fixing bacteria in sugarcane plants since the main contributors to the observed BNF are not known, and in all studies so far reported, there seems to be well established populations of N_2-fixing bacteria within the plants. However, taking into account the different characteristics of *G. diazotrophicus* as promising features to be transferred to sugarcane growers, Lee and Bressan (2005) observed good results when using disease-free clean cane (involving the thermotherapy treatment and meristem tip culture) as the planting material in commercial production of sugarcane. They reported that it was possible to spray nitrogen-fixing bacteria on stem cuttings during planting before covering with soil, and that clean cane was infected more easily than non-treated cane. Therefore, clean cane inoculated with selected nitrogen fixing bacteria could be efficiently used as material for commercial production of sugarcane.

In the context of using PGPB as biofertilizers in agriculturally important corps, fresh produce is increasingly seen as a carrier of food borne pathogens like Shiga toxin-producing *Escherichia coli* (STEC) O157 (Hilborn et al., 1999; Michino et al., 1999). We could expect that PGPB apart from their role as nitrogen fixation could provide protection against undesired bacteria causing food borne disease or spoilage and a shorter shelf life of fresh produce. Considering the current knowledge, they could offer a valid model for studying interactions between plants and food borne pathogens. One could also expect that PGPB like *G. diazotrophicus* by competitive exclusion may perhaps eliminate human pathogens (e.g., *E. coli* O157:H7) that persist in soil and on plant roots (Gagliardi and Karns, 2002).

Although the commercial significance of associative N_2-fixation in many plants remains controversial (because fixation is so variable with the organism, plant, environmental conditions and nutritional status of the soil), the study of biological nitrogen fixing bacteria remains important and necessary. Further experiments will need to be carried out to answer questions about the diazotroph–plant interactions, bacterial establishment, colonization process, BNF (including genetic and regulatory aspects), growth promotion in different plant species, etc., not only with *G. diazotrophicus* but also with the rest of the N_2-fixing AAB so far described.

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