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Review

Nitrogen fixing bacteria in the family *Acetobacteraceae* and their role in agriculture

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For centuries, the *Acetobacteraceae* is known as a family that harbors many species of organisms of biotechnological importance for industry. Nonetheless, since 1988 representatives of this family have also been described as nitrogen fixing bacteria able to plant growth promotion by a variety of mechanisms. Nitrogen fixation is a biological process that guarantees that the atmospheric N_2 is incorporated into organic matter by several bacterial groups. Most representatives of this group, also known as diazotrophic, are generally associated with soil rhizosphere of many plants and also establishing a more specific association living inside roots, leaves, and others plants tissues as endophyte. Their roles as plant growth-promoting microorganisms are generally related to increase in plant biomass, phosphate and other mineral solubilization, and plant pathogen control. Here, we report many of these plant growth-promoting processes related to nitrogen fixing species already described in *Acetobacteraceae* family, especially *Gluconacetobacter diazotrophicus* and their importance to agriculture. In addition, a brief review of the state of art of the phylogenetics, main physiological and biochemical characteristics, molecular and functional genomic data of this group of *Acetobacteraceae* is presented.

Keywords: Biological nitrogen fixation / Plant growth promotion / *Gluconacetobacter diazotrophicus* / Acetic acid bacteria

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Introduction

Bacteria belonging to the Alphaproteobacteria, order *Rhodospirillales*, are known for their agricultural applicability. This order is represented by two bacterial families: *Rhodospirillaceae* and *Acetobacteraceae*. In general, the etymology of members of the *Acetobacteraceae* family derives from the Latin *acetum* or *acidum gluconicum* + *bacter* due to their peculiar main characteristic to produce organic acids during many biotechnological processes, such as vinegar and wine productions. Bacteria of the genus *Acetobacter* are known since 1898 with the description of *A. aceti* [1, 2]. However, only at 1988, it means 100 years after the first species description in this family, is that a diazotrophic species was described in this family [3]. At that time, this discovery raised the possibility that bacteria of many other species could also present nitrogen fixing and plant growth promotion properties for agricultural purpose, similar to the rhizobia inoculant for soybeans. This document brings the trajectory of accumulated knowledge about the species *Gluconacetobacter diazotrophicus* and other diazotrophs genera and species that were described later in this family.

The *Acetobacteraceae* family

Bacteria belonging to the *Acetobacteraceae* family (ex Henrici, 1939) [4] are classified as rods (coccus or ellipsoidal), Gram-negative, mobile, aerobic that conduct an incomplete oxidation of sugars and alcohols to produce organic acids as final product of their metabolism. They have the ability to grow in very acidic environments with pH close to 3.0–3.5, but the optimum range is 5.0–6.5 [5]. They are commonly

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known as "Acetic Acid Bacteria" (AAB) due to their use in the production of vinegar, but this is not the only biotechnological product derived from these bacteria as reviewed in Raspor and Goranovic [6]. Among them, we can mention the production of several fermented beverages, for example, beverages derived from coconut cream, cocoa products of fermentation, in addition to producing microbial cellulose, biotransformation of glucose to gluconic acid and its ketoderivatives, production of non-caloric sugar D-tagalose and shikimate that is an intermediate in the production of many antibiotics. Nonetheless, the AAB may act in the opposite way in a few drinks and pathogenic to diverse cultures being the causative agent of diseases in pineapple and apple. Its presence can influence the quality of beverages such as beer, wine, and vinegar, since certain amount of oxygen and optimum temperature can promote further oxidation of ethanol and organic acids. On the other hand, representatives of the genera *Acetobacter*, *Roseomonas*, and *Granulibacter* have been considered opportunistic bacteria related to chronic granulomatosis, pneumonia, and bacteremia, respectively [7–13].

Acetobacteraceae family initially corresponded to the representatives of the genera *Acetobacter* and *Gluconobacter*, based on morphological, physiological, and biochemical criteria [14, 15]. In 1950, Frateur [16] proposed a classification based on the following physiologic criteria: catalase production, gluconic acid production from glucose, acetic acid oxidation to CO_2 and H_2O, lactic acid oxidation to CO_2 and H_2O, and the oxidation of glycerol to dihydroxyacetone. Later on, hybridization studies of DNA:rRNA indicated that *Acetobacter* and *Gluconobacter* formed a single and independent branch of the RNA superfamily justifying their unification in *Acetobacteraceae* family [4]. In 1984, Yamada and Kondo [17] proposed a new subgenus denominated *Gluconoacetobacter*, which was elevated to the category of genus based on the analysis of 16S rRNA gene sequences [18]. In accordance to this reclassification, species such as *Acetobacter diazotrophicus*, *Acetobacter europaeus*, *Acetobacter hansenii*, *Acetobacter liquefaciens*, and *Acetobacter xylinus* were transferred to the new genus *Gluconoacetobacter*, which then was corrected to *Gluconacetobacter* [19]. The genus *Acetobacter* shows quite unique and exceptional predominance of quinone type Q-9, while quinone type Q-10 is predominant in the other genera [5]. Many of these bacteria are capable of performing a wide variety of biotechnological processes, but those most important commercially are related to the genera *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*.

To date, the genera described and recognized at the LPSN-list of prokaryotic names with standing in nomenclature are *Acetobacter*, *Acidicaldus*, *Acidiphilium*, *Acidisoma*, *Acidisphaera*, *Acidocella*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Belnapia*, *Craurococcus*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Humitalea*, *Komagataeibacter*, *Kozakia*, *Muricoccus*, *Neoasaia*, *Neokomagataea*, *Paracraurococcus*, *Rhodopila*, *Rhodovarius*, *Roseococcus*, *Roseomonas*, *Rubritepida*, *Saccharibacter*, *Stella*, *Swaminathania*, *Tanticharoenia*, *Teichococcus*, and *Zavarzinia* [20]. In addition, *Nguyenibacter* [21], *Rhodovastum* [22], *Sediminicoccus* [23], and *Swingsia* [24] are other proposed genera that remain to be formally recognized and are in the list of prokaryotic names without standing in nomenclature.

Peculiarities of some *Acetobacteraceae* genera

Some peculiarities that deviate from the classical description of AAB can be highlighted on the genera and their species described over these last 50 years.

The genus *Rhodopila* was proposed by Imhoff *et al.* [25] to accommodate a group of purple non-sulfur bacteria that presents vesicular intracytoplasmic membranes, similar to *Rhodobacter* species, but grows at low pH. The genus *Acidiphilium* was described after isolation and characterization of heterotrophic, mesophilic bacteria with requirements for high acidity and unable to use elemental sulfur or ferrous [26]. Later on, isolates from acidic hot springs and mine drainage were characterized on the basis of molecular and phenotypic traits, especially related to ion-chelated chlorophyll a type, in the genus *Acidisphaera*, which differs from other aerobic bacteriochlorophyll-containing (ABC) bacteria by producing zinc-chelated bacteriochlorophyll a (Zn-BChl) [26–28]. In addition to them, other genera known to present bacteriochlorophyll a type were also phylogenetically clustered into the family *Acetobacteraceae*, and they are as follows: *Roseomonas* [7, 29], *Roseococcus* [30], *Craurococcus*, *Paracraurococcus* [31], *Rubritepida* [32], *Humitalea* [33], and *Rhodovastum* (proposed but not yet recognized genus) [22].

The genus *Acidocella* was proposed to accommodate two previously described *Acidiphilum* species that do not present BChl a and that have been classified as monophyletic unit apart from other *Acidiphilium* species according to 16S rDNA sequence [34, 35].

The genus *Stella* is known as a polyprosthecate bacteria characterized by having numerous appendage (from the Greek *prostheca*) [36]. Although it has being classified in this family, further studies based on polar lipids and 16S rRNA genes of the two type strains species indicated its close relationship to members of *Rhodospirillaceae* [37].

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The characterization of a group of acidophilic methanol-utilizing bacteria leads to reclassification of *Acetobacter methanolicus* into a new genus, *Acidomonas* [38]. Afterwards, an emendation of the genus description stated that *Acidomonas* presents acid tolerance, instead of acidophily [39].

Representatives of the genus *Asaia* do not grow in presence of methanol, are characterized by poor or non-existent production of acetic acid from ethanol, and by the absence of growth in presence of 0.35% acetic acid (w/v) [40]. The genera *Asaia*, *Kozakia*, *Swaminathania*, and *Neoasaia* are related phylogenetically to each other; however, *Tsp*509I and *Mbo*II restriction of the 16S–23S rDNA ITS can differentiate *Asaia* species from *Kozakia* and *Neosaia*-type strains [41].

The genera *Muricoccus* and *Theichococcus* were proposed to accommodate isolates that do not produce bacteriochlorophyll a under aerobic conditions and were obtained from building material of a children's day care center, specifically from gypsum liner walls of a children's sleeping room [42]. However, Sanchez-Porro *et al.* had suggest that *T. ludipueritiae* and *M. roseus*, the single species of each genera, should be placed within the genus *Roseomonas* based on pigment color, carbon metabolism, and fatty acids profiles [29].

The genus *Saccharibacter* corresponds to isolates able to grow in the presence of high concentrations of glucose (2–40% w/v—optimum at 10% w/v) but that present negligible or very weak productivity of acetic acid from ethanol. The species type strain *S. floricola* was isolated from pollen collected in Kanagawa, Japan [43].

The genus *Acidisoma* comprises two species, *A. tundrae* and *A. sibiricum*, that were isolated from acidic *Sphagnum*-dominated tundra and Siberian wetlands in Russia [44]. As general characteristic, they are chemoorganotrophic strict aerobes, psychrotolerant, do not possess bacteriochlorophyll a, produce poly-β-hydroxy-butyrate, and are oxidase- and catalase-positive.

Representative of the genus *Nguyenibacter*, *N. vanlangensis*, was isolated from rhizosphere of rice plants in Vietnam [21]. The main characteristics of this genus are oxidation of acetate to carbon dioxide and water but not lactate, no production of acetic acid from ethanol; growth is weakly positive either on 30% D-glucose (w/v) or in the presence of 0.35% acetic acid (w/v). As isolation procedure, the authors used LGI N-free-medium prior to cultivation onto another medium containing glucose and ethanol as carbon source and pH 3.5 but nitrogen fixation was not assessed.

Two genera were proposed during classification of isolates obtained from a flower sample from Thailand, the genus *Neokomagatea* that comprises *N. thailandica* and *N. tanensis* [45] and the genus *Swingsia* that comprises the species *S. samuiensis*, but is not formally recognized [24]. *Endobacter medicaginis*, the first *Acetobacteraceae* found as legume nodules endophytes, was isolated from surface sterilized nodules of *Medicago sativa* grown in an acidic soil at the Province of Zamora, Spain [46].

Several phenotypic and genotypic studies served as the basis for reclassification of several species previously described in the *Gluconacetobacter* genus at the generic level, including nitrogen fixing species [46–50]. The genus *Komagataeibacter* were proposed during separation of *Gluconacetobacter xylinum* group from the *Gluconacetobacter liquefaciens* group.

To date, among all *Acetobacteraceae* genera only some representatives of the genera *Gluconacetobacter*, *Acetobacter, Komagataeibacter*, *Swaminathania*, *Asaia*, and *Acetobacter* are reported as nitrogen fixing bacteria and the strategies used in order to obtain these new species are described in Table 1 [3, 47, 51–55, 59].

The *Gluconacetobacter* genus

Until late 80s, there was not any report of AAB capable to fix nitrogen but Cavalcante and Döbereiner [3] isolated on N-free semi-solid media and described the first N_2-fixing AAB. They succeed to isolate from sugarcane plants a group of acid-tolerant bacteria able to fix nitrogen even at pH below 3.5 using a minimal medium based on LG medium [64], named LGI-P medium, that presents 10% of raw sugar as carbon source and pH around 5.5.

Afterwards, during phylogenetic study of representatives of the genus *Acetobacter*, a new genus was proposed by Yamada *et al.* [18] leading to the elevation of the subgenus *Gluconoacetobacter* to the generic level. Two species previously classified as *Acetobacter* were then renamed into this new genus, *G. liquefaciens* and *G. xylinus*, based on 16S RNA sequence, predominance of Q-10 quinone type, flagella, pigment production, cellulose production, and fatty acid profile. Later on, during the validation, the name has been corrected to *Gluconacetobacter* in accordance with Rule 61 of the Bacteriological Code [19]. Since then, a plethora of species isolated from various environments were described into this genus. Nonetheless, after detailed phylogenetic analysis some of them were reclassified into another genus [47, 51, 55, 65–69].

After 2001, new nitrogen fixing species were described in the genus *Gluconacetobacter*, *G. johannae*, and *G. azotocaptans* [51]. *G. swingsii* and *G. rhaeticus* are cellulose-producing acetic acid bacteria isolated from

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Table 1. Media utilized for isolation of nitrogen fixing *Acetobacteraceae*.

Genus	Bacterial species	Enrichment medium					Initial pH
		N-free LGI[a]	SPYC[b]	EM I[c]	EM IV[d]	GYP[e]	
Gluconacetobacter	*G. diazotrophicus*	+ cane juice	−	−	−	−	4.5
	G. johannae	+	−	−	−	−	5.7
	G. azotocaptans	+	−	−	−	−	5.7
Asaia	*A. siamensis*	−	+	−	−	−	3.5
	A. bogorensis	−	+	−	−	−	3.5
	A. platycodi	−	−	+	+	−	3.5
Swaminathania	*S. salitorerans*	+ NaCl	−	−	−	−	5.5
Komagataeibacter	*K. (kombuchae) hansenii*	+ antifungi[f]	−	−	−	−	4.5
	K. kakiaceti	−	−	+	−	+ antifungi	3.5
Acetobacter	*A. nitrogenifigens*	+ antifungi	−	−	−	−	4.5
	A. peroxydans	+ Y[g]	−	−	−	−	6.0

[a]LGI semi-solid medium containing 10% raw sugar from sugarcane [3].
[b]SPYC − 2.0% D-sorbitol,0.5% peptone, 0.3% yeast extract, 0,0001% cycloheximide.
[c]EM I − 1.0% glucose, 0.5% ethanol, 1.5% peptone, 0.8% yeast extract, 0.3% acetic acid, 0.1% cycloheximide, pH 3.5.
[d]EM IV − 2.0% ducitol, 0.5% peptone, 0.3% yeast extract, 0.1% cycloheximide, pH3.5.
[e]GYP − 1.0% glucose, 1% glicerol, 1% peptone, 0.5%, 0,7% $CaCO_3$.
[f]Antifugi − cycloheximide and/or nystatin (150 mg L^{-1} each).
[g]Y − 0.05% yeast extract. Data based on previous reports [3, 39, 46, 50, 51, 53–62].

apple juice of fruits cropped in the South Tyrol region of Italy [67]. *G. sacchari* was isolated from sugarcane and insects [66]. *G. saccharivorans* and *G. nataicola* were proposed based on a reclassification study of *Gluconacetobacter hansenii* strains [70]. *G. tumulicola* and *G. asukensis* were isolated from biofilms growing on the surface of the plaster walls of the mural paintings of the Kitora Tumulus in Japan [68]. *G. tumulisoli*, *G. takamatsuzukensis*, and *G. aggeris* were isolated from the burial mound soil collected at Takamatsuzuka Tumulus in Asuka village, Nara Prefecture, Japan [69]. As previously occurred to *G. kombuchae*, the species *G. kakiaceti* [62], *G. medellinensis* [71], and *G. maltaceti* [72] were reclassified into the genus *Komagataeibacter* [55] based on previous phylogenetic studies using 16S rRNA sequences [47, 50].

The reasons that corroborated the existence of two phylogenetic groups in the genus *Gluconacetobacter* was discussed by Yamada and Yukphan [13]. According to previous observations, the group 1 included *G. liquefaciens*, *G. diazotrophicus*, *G. sacchari*, *G. johannae*, and *G. azotocaptans*, while group 2 included *G. xylinus G. hansenii*, *G. europaeus*, *G. entanii*, *G. oboediens*, *G. intermedius*, *G swingisii*, *G rhaeticus*, *G. saccharivorans*, and *G. nataicola*. Group 1 species differentiate from group 2 by many physiological and morphological traits, such as flagella and motility, water-soluble brown pigment production, production of γ-pyrone compounds, and 2,5 di-ketogluconic. Species clustered into these groups also showed other common features such as biotechnological applications and habitats. Ecologically, the species of group 1 were found associated with plants, fruits, or flowers, noteworthy group 2 were generally isolated from fermentation process and processed food, such as vinegar, Kombucha tea, and nata de coco [73]. Nitrogen fixation agents of biocontrol and associated to several plant growth promotion were related to species of group 1, while most species in group 2 were related with industrial applications. Cleenwerck *et al.* [74] also contributed with genotypic data to reinforce the separation of *Gluconacetobacter* species at generic level. Recently, *G. xylinum* group (group 2) were separated from the *G. liquefaciens* group (group 1) leading to reclassification of several species in the genus *Komagataeibacter*, including *G. kombuchae*, a nitrogen fixing species later considered heterotypic synonym of previously named *G. hansenii* [48, 50].

In the present scenario, the ability to fix nitrogen has being observed in the following *Gluconacetobacter* species: *G. diazotropicus* [3, 75], *G. johannae*, and *G. azotocaptans* [51].

Gluconacetobacter diazotrophicus

The species *Gluconacetobacter diazotrophicus* (former *Acetobacter diazotrophicus*) was isolated from roots, stems, and leaves of sugarcane not only in Brazil but also in Argentina, Uruguay, Mexico, Cuba, United States, India, Canada, Egypt, beside others [3, 76–78] and then from other agricultural crops such as sugar beet, rice, pineapple, coffee, carrot, and many others [79–81]. It was also isolated from bugs, such as mealybugs commonly found associated to sugarcane crops [82, 83].

This species has been considered as one of the most important diazotrophic bacteria found in high numbers (10^4–10^6 CFU g^{-1} plant fresh tissues) and colonizing the inner parts of roots, stems, and leaves of sugarcane [84].

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It is a nitrogen-fixing bacterium originally classified as *Acetobacter diazotrophicus* but later renamed to the genus *Gluconacetobacter* based on the 16S rDNA sequence and the predominant type of ubiquinone [18, 19].

Physiological characteristics. It is Gram-negative, aerobic but fixes nitrogen in microaerobic conditions. It does not use tricarboxylic acids to grow and is adapted to conditions of high osmolarity and sucrose content (10–30%). In addition, its cultivation in presence of high sugar content revealed that nitrogenase activity is only partially inhibited by the addition of ammonium to the culture medium [85]. It shows high acidity tolerance, fixes nitrogen in presence of nitrate concentrations greater than 10 mM, and reduces the deleterious effect of oxygen concentration to the nitrogenase activity using oxidative metabolism in the periplasmic space at membrane level [3, 86].

Genetic traits. Studies of genetic characterization in *G. diazotrophicus* started in the early 90s. The first report of the chromosomal localization of nitrogen fixation genes and presence of plasmids in *G. diazotrophicus* strains was presented during the 6th International Nitrogen fixation with non-legumes in Egypt [87]. Later on, the *nif* genes, transcription regulatory genes (*nifA* and *ntrBC*) and others related to ammonium sensing and transport (*glnB*, *glnD*, and *amtB*) were sequenced and expression of some of them were studied using transcriptional *gusA* fusion [88–92]. The *nif-fix* gene organization found in *G. diazotrophicus* was similar to that of *Azospirillum brasilense*, but their products were homologous to those observed to representatives of *Rhizobiaceae* family and *R. capsulatus*, considering the GenBank data available at that time [88, 91].

In addition, accessory genes related with nitrogen sensing and metabolism were also studied by sequencing and insertion mutagenesis. At least three copies of genes coding PII-like protein were identified. The copy located upstream to *glnA* (that codes for glutamine synthetase) was named glnB, because of its homology and conserved organization on many others nitrogen-fixing bacteria. The others PII-like coding genes, glnK1 and glnK2, were located upstream of copies of genes that codes for ammonium or methylammonium transportes, *amtB1* and *amtB2*, respectively [92]. Further characterization of single and double mutants containing *gus*-fusion suggested that GlnB and GlnK1 are required as positive signals to efficiently relieve repression by GlnK2. In addition, the authors showed that GlnK2 protein clearly has a function different from those of GlnB and GlnK1, since *nif* gene expression were repressed in glnB glnK1 double mutant under all conditions tested. Based on these studies, the authors suggested that none of the three PII homologs is required for *nif* gene expression indicating novel regulatory features of *G. diazotrophicus* PII proteins. The GlnK2 protein acts primarily as an inhibitor of *nif* gene expression while GlnB and GlnK1 control the expression of *nif* genes in response to ammonium availability, both directly and by relieving the inhibition by GlnK2 [92].

Some years ago a lab consortium, named RIOGENE, announced that the genome of this microorganism was sequenced [60]. Its genome is composed of a 3.9 Mb chromosome and 2 plasmids of 16.6 and 38.8 kb, respectively. The genome size is in the average of others AAB genome already published [93–99]. Further studies of this microorganism functional genomics have been underway by several researchers group and are leading to a comprehension of the role of its gene contents to its general metabolism, nitrogen fixation, and others plant growth mechanisms [100–108]. In addition, several mutants have already been obtained and used for functional genomic characterization [100, 102–107, 109].

The expression of genes related to reactive oxygen species (ROS) detoxification (*sod*, *kat*, and *gor*) was evaluated in *G. diazotrophicus* grown under nitrogen non-fixing (NFIX) and fixing conditions to elucidate the paradox of oxygen consumption during respiration protection and ROS inhibitory effect on nitrogenase activity and *nif* gene expression [110]. They observed that growth of this microorganism under nitrogen fixing condition leads to reduction of ROS accumulation and a strong induction of *sodA*, *katE*, *kat*, *katC*, and *gorA* genes in comparison to cells grown under non-fixing condition. In addition, *sodA* and *katE* gene expression correlates with *nifD* suggesting that reduction of ROS is essential for the homeostasis during respiratory protection and nitrogen fixation in *G. diazotrophicus*. Later on, the participation of *G. diazotrophicus* detoxifying genes during the first step of root colonization was investigated using rice plants [111]. It was shown that ROS accumulates during the first steps of inoculation of *G. diazotrophicus* wild type and mutant strains as response of a plant defense mechanism. Noteworthy, they found out that GR (glutathione reductase) and SOD (superoxide dismutase) mutants of *G. diazotrophicus* could not reduce efficiently the ROS accumulated in the period of 1–7 h after inoculation. In addition, they measured PR genes expression and detected that expression of JA/ET pathway gene increased during wild type–plant interaction, but not to both mutants. These data suggest that *G. diazotrophicus* alters its redox metabolism during BNF by increasing antioxidant transcript levels to circumvent ROS inhibitory effect to nitrogenase activity. Probably this transcriptional regulatory mechanism protects nitrogenase activity during initial stages of plant colonization.

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Genes homologous to those of alternative asparagine biosynthesis pathway and its role during nitrogen fixation were investigated since free asparagine, as well as others amino acids, inhibits nitrogenase activity [112, 113]. Further, genome analyses revealed that genes of asparaginyl-tRNA and asparagine synthetase orthologs are absent in the *G. diazotrophicus* genome. However, the correlation between repression of *nifD* expression and increase of Asparagine level indicated that genes for an alternative route that converts Asp-tRNA^{Asn} into Asn-tRNA^{Asn} by a glutamine-dependent Asp-tRNA^{Asn} amidotransferase B, encoded by the operon *gatCAB*, might be present. In fact, in *G diazotrophicus* the presence of *gatCAB* operon indicates that the ORF GDI2232 encodes an Aspartyl-tRNA synthetase of ND-AspRS type [113]. The role of asparagine and glutamine as N storage molecule in plant tissues, including sugarcane, is a general rule; however, their role in plant bacteria interaction is scarce and requires more investigation. The content of amino acids in sugarcane sap, apoplast, and symplast has been evaluated and shows that Asn is one of the most abundant [114, 115]. It is known that Asn and Gln are amino acids that repress nitrogen fixation *in vitro*. Noteworthy, the presence of ORFs coding for putative L-asparaginase precursor (GDI3138), L-asparaginase II protein (GDI1250), and ORFs coding putative (aspartate) aminotransferase in *G. diazotrophicus* genome may represent an adaptive advantage to its endophytic behavior. Nonetheless, a detailed study is necessary to justify this assumption.

Kerby and Roberts [116] identified in *G. diazotrophicus* genome the presence of two ORFs coding predicted proteins similar to *R. rubrum* CowN (CO weal-nitrogenase) and transcriptional regulator CooA, which belongs to the Crp/Fnr family. The ORFs GDI_3488 and GDI_3487, previously annotated as hypothetical protein [60], share common features of organization and motifs to *cowN* and *cooA*, respectively. Interestingly, the characterization of these genes in *R. rubrum* using PSI-Blast searches revealed that they are widespread and generally presents similar organization in nitrogen-fixing bacteria. The importance of CooA and CowN for Mo-nitrogenase-dependent functioning in the presence of CO was shown to *R. rubrum* and *R. capsulatus*, but not to the Fe-dependent nitrogenase of the latter [116, 117]. The authors suggested that CooA and CowN may act as a two component system that senses and modulates the Mo-dependent nitrogenase activity by protecting it in the presence of CO in *R. capsulatus* and also in many others nitrogen fixing organisms. However, how this protection mechanism works and its relevance to nitrogen fixation is not yet understood.

Here, we presented some examples of the functional genomics studies that have already been published about *G. diazotrophicus*, but many others have already been thoroughly reviewed or are underway [118, 119].

Colonization. It is considered an endophytic bacterium because it has low rate of survival in the soil and was found colonizing the intercellular space of plant tissues of sugarcane [76, 77, 120, 121]. This bacterium is located in different parts of the plant as described by Reis *et al.* [79] and James *et al.* [120]. They demonstrated during "*in vitro*" inoculation studies under controlled conditions, that *G. diazotrophicus* enters into sugarcane micropropagated plants through the tissue of secondary roots then the bacteria penetrated inner tissues and colonize the intercellular spaces (apoplast). Other possible points of infection are wounds and the stomata of sugarcane plants [121]. It also colonizes tip of roots and root hairs of other plants such as wheat, sorghum, and rice as showed using reporter genes [123, 124]. At field conditions, the main route of transmission is by vegetative multiplication of stem pieces of sugarcane, although the trash should also serve as an alternative inoculum source when incorporated into the soil [79]. Another possibility to introduce this bacterium in plants appears to be related to the phloem sap sucking by the insects (mealybugs) presenting this species in the lymph and living within sugarcane leaves sheath pocket [82]. Caballero-Mellado and Martínez-Romero [83] hypothesized that these insects and also micorrhyzal spores could be responsible for local dispersion of this species within short distance while sugarcane setts and bud chips used to propagate sugarcane could carry the bacteria to further distant geographic regions. No further evidence that these insects are responsible for the dispersal of *G. diazotrophicus* species is reported, although it is plausible that this occurs. Unfortunately, ecological studies are underemphasized nowadays and this data is not available to a great number of newer described species.

Oliveira *et al.* [125] observed colonization of micropropagated sugarcane using this species in combination with four other strains of diazotrophs. Promising results were obtained when micropropagated sugarcane plants were inoculated with the type strain PAL5 in combination with small doses of nitrogen as shown by Moraes and Tauk-Tornisielo [126]. Oliveira *et al.* [125] showed that the combined inoculation of five endophytic diazotrophs promotes a synergistic effect when compared with the individual bacterial inoculation in micro-propagated plants of sugarcane in pots and later at field conditions where increases of up to 30% in the accumulation of N via BNF were observed in two varieties of sugarcane planted in three soil types [127].

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Plant growth-promoting strategies. Sevilla *et al.* [128] described the contribution of inoculation with *G. diazotrophicus* in the nutrition of sugarcane and found that other factors influence on plant growth, such as growth regulators production. The ability of *G. diazotrophicus* to fix nitrogen and growth promotion of sugarcane was evaluated by comparing plants inoculated with the wild type (PAL5^T) and an Nif mutant (MAd3A — carry a *nifD* mutation) in two experiments [128]. Both, the type strain and the mutant, colonized sugarcane plants and persisted in mature plants. Under conditions of nitrogen deficiency, plants inoculated with PAL5^T generally grew better and had a higher content of total nitrogen 60 days after planting when compared to plant inoculated with the Nif mutant. These results indicate that the transfer of fixed nitrogen from *G. diazotrophicus* to sugarcane may be an important mechanism for the growth promotion in this association. When nitrogen was not limiting, the stimulation of growth was also observed in plants inoculated with both bacteria suggesting an additional effect of *G. diazotrophicus* inoculation related to growth promotion [128]. This contribution to growth was also observed by Riggis *et al.* [129]] *G. diazotrophicus* was inoculated into maize plants. Among plant-growth substances produced by *G. diazotrophicus*, the indole acetic acid and gibberellins A1 and A3 are phytohormones that act on plant root growth and development of aerial part tissues [130, 131].

G. diazotrophicus synthetize gluconic acid by the extracellular oxidation of the glucose by the action of the enzyme glucose dehydrogenase (GDH-PQQ), localized in the perisplasmic space [86, 132], leading to the production of gluconic acid. This mild non-corrosive acid can, besides lowering the pH, promote chelation and exchange reactions and has been associated with phosphate and zinc solubilisation/chelation by *G. diazotrophicus* [133–138]. Saravanan *et al.* [139] observed that *G. diazotrophicus* grown in Zn-amended broth suffers deformation leading to pleomorphic, aggregate-like cells. Noteworthy, characterization of a mutant unable to grown in the presence of Zn, Co, and Cd salts revealed that the product of *czcA* gene is responsible for *G. diazotrophicus* resistance to these heavy metals [140].

Biological control. Another promising effect of *G. diazotrophicus* inoculation is related to the biological control of other microrganisms, such as *Xanthomonas albilineans* [141, 142], *Colletotrichum falcatum* [143], *Helminthosporium* spp. [144], and *Fusarium* spp. [145]. By cDNA-AFLP analysis, some plant genes (using leaf tissue) involved in biocontrol activity was identified [108]. These results indicate that inoculation stimulates genes involved in plant defense such as genes controlling the ethylene defense pathway. This pathway is activated when sugarcane micropropagated plants are inoculated with endophytic bacteria [146–148]. Even nematodes can be controlled by inoculating *G. diazotrophicus* as demonstrated by Chawla *et al.* [149] that used the isolate number 35–47 to control *Meloidogyne incognita* in cotton.

Gluconacetobacter johannae and *Gluconacetobacter azotocaptans*

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 [51]. Later on, *G. azotocaptans* was also isolated from the rhizosphere of corn [144].

Little information is available about other AAB-N_2-fixing plant interaction. In the case of *G. azotocaptans*, Mehnaz and Lazarovits [144] conducted a trial inoculating plants of four varieties of maize in a greenhouse experiment using sterile soil substrate. The inoculation consisted of *G. azotocaptans*, *Azospirillum lipoferum*, and *Pseudomonas putida*. At 30 days after planting, the authors observed greater root growth and dry mass of the aerial part of the inoculated pots and also observed that some of the strains isolated from the rhizosphere of maize in Canada presented significant plant growth expressed as increased root/shoot mass compared with non-inoculated plants in sand and/or soil, depending on the combination of bacteria and maize variety tested.

Nitrogen-fixing species in *Komagataeibacter* genus

The genus *Komagataeibacter* was proposed to group members of *Gluconacetobacter* species that cluster closely to *G. xylinus* [150]. Most of representatives of this new genus are known to be of industrial application but two of them are also nitrogen-fixing bacteria.

The species *Gluconacetobacter kombuchae* considered a heterotypic synonym of previously named *G. hansenii* [48] was lately reclassified as *Kamagataeibacter hansenii.* It was isolated during a survey of bacteria associated to Kombucha tea together with *Acetobacter nitrogenifigens* [52]. Kombucha tea is a fermented beverage that contains an association of yeast and bacteria that takes 7–10 days to be prepared. The authors utilized aliquots of the final preparation of the tea to inoculate solid plates containing LGI medium described by Cavalcante and Döbereiner [3] but with final pH 4.5 for its isolation. This bacterium also grows in the presence of 30% of glucose or sucrose and can produce cellulose. Sequences deposited at GenBank and described as partial *nifA*

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(EF620555) and *nifH* (DQ141200) coding regions do not share identities/similarities to deduced amino acids of others nitrogen fixing bacteria, as indicated by blast analysis.

Komagataeibacter (Gluconacetobacter) kakiaceti was isolated by Iino *et al*. [62] from traditional kaki vinegar (produced from fruits of kaki, *Diospyros kaki* Thunb). Recently, the genome of the *K. kakiaceti* JCM 25156 was sequenced and revealed presence of genes homologous to *nif* and other regulatory proteins related to N-metabolism. Its whole genome sequence (WGS) is available at GenBank (scaffolds accession number NZ_BAIO01000001-NZ_BAIO01000947). However, up to date no further experimental evidence of N-fixation by this species was reported.

Swaminathania

Swaminathania salitolerans was classified as a new genus and new species by Loganathan and Nair [53]. These authors identified new isolates tolerant to salinity stress using rhizosphere, roots, and stems of mangrove-associated wild rice plants (*Porteresia coarctata* Tateoka). The medium used to obtain these new isolates was a semisolid LGI culture medium without the addition of nitrogen, final pH 5.5 with the addition of 250 mM NaCl. Samples were collected in the city of Tamil Nadu in India, where 41 isolates were obtained and identified as rods, Gram-negative, mobile, and with peritrichous flagella. Strains grew well in the presence of increasing concentrations of acetic acid (0–35%) in a very acid pH, 3.5 and also could grow in the presence of 3% NaCl and 1% KNO_3. Isolates were able to fix nitrogen and solubilized phosphate in the presence of this level of salt, mimicking the location of its isolation. The colonies grown on LGI medium are initially yellow orange but become darker after aging, smooth, and raised margin, characteristics commonly found in this bacterial family. In this case, there is a description of fixing species using Acetylene Reduction Assay (ARA) to estimate nitrogenase activity during growth in semi-solid LGI medium, besides PCR amplification of *nifD*. But no further works using this bacterial species were published and therefore its agricultural importance is unknown.

Asaia

The *Asaia* genus was first described with a single species *A. bogorensis* and then six more species were included: *A. siamensis*, *A. krungthepensis*, *A. lannaensis*, *A. platycodi*, *A. prunellae*, and *A. astilbes* [40, 61, 151, 152]. These strains produce low quantities of acetic acid from ethanol and grew in medium with dulcitol as the sole carbon source, indicating that they belong to the genus *Asaia* [54]. The isolates also grow on LGI containing 10% sucrose as carbon source (LGI-P), but in this case it is acidified to pH 5.0 with acetic acid. Interestingly, this genus includes species described from samples taken from the interior of insects as mosquito *Anopleles* and *Plasmodium* that are vectors of malaria and dengue fever and cause of its sanitary importance, many studies describe the presence of various species of mosquitoes in association with this group of bacteria [153].

In India, a study was performed in order to isolate diazotrophs from three different samples: one flower called *Michalia champaca*, from the *Anopheles* mosquitoes, and from ants, such as in *Tetraponera rufonigra*, *Pseudomyrmex*, *Cephalotes*, and *Paraponera* [54, 154]. It is important to emphasize that these isolates where obtained using N-free LGI medium as described by Magalhães *et al*. [155] to isolate *Azospirillum amazonense* modifying the final pH of the medium from 6.0 to 4.5 replacing sulfuric acid by acetic acid. The same medium was used to characterize the *G. diazotrophicus* species in 1988 [3]. Samaddar *et al*. [54] isolated *Asaia* spp. as endophytes based on the surface disinfection of the plant tissue and confirmed their ability to fix nitrogen by using ARA to estimate nitrogenase activity, amplification, and sequencing of *nifH*-like gene. *Asaia bogorensis* (MTCC 4041^T), *A. siamensis* (MTCC 4042^T), and *A. platycodi* AS6 strains were positive to these tests. They formed pink colonies, shiny, smooth, with entire margin in agar plates containing AG medium composed of D-glucose (0.1%), glicerol (1.5%), peptona (0.5%), yeast extract (0.5%), malt extract (0.2%), $CaCO_3$ (0.7%), and agar (1.5%) as described by Yamada *et al*. [40]. This pigmentation increased after prolonged incubation at 4 °C. All of these isolates were classified as aerobic grow at pH 4.5 and 30 °C. Acetate and lactate are oxidized to carbon dioxide and water but the activity was considered low. Partial sequences of *nifH*-like genes from several isolates and *Asaia* type strains were obtained and, as well as their genomes [63], are deposited at GenBank [54]. Noteworthy, up to date blast analysis revealed that these sequences blast only among them and with several partial *nifH*-like sequences from unculturable clone or chlorophyllide reductase subunit X (*bchX*) partial gene sequences from several microbial genome or unculturable clones (Fig. 1).

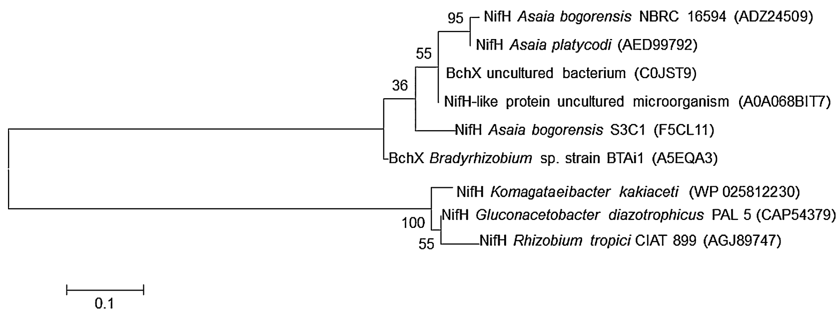

Figure 1. Maximum Likelihood tree of partial NifH and BchX proteins selected from protein sequences blast analysis using *nifH* gene deduced protein from *G. diazotrophicus* and *Asaia bogorensis* as query. Maximum Likelihood method was based on the Dayhoff matrix model in MEGA5 [156]. The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrap values).

Acetobacter

Although this genus is the oldest of *Acetobacteraceae* family, the description of diazotrophs representatives was first raised by the description of *Acetobacter diazotrophicus* in the 80s. However, based on detailed taxonomic and phylogenetic studies, this species was reclassified and renamed into the genus *Gluconacetobacter*. No other species of the genus *Acetobacter* had been described as diazotrophic until 2005 when Muthukumarasamy *et al.* [57] presented various isolates belonging to *A. peroxydans* that fix nitrogen. Most of the isolates were obtained from samples of flooded rice cultivated in India but studies of *nifH* amplification and ARA confirmed that even in the type strain of *A. peroxydans* LMG 1635^T these characteristics were present [57]. Shortly thereafter, another study presented the description of the second species of nitrogen-fixing *Acetobacter* named *A. nitrogenifigens* based on isolates obtained from Kombucha tea in India [52].

The nitrogen fixing *Acetobacter* species *A. nitrogenifigens* shows polar flagella similar to those of *Gluconobacter* [52]. It also produces brown pigment and γ-pyrone compounds, suggesting that 2-ketogluconate and 2,5-diketogluconate are also produced as found in *Gluconobacter* and *Gluconacetobacter* [18, 86, 157]. Although claimed as positive for ARA, the *A. nitrogenifigens* RG1 partial *nifH* sequence deposited at GenBank (AY952470) do not blast with any *nifH* coding protein as also observed to *K. hansenii* (RG3).

An overview of the source of isolation and data about nitrogenase activity and *nif* genes to all these species are shown in Table 2.

Table 2. Source of isolation and characterization of nitrogen fixing bacteria belonging to *Acetobacteraceae* family.

Genus	Species	Source of isolation	ARA	Molecular data
Gluconacetobacter	*G. diazotrophicus*	Sugarcane, roots, and stems	+	WGS
	G. johannae	Coffee plants, rhizosphere, and rhizoplane	+	*nifH* sequence
	G. azotocaptans		+	
Asaia	*A. siamensis*	Tropical flowers	+	*nifH* sequence[b]
	A. bogorensis	Flowers (*B. purpurea* tree and plumbago), fermented glutinous rice	+	
	A. platycodi	Ballon flower (*Platycodon*grandiflorum)	+	
Swaminathania	*S. salitorerans*	Mangrove associated wild rice rhizosphere, roots, stems, and leaves	+	*nifH* amplification
Komagataeibacter	*K. (kombuchae) hansenii*	Kombucha mat suspension	n.s.	*nifH* sequence[c]
	K. kakiaceti	Sample of vinegar suspension from kaki fruit	n.d.	*nifH* blast analysis
Acetobacter	*A. nitrogenifigens*	Aliquots of Kombucha mat suspension	n.s.	*nifH* sequence[c]
	A. peroxydans	Wetland rice varieties, rhizosphere, roots and stems	+[a]	*nifH* amplification

n.d., not determined; n.s., data not shown.
[a]Observed values were low and inconsistent.
[b]Blast among *Asaia* spp *nifH* sequences but not to other *Acetobacteraceae*.
[c]Do not blast with any *nifH* sequence in GenBank. Data based on previous reports [3, 39, 46, 50, 51, 53–63].

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Agricultural applications

The agricultural application of species and strains belonging to *Acetobacteraceae* family will be based almost entirely in a single species, *G. diazotrophicus*. This species is the oldest described and characterization of its agricultural potential to important crops like sugarcane was quite widespread in Brazil and other countries. For the other nitrogen fixing species descriptions of use are rare or no report of agricultural application is available.

The use of diazotrophs in agriculture has been explored for over 30 years and its apex in the past century, the decade of 80–90, then the description of *G. diazotrophicus*. One of the first reports that populations of diazotrophs could be affected by increasing doses of N-fertilizer was made by Vose *et al.* [158] in sugarcane which showed that high levels of mineral N caused a significant reduction in the acetylene reduction activity, very popular method which measures the indirect activity of the nitrogenase enzyme acting as a competitive inhibitor. This effect was believed to inhibit this enzyme synthesis. In 1993, after the description of *G. diazotrophicus*, studies conducted in Mexico by Fuentes-Ramírez *et al.* [159] reported that the association between *G. diazotrophicus* and sugarcane could be severely limited by high N-fertilization, which would explain the decrease in acetylene reduction activity. In their study, the crops fertilized with 120 kg N ha^{-1} showed higher number of isolates than the plots fertilized with 300 kg N ha^{-1}, levels not applied in Brazil. Muthukumarasamy *et al.* [160, 161] obtained similar results in India for *G. diazotrophicus*. They suggest that this effect was not directly related to the presence of high levels of nitrogen fertilizer in sugarcane crop since this bacterium is able to grow and fix nitrogen "*in vitro*" in presence of high concentrations of NO_3 (60 mM). It is more likely that at these high N doses the physiological state of the plant undergoes changes and subsequently influences negatively the population of this organism. Muñoz-Rojas and Caballero-Mellado [162] observed a negative effect on *G. diazotrophicus* population in the presence of high doses of nitrogen appled in sugarcane planted in Mexico. These results were confirmed by Reis Jr. *et al.* [163] using two sugarcane varieties planted in a sand soil fertilized with 300 kg de N ha^{-1} in comparison with the control without N application. Only the variety SP792312 presented plant with high levels of total N in the fertilized plots and lower numbers of *G. diazotrophicus*. Medeiros *et al.* [164] utilized different sources of nitrogen and observed that *G. diazotrophicus* reduced acetylene reduction activity in the presence of high levels of N.

Studies conducted in India with the application of *G. diazotrophicus* were repeatedly evaluated by Suman *et al.* [153–155]. They reported that the population of *G. diazotrophicus* was influenced by increased doses of N-fertilizer and that N efficiency in sugarcane increased in the presence of *G. diazotrophicus* inoculation in greenhouse experiments [166]. Later on, Suman *et al.* utilized one strain of *G. diazotrophicus*, named IS100, besides strains of *A. brasilense* and *Azotobacter chrococcum* to evaluate nitrogen efficiency applied in increased doses on sugarcane planted in India [167]. *G. diazotrophicus* showed the best results of crop yield, followed by its combination with *A. chrococcum* and *A. brasilense*.

Application of *G. diazotrophicus* was also evaluated in the germination of stem pieces of sugarcane by De la Cruz *et al.* [168] in Philippines. These authors tested inoculation with different cell densities (10^8, 10^{10}, and 10^{12} cells ml^{-1}) and methods of application (spray, immersion for 2 h and dipping during 2 min). They observed that inoculation led to increase in percentage survival plant height and shoot/root biomass when compared to the control at 45 days after planting. Introduction of microbial inoculant in 10^{12} ml^{-1} cells by immersion method produced taller plants with greater biomass and root compared to other treatments and uninoculated control.

Strains of *Gluconacetobacter diazotrophicus*, *Azospirillum amazonense*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, and *Burkholderia tropica* species were applied in sugarcane using pots filled with 60 kg of soil and also field experiments planted in three different soil types in São Paulo and Rio de Janeiro states of Brazil showing contributions of the biological process with higher crop yields of different varieties SP70-1143, SP81-3250, RB72454, RB867515 [125, 127, 169, 170]. Oliveira *et al.* [127] used the technique of $\delta^{15}N$ (natural abundance of ^{15}N in the soil) and tested seven types of inoculants and found that the inoculant containing five strains described above showed the best results. These authors also quantitated the contribution of BNF showing that the mixture of five strains obtained 29.2% of the accumulated N derived from the air. Schultz *et al.* [170] utilized the same five strains and modifications of the $\delta^{15}N$ method of BNF quantification of soil applied in the sugarcane yield of RB72454 and RB867515 varieties and showed that plant biomass increased, but found no contributions of nitrogen fixation process by the inoculation. In order to understant how the sugarcane was colonized by this mixture, a fluorescent in situ hybridization (FISH) analysis based on rRNA-targeted oligonucleotide probes confirmed that in micropropagated sugarcane inoculated with this

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mixture of five species reached the endophytic habitat of micropropagated sugarcane plantlets through active infection of the root cap and emerging zone of secondary roots, although with different efficiencies due to apparently different competitiveness for colonization [133].

Maheshkumar *et al.* [133] observed that this species was able to solubilize rock phosphate "*in vitro*," and it could be one of several effects that can promote plant growth after inoculation. Sugar beet has also been used to check the response of *G. diazotrophicus* inoculation as described by Jambukar and Wange [155]. In 2009, Tian *et al.* [171] observed the effect of *G. diazotrophicus* inoculation in different maize genotypes, 17 hybrids, and 10 sweet corn varieties planted in Canada. Colonization of 11 hybrids and 9 sweet corn varieties by *G. diazotrophicus* was confirmed using species specific primers, but populations were quantified only in the order of 200–3000 cells g^{-1} of plant tissue.

G. diazotrophicus is known worldwide for nitrogen fixation but this is only one of its mechanisms of interest for agriculture and other industrial processes. For example, *G. diazotrophicus* strain SRT4 has genes for the production of levan-sucrase both endo and exo levanases which are expressed under stress conditions [172, 173]. Another product of its growth is bacteriocins that may act to control growth of other microorganisms or even other strains of the same species [174]. This bacteriocin is constitutively expressed in different conditions of culture medium and dependent on the strain tested.

However, is there *G. diazotrophicus* as commercial product for use in agriculture? The answer is yes. Descriptions of products containing *G. diazotrophicus* can be found elsewhere. In Argentina, the ene-2 Endophyte-Plus® sold by the company ARBO SRL Laboratory, is recommended to be applied as an inoculant for wheat, maize, soybean, and tomato (http://www.arbolab.com.ar/es/productos/2lvl/prom.html). In Mexico, the company Agro Organics GAIA sells a product containing a mixture of *G. diazotrophicus* and the fungi *Penicillium* called Glubac® (http://www.organicosgaia. com.mx/biotransferentes-de-nutrientes.html).

In Brazil, origin of the *G. diazotrophicus* description, a patent based on a microorganism species or strain is not allowed, but to several other countries it is. In the United States, there is a patent for use of several nitrogen fixing bacteria, including *G. diazotophicus*, to enhance plant growth in cereals (US 7393678 B2), and other claiming its use to reduce N fertilization in sugar rich plants, especially sugar beet (20110225679), showing that it can be part of a product for agricultural use. In Brazil, bioprocess can be a matter of patent claims, such as the growth conditions of this bacterial to the production of biomass and fermentation products (PI0917666-7 A2).

However, a good product for the industry needs to possess a long shelf-life in order to reduce the costs and facilitates the distribution. Unfortunately, a few studies have developed vehicles and protective substances that increase the longevity of cells of this species. Nita *et al.* [175] tested several substances such as cell protective for *G. diazotrophicus* under different temperatures (4 and 25 °C). Efficacy was evaluated in tests of wheat inoculation under greenhouse and field conditions. The best method tested was the application of molasses (cane syrup) with 0.1% (w/v) of NH_4Cl. Trehalose, Arabic gum, and Polyethylene glycol 300 (PEG 300) presented the best results. Addition of L-ascorbic acid (0.02% w/v) to the preservation medium also enhanced the efficacy of the substances used as protectors. After 8–9 months of stock ay 4 °C, *G. diazotrophicus* (strain L1) showed the best results of shelflife in the presence of arabic gum (5% w/v) and PEG 300 (5% w/v), respectively, and also keeping the growth promotion effect. Silva *et al.* [176] tested a polymer based on carboxymethylcellulose on the survival of *G. diazotrophicus* strain PAL5^T with a shelf life of 10^9 cell ml^{-1} for 120 days.

To date, data about agricultural application of other nitrogen fixing *Acetobacteraceae* are restricted to *Asaia* and it is based on a single report of Weber *et al.* in 2010 [177] that utilized a single strain of *A. bogorensis* (AB 219) as inoculant for pineapple and monitored colonization by using agar plates of JNFb medium (malate as a carbon source and final pH 5.8) and population by the Most Probable Number (NMP). The growth and fruiting of pineapple were benefited from the inoculation of *A. bogorensis* (strain 219) associated with irrigation and increasing doses of organic fertilizer (compost).

Final considerations

Since the discovery of *Gluconacetobacter diazotrophicus* in 1988, many other diazotrophs belonging to the family *Acetobacteraceae* were described as nitrogen fixing species, but this number can increase. The strategy to isolate and identify new species generally is not based on criteria of biological nitrogen fixation ability and this character is not a discriminatory one. Interestingly, we observed that some researcher groups used the nitrogen free LGI medium as a strategy to obtain new isolates. It is expected that using N-free medium during isolation process can enrich populations of nitrogen fixing

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bacteria leading to recovery of many of them from environmental samples. In addition, the original pH of LGI was 5.5, but several new species were described with a simple modification of the final pH to levels lower than 5.0. Since representatives of this family are adapted to acidic environment, lowering pH can be considered another strategy used by many authors to isolate and describe new species of nitrogen fixing *Acetobacteraceae*.

It is noteworthy that as many as new species have been described over the last 25 years, publications containing studies of their ecology and distribution diminished considerably. *G. diazotrophicus* is the most studied nitrogen fixing bacteria of this family for agricultural application. Since the beginning, several studies describing its survival, habitats, mode of plant colonization, and transference to new hosts have accumulated in the literature. Based on them, the description of *G. diazotrophicus* as a true endophyte was proposed and accepted. The endophytic behavior of *G. diazotrophicus* is based on many ecological surveys and studies while these data are lacking to other species described. Actually, nowadays publications of ecological research are an exception when we compare with the increasing numbers of species description mainly based on a set of physiological and molecular data, small numbers of specimens or even only one representative.

Nitrogen fixation is a biological processes well characterized and understood, at to some points, in pure culture and *in vitro* that occurs when an appropriate energy source is available in combination with the optimal temperature, pH and controlled O_2 concentration. Nonetheless, it is not an easy task to really prove that a single strain is responsible for part of the assimilated nitrogen in plant, especially under field conditions.

In general, the main effects that are easily identified in plants that establish association with non-symbiotic nitrogen fixing bacteria are root surface enhancement, increased grain production, and early maturation. Besides nitrogen fixation ability, *G. diazotrophicus* also produces growth hormones such as auxins and gibberellins and also can be considered PGPB when compared to *A. brasilense*. In agricultural perspective the application of *Acetobacteraceae* species can contribute to plant growth and improve nitrogen assimilation of the host plant. However, under field conditions the effect of the number of bacteria in the plant versus the contribution of biological nitrogen fixation and/or plant growth promotion is not clearly established. It is already reported that introduced populations can undergo changes not only on their physiological aspects, but also in genomic aspects. So far, over more than 30 years of studies most of the knowledge of these aspects of the bacterium–plant interaction is based on analysis conducted under controlled laboratory conditions.

Although the centennial knowledge of this versatile bacterial family to industrial application, a lot has to be done about the potential of the nitrogen fixing *Acetobacteracea* to agricultural application and even to many other industrial biotechnological processes is limited yet. To improve agricultural use or even to broaden the industrial purpose of these nitrogen fixing *Acetobacteraceae* species depends on development of new biotechnological data.

For development of new biotechnological application and products, it will be necessary to increase knowledge and exploit the genomic potential for adaptation, competition, and survival of these bacteria. Introdution of certain species to different plants and/or environmental conditions has to be explored. Besides, the demand of development of methods, easily applied under field conditions, for bacterial inoculation, monitoration, and validation must be constantly considered. In addition, further efforts to characterize the ecology related to these microorganisms and plant relationship is essential. Selection or development of genetically modified bacteria adapted to field competition, stress, and interaction with other components of the microbiota will be one of the goals to improve the inoculation technology worldwide.

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