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Non-nodular Endophytic Bacterial Symbiosis and the Nitrogen Fixation of *Gluconacetobacter diazotrophicus*

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Abstract

There is a need to reduce the negative polluting influence of mineral nitrogen fertilizers and to develop a more sustainable climate smart agriculture capable of meeting our future food security needs. Biological nitrogen fixation can have a role in this if it can be applied to the major food crop plants. Certain strains of the obligate nitrogen-fixing bacterial endophyte *Gluconacetobacter diazotrophicus* have the necessary attributes for this role. An 'extra-ordinary endophyte' this bacterium is one of relatively few that has mechanisms to cope with high levels of sucrose, an acidic pH, a wide range of oxygen environments, nitrogen fixation, as well as having respiratory chain attributes that make it a possible candidate eukaryote proto-mitochondria. Having a small genome relative to other endophytes, it is typical of facultative intracellular colonizers, with a life cycle that involves horizontal transfer to other high sucrose species via insects and potential vertical transfer through seeds. Every method used for demonstrating nitrogen fixation in rhizobia have been used to demonstrate nitrogen fixation in *G. diazotrophicus* both *in vitro* and *in planta*, and field trials demonstrate yield increases and the potential to reduce nitrogen fertilizer use, meeting both food security and climate smart agriculture needs.

Keywords: nitrogen fixation, *Gluconacetobacter diazotrophicus*, bioenergetic systems, cereals, non-nodular symbiosis, facultative colonization, intracellular colonization, endophyte, yield impact, food security, climate smart agriculture

1. Introduction

Sugarcane, *Saccharum officinarum*, is grown in many parts of the world for processing into cane sugar. In Brazil, a primary driver for growing sugarcane has been ethanol production for use as a sustainable substitute fuel for petrochemicals. For many years, and in deed for decades,

Brazilian sugarcane had been produced in the same regions with little use of nitrogen fertilizers, without any apparent loss in yield [1]. This led to speculation that the crop was benefiting from biological nitrogen fixation (BNF). In an experiment using labeled nitrogen, it was demonstrated that the sugarcane variety CB 47-89 derived around 60% of its nitrogen from a biologically fixed source [2]. Subsequent to this, studies confirmed that some varieties of Brazilian sugarcane were capable of obtaining 60–80% of their nitrogen requirements from BNF, highlighting the possibility that under the right conditions, it might be possible to dispense altogether with nitrogen fertilizers for these varieties [1, 3]. The bacteria thought to be responsible for the BNF was a new species, *Acetobacter diazotrophicus* [4] discovered in 1988 by Vladimir Cavalcante and Joanna Döbereiner in Alagoas, Brazil [5]; initially named *Saccharobacter nitrocaptans* and later renamed *Gluconacetobacter diazotrophicus* [6].

2. A review of the key aspects of the symbiosis of the endophyte *Gluconacetobacter diazotrophicus*

The nature of the symbiosis of the endophytic nitrogen-fixing bacteria *G. diazotrophicus* has increasingly become a subject of scientific inquiry because of its potential for reducing nitrogen fertilizer use in cereals and other major food crops, its extra-ordinary attributes and capabilities relative to other endophytes and nitrogen fixers, its life cycle and its ability to fix nitrogen under a range of circumstances.

2.1. Demonstrated impact of *G. diazotrophicus*

The ability of *G. diazotrophicus* to fix up to 80% of the sugarcane plants nitrogen requirements is significant in agriculture terms, not least if this capability could be transferred to other grass and cereal species. The drive to find a means of introducing BNF in non-legumes, particularly through the ability to transfer nodulation to non-leguminous cereal crops, had been an important focus of research since the 1970s [7]. The primary reason for this was the need to produce more climate smart, sustainable systems of agriculture that are less reliant on inorganic nitrogen fertilizers produced via the Haber Bosch process.

The 500 million tonnes of ammonia produced each year through this process in order to meet the needs for nitrogen fertilizer account for 1% of the world's energy usage and 3–5% of natural gas usage [8]. However, crops use only an estimated 30–50% of the nitrogen fertilizer applied to the soil. The remainder is lost, either to the atmosphere as nitrous oxide gas or into waterways as nitrate run-off. Nitrogen fertilizer use accounts for around 66% of UK agricultural nitrous oxide emissions contributing to climate change [9], while nitrate run-off contaminates drinking water, with 5% of the European population exposed to unsafe levels [10].

Despite the obvious need to find sustainable solutions for future more climate smart agriculture, it is now generally acknowledged that the promise of BNF through rhizobial-based root nodulation in non-leguminous plants has not been realized [7]. Unfortunately, it is also not currently considered possible in cereals without further years of genetic manipulation [11]. Alternative approaches however, based on the findings relating to *G. diazotrophicus* in Brazil

in sugarcane offer some prospect for the development of non-legume crop symbiotic nitrogen fixation, not only to increase crop yields but also to potentially reduce nitrogen fertilizer use, and this prospect is now beginning to be realized [12].

Apart from fixing atmospheric nitrogen, diazotrophic bacteria such as *G. diazotrophicus*, can affect plant growth directly by the synthesis of phytohormones and vitamins, improved phosphate and nutrient uptake and enhanced stress resistance [13]. It has been demonstrated that strains of *G. diazotrophicus* differentially affected growth parameters of sugarcane, with some strains improving germination, tiller number and plant height relative to others [14] and there is also evidence that *G. diazotrophicus* improves tolerance to the sugarcane pathogen *Xanthomonas albilineans* as a result of production of bacteriocin; as well as reducing galling caused by root knot nematodes (*Meloidogyne incognita*) in bottle gourds and cotton [15]. *G. diazotrophicus* has also been shown to enhance photosynthetic capability and water use efficiency [16] and in sorghum increased chlorophyll and leaf nitrogen [17].

Inoculation of crop plants with *G. diazotrophicus* has been shown to increase crop yields in tomato [18], in sugar beet [19] and increased both the shoot and root dry weight of sorghum [20]. However, more significant yield enhancement has been demonstrated in recent independent field trial research utilizing proprietary NFix® technology (Patent Number: WO2016/016629) based on *G. diazotrophicus* of around 1 tonne per hectare in both maize and wheat (**Figures 1** and **2**) at any level of nitrogen fertilizer [12, 21].

These levels of plant yield improvement are somewhat surprising and suggest a close symbiotic relationship and multiple plant benefits from the association with *G. diazotrophicus*. Joanna Döbereiner even referred to *G diazotrophicus* as "this extra-ordinary endophyte" but perhaps even Döbereiner would be surprised by the level to which the bacteria she was jointly responsible for discovering [5], is truly extra-ordinary.
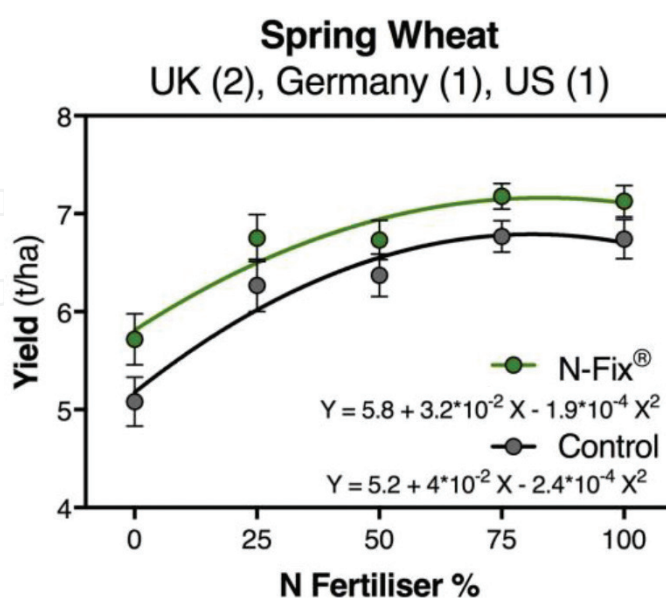

Spring Wheat
UK (2), Germany (1), US (1)

N-Fix®
$Y = 5.8 + 3.2*10^{-2} X - 1.9*10^{-4} X^2$
Control
$Y = 5.2 + 4*10^{-2} X - 2.4*10^{-4} X^2$

Figure 1. For spring wheat across sites (2015: UK, 2016: UK, 2017: Germany, US) and N levels, N-fix® inoculated seed increased yield by 7% (460 kg/ha) and demonstrated a potential to N-fertilizer savings of up to 61% with no reduction in yield [21].
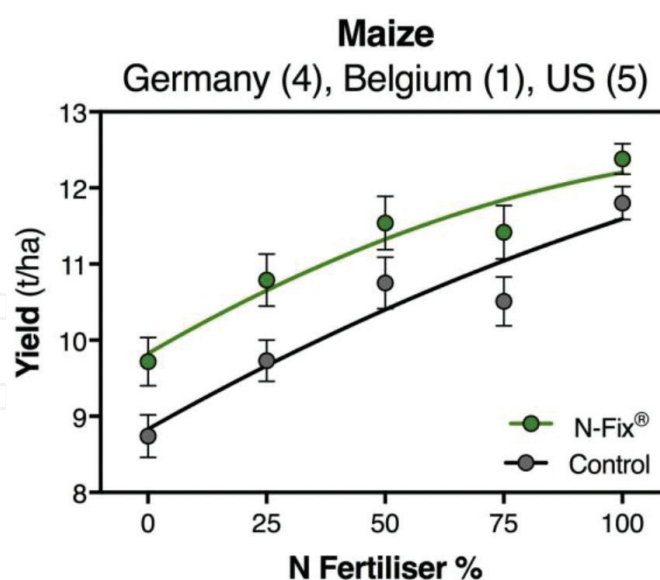
Figure 2. Combined data from 10 maize trials (2014: 4 Germany, 1 Belgium, 2015: 3 US, 2016: 2 US) demonstrated an overall increase in yield of 8% (830 kg/ha; **Figure 2**A). Estimation from second order polynomial fit, predicts that N-fix® can replace 27% of the nitrogen fertilizer inputs without yield penalty [21].

2.2. *G. diazotrophicus*: an "extra-ordinary endophyte"

G. diazotrophicus is a Gram-negative, non-spore forming, non-nodule producing, endophytic nitrogen-fixing bacterium. This bacterium belongs to the phylum Proteobacteria, the class Alpha-Proteobacteria, the order Rhodospirillales, the family Acetobacteraceae (Acetic acid bacteria; AAB), within the genus of *Gluconacetobacter* [22]. Such a phylogeny does not suggest anything particularly remarkable about the species—*G. diazotrophicus*. However, there are a number of key attributes that distinguish this bacterium from others and point to the reasons why it is able to achieve the types and levels of impact demonstrated in **Figures 1** and **2**, when colonizing crop plants. Among these attributes *G. diazotrophicus* has the ability to cope with high sucrose concentrations, low oxygen and pH levels and the ability to intracellularly colonize and fix nitrogen in a wide range of crop plants [12, 23, 24].

The availability of water is essential for the functioning of living systems and relatively few bacteria can survive and reproduce at water activity levels below 0.90 aw [25, 26]. The presence of solutes such as, salts or sugars can create an osmotically stressful environment for bacteria and relatively few species have mechanisms that allow cell multiplication under extreme conditions of <0.70 aw [26, 27]. Plant sap generally has water activity values between 0.99 and 0.96 aw (and pH 4.4–8.0); levels that are able to support a phylogenetically diverse groups of micro-organisms, including plant pathogens, plant and insect bacterial and fungal endosymbionts [27].

G. diazotrophicus is one of the relatively few bacteria capable of being cultured at very high sucrose concentrations (876 mM sucrose [28]; 30% [29]) and can tolerate a water activity level of 0.892 aw [26]. This is perhaps not surprising given its host plant, sugarcane and other high sucrose content host plants from which it has been isolated (**Table 1**), but for *G. diazotrophicus* to tolerate sucrose-induced stress, it has to have the mechanisms with which to cope. In general for bacteria, a number of osmotolerant mechanisms exist and most of these exist in *G. diazotrophicus*,

Plant family	Host plant	References
Amacariaceae	Mango	[146]
Amaranthaceae	Beet root	[146, 147]
Apiaceae	Carrot	[146, 147]
Arecaceae	Oil Palm	[148]
Brassicaceae	Radish	[147]
Bromeliaceae	Pineapple	[149]
Cactaceae	Forage cactus	[150]
Convolvulaceae	Sweet potato	[151]
Euphorbiaceae	Cassava	[146]
Musaceae	Banana	[94]
Myrtaceae	Guava	[146]
Poaceae	Cereals and grasses	[5, 90, 112, 137, 151–155]
Rubiaceae	Coffee	[94] [156]
Solanaceae	Tomato	[157]
Theaceae	Tea	[94]

Table 1. The natural host range of *G. diazotrophicus* is restricted to 19 plant species representing 15 plant families.

but also in bacteria that do not live with such high levels of sucrose. Therefore, additional mechanisms that protect *G. diazotrophicus* specifically against high sugar concentrations may also act in this species [30].

G. diazotrophicus lacks a sucrose transport system and depends on the secretion of a constitutively expressed levansucrase (LsdA), a fructosyltransferase exoenzyme with sucrose hydrolytic activity, in order to utilize plant sucrose [31, 32, 33]. Levan is implicated in sucrose tolerance in *G. diazotrophicus*. A levansucrase defective mutant of *G. diazotrophicus* demonstrated a significant decreased tolerance to sucrose compared to the wild type [33]. Osmotic pressure is regulated in many bacteria by the movement of potassium ions in to and out of the cell [34]. In *G. diazotrophicus* sucrose tolerance is, at least partially, achieved through genes encoding for the KupA protein [27]. Interestingly, however, this gene is considered only a secondary low affinity potassium transporter for bacteria generally and certainly has not been implicated in the regulation of osmotic stress [35]. Hence, this high-affinity potassium transport role of the KupA protein by which *G. diazotrophicus* regulates osmotic stress in high sucrose concentrations, is different from other bacterial species [27]. *G. diazotrophicus* seems to have a larger number of isoforms of enzymatic systems involved in osmotolerance [30].

High sucrose concentrations occur in a range of environments that may be associated with bacterial endosymbionts. In addition to the sap of the host plant other sites of high sucrose include floral nectar, plant fruits and fruit juices as well as the guts of sugar-feeding insects and the rhizosphere [27]. Studies of bacterial-insect symbiosis have demonstrated that the AAB

are capable of establishing symbiotic relationships with insects that rely on a sugar-based diet [36]. The AAB form symbiotic associations within the mid-gut of insect species representing a diverse range of Orders namely Diptera, Hymenoptera, Hemiptera and Homoptera. This insect habitat is characterized by the presence of sucrose or other diet related sugars, low oxygen concentrations and a low pH. Symbiotic associations of species of *Gluconacetobacter* have been found in fruit flies, *Drosophila melanogaster*; bees, *Aphis mellifera* and for *G. diazotrophicus*, within the gut of the sugarcane mealybug, *Saccharicoccus sacchari* [36].

While the insect gut may suit the ability of *G. diazotrophicus* to tolerate sucrose rich environments, as an aerobe, the oxygen levels in the guts of many insects may be less suitable, varying as they do from aerobic to completely anoxic [37]. However, the presence of *Gluconacetobacter* species within insect guts and of *G. diazotrophicus* in *S. sacchari*, would suggest some ability to cope with a range of oxygen environments. In a genomic analysis of 14 AAB to assess traits associated with insect symbiosis, the presence and distribution of the oxygen-reacting systems of the electron transport chain (terminal oxidases) were studied [38]. It was found that the operons of both cytochrome bo3 (CyoA-D) and bd (CydAB) ubiquinol oxidase, which have a high affinity for oxygen, were present in the genomes of all of the AAB studied, including *G. diazotrophicus*. The high oxygen affinity cytochrome bd oxidases are typically expressed by enterobacteria, intracellularly colonizing animal cells (e.g. *Brucellar suis*; [39]), which have oxygen concentrations lower than those found in the extracellular environment. Although, AAB are typically considered aerobes the capacity to live in low oxygen concentrations conferred through the ubiquinol oxidases enables endosymbionts such as *G. diazotrophicus* to survive in a range of environments, including the micro-oxic environment of the insect gut [37]. Phylogenetic comparisons demonstrate that these terminal oxidases were present in the common ancestor of AAB, thereby constituting an ancestral character [38]. In addition, the presence of reactive oxygen species (ROS) detoxifying genes in *G. diazotrophicus*, have a high similarity to related enzymes from phylogenetically distant symbiontic organisms [40]. This could be an indication that nitrogen fixation is an ancient process in *G. diazotrophicus* and was probably acquired before the adaptation to the endophytic lifestyle [30]. An obligate symbionts lifestyle necessitates a close metabolic association with its host plant. *G. diazotrophicus* antioxidant catalase genes that act to reduce the toxicity of oxygen during nitrogen fixation [40] are related phylogenetically to distant organisms that are normally isolated from plant leaves with the ability to promote the growth of various plant seedlings [41, 42]. The enzyme pyruvate decarboxylases (PDC) are rare and found in bacteria that are strongly plant associated, in which the environment contains ethanol and a low pH [43]. Their rarity suggests that the PDCs have a significant and specific metabolic role in these environments. PDCs are expressed in plants as part of the pathway of fermentation converting sugars into cellular energy under conditions of low pH caused by oxygen stress, when normal aerobic energy metabolism is not possible, for example, root water logging [44]. In *G. diazotrophicus*, PDC expression is regulated and is not constitutively expressed and it is possible that the expression of *G. diazotrophicus* PDC is also pH or oxygen dependent. It is conceivable that *G. diazotrophicus* PDC could perform a role outside the bacterial cell in support of plant cell metabolism under oxygen stress and in doing so would further deepen the symbiotic relationship between the plant and the bacterium to the point where *G. diazotrophicus* could almost be considered a "plant organelle" [43].

The study of bioenergetic systems associated with terminal oxidases, and the ability to fix nitrogen and function under a wide range of oxygen concentrations has also raised the prospect of *G. diazotrophicus* having been associated in evolutionary time scales with a key eukaryote cell organelle—the mitochondria. It has been postulated that proto-mitochondria 'bacteria' were adapted to different levels of environmental oxygen of the anoxic proterozoic oceans [45], exploiting also the terminal oxidases of facultatively anaerobic bacteria to obtain bioenergy [46].

It is logical to argue that the mitochondrial systems that generate most cellular bioenergy must define the minimal bioenergetic capacity of proto-mitochondria. Ubiquinol in the mito-chondrial respiratory chain produces most bioenergy in eukaryotic cells and shows strong similarity with that of aerobic proteobacteria [47, 48]. On this basis the maximum number of bioenergetic systems carrying out the oxidation of ubiquinol includes the bc1 complex, cyto-chrome c, cbb3, aa3, bo and bd as well as nitrogen metabolism since nitrogen compounds can function as electron acceptors for the oxidation of dehydrogenases [49, 50].

Analysis of all of the available genomes of the Alpha-proteobacteria and using a model based upon the pathways of differential loss of the six bioenergetic systems leading to the reduced subset of current mitochondria, concluded that those subsets lacking the cbb3-type oxidases probably represents the closest match for the bioenergetic capacity of the distal ancestors of mitochondria [49]. Alpha-Proteobacteria lacking the cbb3 type oxidase is typified by methy-lotrophs and the genus *Gluconacetobacter*.

2.2.1. G. diazotrophicus in comparison with other bacterial endophytes

Bacterial genomes vary a great deal in size ranging from 0.16 megabases (Mb) in *Carsonella ruddii* [51] to approximately 9.7 Mb in *Burkholderia xenovorans* [52]. Among the nitrogen-fixing endophytes the rhizobia are the most well studied, and soybean a key leguminous crop. In a systematic comparative genomic analysis of soybean micro-symbionts and other rhizobia sampled from a range of ecological zones, it was found that the average genome size of *Bradyrhizobium* strains was 9.8 ± 0.87 Mb which was significantly (P < 0.001) larger than that of nine *Sinorhizobium* genomes—6.6 ± 0.30 Mb [53]. Similarly the genome size of 48 strains of *Sinorhizobium* varied between species and strains from 6.2 to 7.8 Mb [54]. The key requirement in assessing these differences among the rhizobia has been the need to gain an understanding of the types of genome essential for nodulation and nitrogen fix-ation. In trying to define these core characteristics, the genome size of 14 strains of the Rhizobiales ranged between 4.9 Mb, exemplified by *Mesorhizobium* species, up to 9.1 Mb in *Bradyrhizobium japonicum* [55].

The intracellular environment is the main factor that correlates to genome size in bacteria [56, 57]. An analysis of 350 bacterial species genomes comparing the nature of their association with their host (early, advanced and extreme stages of adaptation) demonstrated a decreasing genome size with increasing levels of host adaptation [56]. Bacteria in an early facultative intracellular stage of adaptation tend to have a median genome size ca. 3.1 Mb, advanced obligate intracellular stages a median genome size ca 1.3 Mb and an extreme obligate intracellular mutualist, a median genome size ca. 0.7 Mb.

For plant endosymbionts a comparison of genome sizes of nine bacteria (*Burkholderia phytofirmans* PsJN, A*zospirillum* sp. B510, Klebsiella pneumoniae 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Pseudomonas stutzeri* A1501, *Enterobacter* sp. 638, *Azoarcus* sp. BH72, *Gluconacetobacter diazotrophicus* Pa15) with differing lifestyles exhibited a range in size from 7.6 to 3.9 Mb (**Table 2**), with *G. diazotrophicus* having the smallest genome ([58]; 3.9 Mb [30]). The genome size of 3.9 Mb places *G. diazotrophicus* firmly in the facultative intracellular colonizer category [56]; an intracellular colonization capability that was first demonstrated in 2006 [24]. Certain strains of *G. diazotrophicus* are capable under the right conditions to intracellularly colonize a range of crop species and this ability has subsequently been demonstrated for a range of other bacteria and host plants [59–63].

Facultative intracellular symbionts are characterized by their adaptive flexibility which is reflected in the relatively greater number of mobile genetic elements compared with obligate intracellular symbionts [56]. *G. diazotrophicus* has 4–5 times more mobile elements than other endophytes, for example, 109 transposases [30, 58], reflecting a high degree of adaptive flexibility. Such flexibility is needed to overcome constraints that include the ability to attach to host cells, entering the cytoplasm, multiplying, exiting and being transmitted to new host individuals without being recognized by the host immune system [56].

Genetic diversity and adaptive flexibility is also achieved though bacterial plasmids with genes controlling important functions such as nitrogen fixation, sulfur utilization and hydrocarbon degradation. Nitrogen-fixing genes can be conserved in chromosomal DNA and within plasmids [64]. The symbiotic bacterium of genus Rhizobium carry high molecular weight plasmids (90–350 $Å\sim 106$) and in *R. leguminosarum* plasmids have a role in nodule formation, symbiosis as well as carrying nitrogen fixation (nif) genes [65]. Plasmids occur in *G. diazotrophicus* but their number and size varies between strains, with for example *G. diazotrophicus* UAP8070 and UAP5665 each having three plasmids of 93, 22 and 22 kb in size [66], PR2 has two plasmids one particularly large at 170 kb and a smaller one at 24 kb [66], whereas Pal5 has two plasmids of 38.8 and 16.6 kb, [30] and strain UAP5541 has no plasmids at all [66–68].

Genes responsible for nitrogen fixation in *G. diazotrophicus* are located on the chromosome [30, 66]. However, plasmid genes will have other key roles and it has been speculated that for *G. diazotrophicus* they contribute to an improved fitness of the colonized host plant or the insect symbiosis for the bacterium [66]. Strain differences in *G. diazotrophicus* are complex with a mix of highly conserved regions and highly variable groups of genes [30]. A considerable number of coding sequences on 20 genomic islands across a range of 19 strains of *G. diazotrophicus* encode genes involved in processes that could confer intra-specific differences such as, responses to oxidative stress, proteases, biosynthesis of antimicrobial agents, amino acid metabolism and secondary metabolites, as well a large number of transport systems and transcriptional regulators [30]. Strain differences in *G. diazotrophicus* have been observed for a range of key attributes including expression of cell wall degrading enzymes [69], intracellular colonization [24], responses to nitrates [70, 71], siderophore production [72], as well as bacterocin production [73].

The presence and expression of nitrogen-fixing nif genes, are key to the ability of *G. diazotrophicus* to fix nitrogen. In 2000, a major and unique 30.5-kb cluster of nif and associated genes of *G. diazotrophicus*, was sequenced and analyzed [30, 74]. This cluster represented the largest

Endophyte functions	Range	Gd value	Implications
Motility and Chemotaxis	- or +	+	MCP, a transmembrane sensor protein permits *G. diazotrophicus* to detect concentrations of molecules while Che proteins enable orientation and movement.
Type IV pilli & flagella	9–88	9	
Methyl accepting proteins	12–73	12	
Che-protein response regulators			
Plant polymer	26–68	35	GHs facilitate plant entry, sugar metabolism, bacterial cell wall metabolism, and host-microbe interaction [158]. *G. diazotrophicus* has specific cell wall degrading enzymes [69].
Degradation (PPD)	23–63	23	
Glycoside hydrolases (GH)			
% putatively PPD			
Detoxification	8–21	12	Endophyte survival requires the ability to detoxify or manage movement of xenobiotics using efflux pumps. *G. diazotrophicus* has poor survival in the rhizosphere [89, 92, 93].
Antioxidative enzymes	209–681	209	
Efflux pumps			
Fe uptake	6–22	22	Biologically available Fe is limited in plants and endophytes. Uptake of ferric siderophore complexes is achieved via TonB-dependent receptors [159]. Endophytes with large numbers of these receptors may compete with plants or fungi for iron acquisition.
Ton-B dependent receptors			
Degradation	0–16	0	*G. diazotrophicus* is at the extreme low end of the ability to degrade complex plant metabolites.
Dioxygenases			
Transporters	510–1196	510	*G. diazotrophicus* has a relatively high number of transporter genes enabling transport of nutrients and excretion of toxins. Low numbers of the ABC family of transporters, porin genes and the lack of putrescine transporters perhaps suggests poor rhizosphere competence.
Total number	105–183	131	
No./Mbp genome	95–126	95	
No. transporter types	3–53	7	
Porin	142–477	142	
ABC transporters	- or +	-	
Putrescine			
Secretion systems	- or +	+	*G. diazotrophicus* in common with many other endophytes has available key secretion systems
Type I & IV	- or +	-	
Type II, III, Va, Vb, VI			
Signaling	87–272	87	Complexity of signaling systems correlates with the genome size, phylogeny, ecology and metabolic activities of the bacteria [160]. Bacteria living in diverse habitats encode more ECF sigma factors than in stable niches [161].
Two component systems	65–142	96	
Bacterial IQ	2–17	3	
ECF Sigma factors			

Table 2. Compiled from the survey and analysis of nine endophytes: *Burkholderia phytofirmans* PsJN, *Azospirillum* sp. B510, *Klebsiella pneumoniae* 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Pseudomonas stuzeri* A1501, *Enterobacter* sp. 638, Azoarcus sp. BH72, Gluconacetobacter diazotrophicus Pa15, with particular reference to G. Diazotrophicus (Gd) [58].

single grouping of genes required for nitrogenase structure and function, found in any diazo-troph at that time [74]. Interestingly, the overall arrangement of genes was similar to the nif-fix cluster in *Azospirillum brasilense*, while the individual gene products most closely resembled those in species of Rhizobiaceaor, proteobacteria comprising multiple subgroups that can both enhance or hinder plant development [75]. The individual *G. diazotrophicus* gene products are generally similar to those found in other groups of proteobacteria, with 17 gene products being most like those in members of the Rhizobiaceae and 9 gene products being most closely related to *Rhodobacter capsulatus* proteins. NifU and NifS were most similar to the gene products of *Azotobacter* species [74].

2.3. Life cycle of *G. diazotrophicus*

As a Gram-negative bacteria, *G. diazotrophicus* has no spore or resting stage; it reproduces asexually through binary fission. *G. diazotrophicus* is also an obligate endophyte [23], which means it is a bacterium requiring internal as opposed to external plant tissues to complete its life cycle. *G. diazotrophicus* primarily inhabits intercellular apoplastic spaces, the xylem and the xylem parenchyma [76, 77]. However, studies using β-glucuronidase (GUS)-labeled *G. diazotrophicus*, demonstrate that this bacterium is also capable of intracellular coloniza-tion within membrane-bound vesicles in its host plant [24]. Some strains of *G. diazotrophicus* have this intracellular colonization capability in common with a number of other bacteria, for example a phylotype related to *G. diazotrophicus* in *Pinus flexilis* (limber pine) and *Picea engelmannii* (Engelmann spruce) [62] and *Methylobacterium extorquens* in *Pinus sylvestris* [78].

The symbiosome is the unifying feature of all endosymbiosis [79]. The symbiosome is created by the engulfment of the microorganism by a plant-derived membrane in a manner that resem-bles phagocytosis in animal cells [80]. In legume symbiosomes, bacteriods are enclosed within such a plant-derived membrane. The challenge for any other nitrogen-fixing endosymbiont is first to establish intracellularity within living plant cells and within symbiosome-like struc-tures. All carbon and nitrogen sources and oxygen must cross the symbiosome and bacteriod membranes making them crucial to the establishment and maintenance of symbiosis [81].

The UAP5541 strain of *G. diazotrophicus* is known to constitutively produce three hydrolytic enzymes such as endoglucanase, endopolymethylgalacturonase and endoxyloglucanase that facilitate bacterial penetration of plant cell walls [69]. After cell wall penetration, when *G. diazo-trophicus* is present at the surface of the plasma membrane, uptake into vesicles may be triggered by sucrose-induced endocytosis [82]. *G. diazotrophicus* is known to produce large amounts of IAA. At low concentrations, IAA can function as a reciprocal signaling molecule in bacterial-plant interactions [83]. Once intracellular, the enzymes enable *G. diazotrophicus* to colonize cell walls, intercellular spaces and to be transmitted cytoplasmically to daughter cells in actively dividing plant cells thereby spreading systemically throughout the roots and shoots [84]. The plant will not be passive in this process of colonization by the endophyte; plants have evolved molecular mechanisms to deal with challenges imposed by colonizing bacteria [85]. In sugarcane a number of genes have been found to be differentially expressed in the presence of bacteria [86]. The shr5 gene was differentially expressed after inoculation of sugarcane with *G. diazotrophicus* and other nitrogen-fixing bacteria [87]. This gene encodes a protein involved in plant signal transduction during establishment of plant-endophyte interactions. Down regulation of shr5

was evident when the plants were colonized by *G. diazotrophicus*. This suggests that the initial steps of endophytic colonization are actively monitored and possibly enhanced or diminished by the plant [88].

Obligate endophytes such as *G. diazotrophicus* are thought to spread from plant generation to plant generation via seeds, vegetative propagation, dead plant material and possibly by insect sap feeders [89].

2.3.1. Horizontal transmission of G. diazotrophicus

G. diazotrophicus is a non-invasive, obligate, endophytic species [90]. Hence, its ability to survive outside its plant hosts is likely to be poor and its infection capability will be low [91]. There is certainly little evidence of its survival in soil [89, 92, 93]. In host range studies, *G. diazotrophicus* has only been isolated from the rhizosphere of plants in two cases, in banana [94] and rice [95] (**Table 1**). Studies involving immunocapture and PCR have failed to find *G. diazotrophicus* in soil collected between rows of sugarcane plants grown in the field (Santos et al., unpublished data; source [93]). When PCR was used, fragments of the same size as those from *G. diazotrophicus* genomic DNA were detected in soil samples from sugarcane fields, however, the bacterium could not be re-isolated from micro-propagated sugarcane plants used as a trapping host [92]. *G. diazotrophicus* has been isolated from arbuscular mycorhizal fungi (AMF) associated with sweet potato and sweet sorghum [96] and sorghum [17] but survival of *G. diazotrophicus* in soil appears to be limited. Populations of *G. diazotrophicus* residing in plant debris could, following release into the soil, potentially gain entry into a new host plant through the roots, tips and cells of the root cap and meristem, at areas of lateral root emergence and through root hairs [77, 97, 98]. This process would be facilitated by the release from the bacteria of their hydrolytic enzymes in the presence of root exudates containing suitable sugars. Within the stems of host plants, specifically sugarcane, the bacterium is capable of entering at breaks caused by the separation of plantlets into individuals [77].

The ability of *G. diazotrophicus* to survive in the soil long enough to multiply and find a potential host plant is probably limited given its lack of putrescine transporters, because of restricted carbon availability (as sucrose/glucose), and competition from free-living soil bacteria. Hence, the *G. diazotrophicus* must have a means of horizontal transmission that does not rely solely on soil-mediated transfer.

Surveys have indicated that the *G. diazotrophicus*, although present at all sites, in all parts of the sugarcane plant and in all trash samples examined, it was not present in samples taken from associated forage grasses, cereals or weed species within the sugarcane fields [99]. *G. diazotrophicus* has only been found to occur naturally in a total of 19 plant species, mainly crops, across 15 plant families including, Poaceae, Convolvulaceae, Rubiaceae and Bromeliaceae (**Table 1**). Given the bacterium thrives in an intercellular environment rich in sucrose which it uses as a carbon source the number of candidate host species for natural colonization is low. However, despite difficulties in achieving colonization [100], *G. diazotrophicus* has been intentionally inoculated into cotton, calabash (*Lagenaria siceraria*) [15], maize [101] sugarcane, wheat, rice, oilseed rape, tomato, white clover [24, 102], sugar beet, common beans [103] *Arabidopsis* [24] and sorghum [104].

Another potential means of horizontal transmission is through the uptake and distribution via plant feeding insects. The symbiotic association of AAB with insects has been reviewed [36] and the genus of *Gluconacetobacter* has been identified in the guts of fruit flies (*G.* 'munehiro' [105] and *G. europaeus* [106]) and honeybees (*Gluconacetobacter* sequences [107] and *Gluconacetobacter* clone sequences [108]), while in sugarcane *G. diazotrophicus* has been isolated from the gut of the pink sugarcane mealybug (*Saccharicoccus sacchari*) [70, 109–111] a plant sap-sucking insect. This would suggest that horizontal transmission of *G. diazotrophicus* is possible through sap-sucking insect vectors, such as the pink sugarcane mealybug.

> The insects might become colonized during sap-feeding and then re-inoculate the bacteria to stems of other plants. It has been suggested that G. diazotrophicus is imbibed from sugarcane by S. sacchari and the population within the insect is a subset of the sugarcane population [70]. Alternatively, G. diazotrophicus may be an autochthonous microbiota of mealybugs associated with sugarcane [109]. An investigation of the frequency of strains of G. diazotrophicus isolated from cane internodes and sugarcane mealybugs in Cuba indicated a higher frequency of isolation from the plant than from the insects [110]. This would suggest that the primary host of G. diazotrophicus is the plant rather than the insect: the latter acting only as a transmission vector. It may also imply that the insects do not provide the optimal conditions for multiplication or survival of the G. diazotrophicus [110]. If the strains differ due to whether they are isolated from the plant or the insect host, the function of the insect as a transmission vector [109] would be unlikely. Given that G. diazotrophicus was recovered from mealybugs in 1 out of 20 insect colonies associated with plants from 11 varieties growing in 4 localities; if G. diazotrophicus were an autochthonous microbiota of mealy bug then the recovery of G. diazotrophicus from the insect would be more frequent [110].

Successful transmission of bacterial endophytes by insects depends on host and cultivar preferences of the vector and on the vector inoculation efficiency and how rapidly the insect can effectively transmit the bacterium to another host plant. From the limited information available, the vector inoculation efficiency is at best 5%, which would imply a low chance of successful insect transmission. This low figure is supported by the natural plant host range of *G. diazotrophicus* (see **Table 1**), which is restricted to 19 plant species. In addition, the important role of the host and cultivar preferences is supported by surveys in sugarcane that have indicated that the *G. diazotrophicus*, although present at all sites, in all parts of the sugarcane plant examined, the bacteria was not present other plant species within the sugarcane fields [99].

Horizontal transmission of *G. diazotrophicus* has most likely occurred through vegetative propagation of crops (particularly sugarcane) with interspecies transmission potentially having occurred via vesicular-arbuscular-mycorrhizal fungi [17, 112], or more likely, sap-feeding insects. *G. diazotrophicus* has been isolated from *Saccharicoccus sacchari*, the sugarcane mealybug [70, 109]—which has a host range including many species of grasses (including sorghum, rice and miscanthus as well as sugarcane) and pineapple (CABI Invasive Species Compendium; http//www.cabi.org), which through horizontal transmission, could explain the presence of *G. diazotrophicus* in these plant species (**Table 1.**).

2.3.2. Vertical transmission

Plant endophytes may be vertically transmitted through plant seeds either endophytically or epiphytically. Bacteria have been isolated from the seed of a diverse range of plant species [112]. Genomic adaptation of bacterial endophytes for a symbiotic life cycle may include strategies for vertical transmission via the seed at the expense of competitiveness and ability to survive in most environments outside the plant. The rich diversity of bacteria in the seed

of *Miscanthus* indicated the bacteria are not only able to avoid plant defenses, but potentially have a more active role, acting primarily during germination and seedling establishment [113]. *G. diazotrophicus* has not been isolated from the seeds of its host sugarcane [66]. However, the intracellular capability of some strains of *G. diazotrophicus* means they have the potential for vertical transmission through intracellular colonization of the seed [24]. Certainly, the ability of *G. diazotrophicus* to fix nitrogen and produce plant growth hormones may aid initial seedling establishment and growth but there is little recorded evidence to date of vertical transmission for *G. diazotrophicus*. Vertical transmission has been demonstrated in seeds of OSR at ca. 15% and seed treated and field grown Barley of 1–3%, but the presence of *G. diazotrophicus* in S1 wheat seed from colonized plants, either in the laboratory or under field conditions, has not been possible (unpublished data Azotic Technologies Ltd.).

2.3.3. Nitrogen fixation in G. diazotrophicus

Although most often associated with rhizobial symbiosis in the root nodules of legumes, BNF occurs in species of more than 100 genera distributed among several of the major phylogenetic divisions of prokaryotes [114, 115]. The principles are the same, whichever bacteria and wherever it may be located in the plant. BNF is simply a process by which atmospheric dinitrogen (N_2) is reduced into two molecules of ammonia (NH_3) by the enzyme nitrogenase with 8H+, 8e− and 16 Mg ATP [116]. The process in *G. diazotrophicus* is catalyzed by nitrogenase which is a molybdenum-dependent system that consists of two proteins, dinitrogenase reductase (Fe protein containing the ATP-binding sites) and dinitrogenase (MoFe protein containing the substrate binding sites) [117–119]. Both of these proteins are irreversibly inactivated by oxygen but with dinitrogenase reductase being the more sensitive of the two. However, because nitrogen fixation is a very energy demanding process, it requires oxygen for aerobic respiration for ATP synthesis. This creates what is known as the "O_2 Paradox" [120] whereby nitrogen-fixing bacteria need to respire to generate the energy for nitrogen fixation, while minimizing O_2 to enable the nitrogenase to function.

Rhizobia manage the O_2 paradox by creating a micro-aerobic environment within a root nodule (providing a barrier to O_2 diffusion) that involves a specific O_2-delivering leghemoglobin combined with a highly efficient respiratory pathway. The large energy demands for fixing nitrogen are generated through respiration utilizing the extremely high O_2 affinity cyt cbb3 terminal oxidases [88, 121]. Interestingly, *G. diazotrophicus* lacks the cytochrome cbb3 that allows respiration at very low levels of oxygen [122] in rhizobia, and does not fix nitrogen within nodules or have the benefit O_2 delivery by leghemoglobin. However, in *G. diazotrophicus* a number of other factors appear to be involved in providing the necessary protection; sucrose, the colony structure and the extrapolysaccharide levan, detoxification of reactive oxygen species as well as control of oxygen through its respiratory pathway.

Firstly, sucrose: *G. diazotrophicus* has no sucrose transport system and in high sucrose concentration environments of around 10% the sucrose has a positive effect on nitrogenase activity protecting nitrogenase against inhibition by oxygen [123]. Secondly, the fructo-oligosaccharide levan; this enables an unusual feature of *G. diazotrophicus*, namely its ability to fix nitrogen in colonies grown on both semi-solid and solid media [124–126]. This is achieved because of the levan mucilage in culture, is capable of limiting oxygen diffusion. It does this to the extent of enabling *G. diazotrophicus* to fix nitrogen even when the pO_2 is not much lower than tropospheric levels [127].

In addition, the levan also increases tolerance to reactive oxygen species (ROS) that may be increased under conditions of high respiration rates causing oxidative stress [40, 128, 129]. There is some evidence for a nitrogenase protection mechanism in fluctuating levels of oxygen [126], possibly involving a putative FeSII Shethna protein, which forms a complex with the nitrogenase during sudden increases in oxygen pressure. This process renders the enzyme temporarily inactive but protected from oxygen damage, similar to the situation in the species, *Azotobacter vinelandii* [130]. However, it has been suggested that other FeSII proteins, rather than Shethna proteins represent more appropriate candidates for this role [30, 131].

One of the remarkable features of *G. diazotrophicus* is its respiratory system whereby its extremely high respiratory rates are among the highest ever reported for aerobic bacteria [132, 133] underpinning *G. diazotrophicus*'s candidature in evolutionary terms, as a potential proto-mitochondrion [49]. Glucose provides the principle energy source to meet the high-energy demand associated with the conversion of dinitrogen by nitrogenase [134, 135] via the pyrroloquinoline quinone-linked glucose dehydrogenase in the periplasmic membrane.

G. diazotrophicus is able to change its electron transport chain composition during nitrogen fixation. In well-aerated cultures, cytochrome a1 and cytochrome bb are expressed as the main terminal oxidase, whereas when nitrogen fixation is repressed, cytochrome a1 diminishes dramatically concomitantly with the appearance of cytochrome bd [132]. Oxidase activities are also much higher in membrane preparations obtained from cultures under nitrogen-fixing conditions than in those from cultures under non-nitrogen-fixing conditions.

The combination of the sucrose environment in natural host plants (**Table 1**), the barrier formed by the extrapolysaccharide levan and the enhanced tolerance this provides to ROS, the very high respiration rates and the ability of *G. diazotrophicus* to change its electron transport pathway during nitrogen fixation plus the extra energy provided by the pyrroloquinoline quinone-linked glucose dehydrogenase, provides all of the conditions necessary for effective nitrogen fixation in this bacterium.

The methodology for determining nitrogen fixation by endophytic bacteria is now well established and every method used to determine nitrogen fixation in rhizobia root nodules has been used to demonstrate nitrogen fixation in crop plants by *G. diazotrophicus* [12]. These techniques include chlorophyll levels and leaf percentage nitrogen [17], nitrogenase activity measured through an acetylene reduction assay (ARA) [136, 137], nif gene mutant studies [138], labeled nitrogen 15 N2 studies [137–138], enhanced photosynthetic rates [16] and plant growth and yield benefits [12, 19, 139, 140].

There are two key characteristics of *G. diazotrophicus* with regard to its nitrogen-fixing capability: (i) its ability to excrete almost half of the fixed nitrogen as ammonium which is potentially available to plants [141, 142] and (ii) its lack of a nitrate reductase protein which suggests that the ability of *G. diazotrophicus* to fix nitrogen is independent of the amount of nitrate in its environment [124]. With regard to the latter, laboratory studies have indicated that nitrogenase activity was not inhibited or repressed by nitrates [141] and was only partially inhibited by ammonia [23, 141, 143]—which is consistent with the possibility of having a feedback mechanism for ammonium—the form in which nitrogen may be excreted by the

bacterium [93, 141], but not nitrate for which there may be no nitrogen reductase feedback mechanism. Studies with different sugarcane varieties comparing ammonia versus nitrate sources of nitrogen have demonstrated their effects (using both ARA and bacterial counts) to be plant variety dependent, but with ammonia having a greater negative impact on nitrogen fixation than nitrate, and the reverse true of counts of colonized bacteria [144, 145]. Growth of *G. diazotrophicus* in culture was not affected by nitrate but was reduced in sugarcane plants treated in the field with high levels of nitrate fertilizers [68]. **Figures 1** and **2** clearly demonstrate that the *G. diazotrophicus* treated maize and wheat crops generated higher yields relative to the controls, irrespective of levels of nitrogen fertilizer applied.

3. Conclusions

G. diazotrophicus is an extra-ordinary nitrogen-fixing endophyte; a bacterium with important ancestral attributes, the significance and value of which are increasingly becoming apparent as research to facilitate its use in climate smart agriculture is undertaken. Typical of a facultative intracellular symbiont, *G. diazotrophicus* retains genetic flexibility through its genome and plasmids and can respire under a wide range of oxygen concentrations suitable for both an intracellular plant and insect habitat. With a respiratory system that enables extremely high respiratory rates, as well as large groups of genes associated with nitrogenase structure and function and a range of mechanisms that protect the nitrogenase from oxygen, the bacterium combines these factors to ensure symbiotic nitrogen fixation *in planta*. A highly adaptive obligate endophyte, with different strains demonstrating a range of attributes, including both inter- and intracellular colonization capability, *G. diazotrophicus* has the potential to reduce nitrogen fertilizer use while maintaining crop yields.

Acknowledgements

Conflict of interest

The author declares a role in the development of the proprietary NFix® formulation cited above in this publication and its commercial utilization but no other competing or conflict of interests exist.

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