

## POLYPHASIC CHARACTERIZATION OF *GLUCONACETOBACTER DIAZOTROPHICUS* ISOLATES OBTAINED FROM DIFFERENT SUGARCANE VARIETIES

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### ABSTRACT

A polyphasic approach was applied to characterize 35 *G. diazotrophicus* isolates obtained from sugarcane varieties cultivated in Brazil. The isolates were analyzed by phenotypic (use of different carbon sources) and genotypic tests (ARDRA and RISA–RFLP techniques). Variability among the isolates was observed in relation to the carbon source use preference. Glucose and sucrose were used by all isolates in contrast to myo-inositol, galactose and ribose that were not metabolized. The results of the analysis showed the presence of two groups clustered at 68% of similarity. The genetic distance was higher when RISA-RFLP analysis was used. Analysis of 16S rDNA sequences from isolates showed that all of them belonged to the *G. diazotrophicus* species. Neither effect of the plant part nor sugarcane variety was observed during the cluster analysis. The observed metabolic and genetic variability will be helpful during the strain selection studies for sugarcane inoculation in association with sugarcane breeding programs.

**Key words:** biological nitrogen fixation, genetic diversity, plant-bacteria interaction

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### INTRODUCTION

The endophytic bacterium *G. diazotrophicus* has been found colonizing different crop plants such as sugarcane, pineapple, coffee, sweet potato, *Pennisetum purpureum*, *Eleusine coracana*, *Erianthus* and insects such as mealy bugs (21). In addition to being able to fix nitrogen it has been shown to possess many other characteristics potentially valuable in the area of agriculture such as production of substances responsible for plant growth-promotion (10), antifungal and antibacterial properties (15,18).

One pre-condition to exploit the benefits derived from an efficient association, mainly related to the biotechnological potential for agriculture including biological nitrogen fixation, is to identify endophytic associations of microorganisms with different plants (8). It is already known that the genetic diversity within a species can be correlated with the spatial distribution, environmental effects and specificity of host plant interaction

(12,26). These aspects could also be considered during the strain selection process concerning the inoculation efficiency within the process of host plant interaction and environmental conditions (6,16).

Previous studies (4,5) found that *G. diazotrophicus* isolated from sugarcane plants has a very low genetic diversity. In addition, the authors observed that the strains isolated from the Brazilian sugarcane varieties are more diverse than those isolated from the Mexican sugarcane plants. Nevertheless, the majority of the bacteria were isolated from few sugarcane varieties cultivated in both countries, which may have biased the results. In contrast, other studies using RAPD molecular analyses for *G. diazotrophicus* strains isolated from Indian sugarcane varieties showed a substantial genetic diversity (28). Since the first studies on the genetic diversity of *G. diazotrophicus*, the main factors that have been assigned as responsible for the limited genetic diversity are the limited distribution of host plants and its propagation by vegetative

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means as in the case of sugarcane using stem cuttings (setts). In this model of endophytic interaction, the bacteria is maintained from one generation to the next within the host plant environment that may protect the microbial natural population from competition for nutrient availability, survival in different soil types or other physico-chemical stress. The analysis of the phylogenetic micro diversity of the bacteria in association with the host plant may contribute to understand the “functional diversity of microbial communities and ultimately the ecosystem function as a whole” (26). These aspects may help to achieve a better understanding of the plant-bacteria relationship and perhaps to answer “why certain plant genotypes obtain much higher contribution from Biological Nitrogen Fixation than others”, question raised by Döbereiner *et al.* (7).

The aim of this study was to use the polyphasic analysis to characterize *G. diazotrophicus* isolates obtained from inner tissues of roots and leaves of different sugarcane varieties maintained in the Germoplasm Bank in Brazil.

## MATERIALS AND METHODS

### Isolates

A total of 35 isolates were tested (Table 1) and all of them were obtained as described by Barbosa *et al.* (3). Six reference strains (BR11334, BR11208, BR11284, BR11281, BR11280, BR11249) representing the different groups as described by Caballero-Mellado *et al.* (5) using multilocus enzyme electrophoresis analysis were also used for comparison.

### Carbon source metabolism

The main carbon source of the medium was omitted and the following carbon sources were tested: glucose, fructose, sucrose, arabinose, ribose, galactose, myo-inositol, sorbitol and adonitol (all provided by Sigma Chemical, St. Louis, MO, USA) as described by Barbosa *et al.* (3).

### DNA extraction

The DNA of the strains was extracted either from liquid cultures or from colonies according to the methodology adapted from Audy *et al.* (1).

### PCR amplification of the 16S rDNA and 16S-23S rDNA intergenic region

The amplification of 16S rDNA region followed the methodology described by Young *et al.* (31), using the pair of primers Y1 and Y3. The amplification of the 16S-23S rDNA intergenic spacer region was performed with primers pHr and p23Suni322anti (9,14).

### Restriction of amplified DNA

Five microlitres of each PCR amplified products was digested with restriction endonucleases *Hae* III, *Cfo* I, *Alu* I, *Msp* I, *Taq*

**Table 1.** Characteristics of the bacterial isolates and reference strains used in this study.

Isolates	Plant part	Sugarcane variety	Country of origin
BR11367	roots	SP71-1406	Brazil
BR11368	Roots	SP70-443	Brazil
BR11392	Roots	SP70-1284	Brazil
BR11373	aerial part	SP70-443	Brazil
BR11514	Roots	PB-4672	Brazil
BR11394	Roots	PB-4612	Brazil
BR11513	Roots	CB-7437	Brazil
BR11404	aerial part	CB-7437	Brazil
BR11509	Roots	RB 739359	Brazil
BR11369	Roots	RB 739735	Brazil
BR11383	aerial part	Co 622	India
BR11386	Roots	Co 1007	India
BR11950	Roots	Co 740	India
BR11397	aerial part	Co 453	India
BR11951	Roots	Ragnar	India
BR11376	aerial part	Ragnar	India
BR11379	aerial part	Azul Casa Grande	India
BR11388	Roots	CP 5376	USA
BR11396	aerial part	CP 5376	USA
BR11316	aerial part	CP 5323	USA
BR11390	Roots	CP 5330	USA
BR11398	aerial part	CP 5330	USA
BR11952	Roots	CP 5480	USA
BR11953	Roots	CP 5950	USA
BR11391	aerial part	CP 5950	USA
BR11954	Roots	CP 601	USA
BR11387	Roots	CP 5268	USA
BR11401	aerial part	CP 5268	USA
BR11955	Roots	H 493533	USA
BR11377	aerial part	B 4098	Barbados
BR11384	Roots	Pepe Cuca	Cuba
BR11393	aerial part	Pepe Cuca	Cuba
BR11956	aerial part	PR 1124	Porto Rico
BR11378	aerial part	Na 5679	Argentina
BR11370	Roots	Mex 5219	Mexico
BR11280*	Stems	ND	Brazil
BR11281*	Stems	ND	Brazil
BR11284*	Roots	ND	Brazil
BR11208*	Stems	SP70-1143	Brazil
BR11249*	Rhizoplane	Na5679	Brazil
BR11334*	mealybug insect	ND	Mexico

ND: Not determined; \* reference strains.

I and *Hinf*I (Invitrogen, SP, Brazil) for the 16S rDNA region (ARDRA-RFLP) and *Hae* III, *Alu* I, *Msp* I, *Rsa* I, and *Pvu* I (Invitrogen, SP, Brazil) for intergenic spacer region (RISA-RFLP). The banding pattern of digested products were separated by electrophoresis on a 3% agarose (Sigma Chemical, St. Louis, MO, USA) gel on 1 x TAE buffer (Trizma base - Promega Madison, Wisconsin, USA; Glacial Acetic Acid - Merck, Darmstadt, Germany; EDTA - Sigma Chemical, St. Louis, MO, USA), carried out at 60 V for 2 h 30 min, stained with ethidium bromide (Sigma Chemical, St. Louis, MO, USA), photographed using a Kodak Logic 100 (Kodak®, San Francisco, USA) equipment and size estimated by comparison against a 100 pb DNA ladder (Invitrogen, SP, Brazil).

#### Polyphasic analysis based on carbon source utilization and genetic diversity (ARDRA and RISA - RFLP)

The clustering analysis of the isolates was based on the qualitative characteristic of presence (+) or absence (-) of pellicle veil formed in the semi-solid medium (carbon source) and bands in the gel (ARDRA and RISA - RFLP). According to these data, a binary matrix was constructed for each data independently prior to the consensus analysis using the polyphasic approach. The similarity among the isolates was estimated by UPGMA indices using the Jaccard coefficient (22) and represented graphically through a dendrogram generated with the NTSYS-pc (version 1.8, Exeter Software, Setauket, New York, USA).

#### 16S rDNA sequencing and phylogenetic analysis

The PCR products were directly sequenced using a TE Dynamics Kit in the MegaBACE 1000 System (GE-Amersham Biosystem, Chalfont St. Giles, UK) with the primers designed by (27). The 16S rDNA nucleotide sequences were used to determine the phylogenetic position of the isolates among other *G. diazotrophicus* strains in the phylogenetic tree. Multiple sequence alignments were carried out with ClustalW (29) and the phylogenetic trees were constructed by the neighbour-joining method (25). To dendrogram construction the TreeCon (30) package, with the distance of Jukes and Cantor, was used (11). Others program parameters were maintained unchanged. Bootstrap analyses of 1.000 replicates were established.

#### Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rDNA genes obtained were submitted to deposit at the Genbank. The identities of several nitrogen-fixing bacteria sequences obtained from this deposit that were used to construct the phylogenetic trees are represented by their accession numbers.

## RESULTS AND DISCUSSION

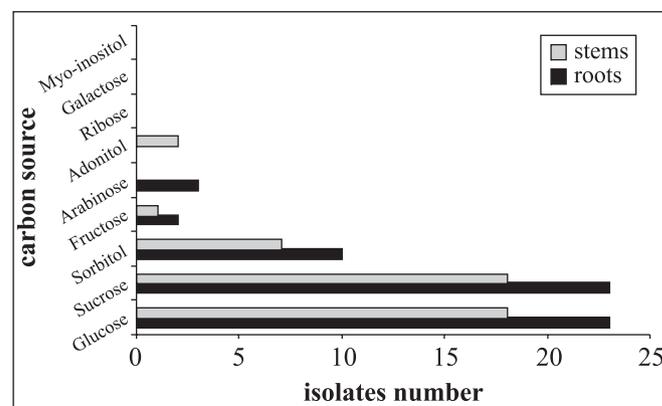
All of the 35 isolates and the six reference strains of *G. diazotrophicus* were able to grow in the semi-solid nitrogen-

free medium containing glucose or sucrose as carbon source (Fig. 1). Sorbitol was only used by 17 isolates originated either from the aerial part or the roots of the sugarcane varieties. On the other hand, arabinose was only used by three isolates, adonitol was used by the type strain BR11281 (PAL5) and the isolate BR11398 while fructose was used by two reference strains and one isolate. The carbon sources myo-inositol, galactose and ribose were not used by any isolates as well as by the reference strains tested. Similar result was also observed by Barbosa *et al.* (3) where a lower number of carbon sources were used by strains of *G. diazotrophicus* under nitrogen fixation condition.

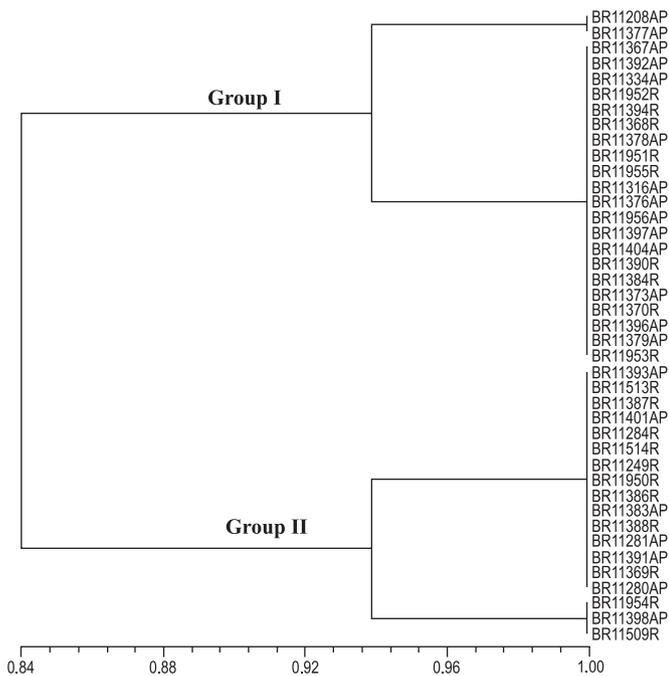
The cluster analysis using the ARDRA technique showed the isolates randomly distributed forming two groups at 84% similarity (Fig. 2). Among the tested restriction enzymes, only *Hae*III and *Hinf*I were able to generate differential polymorphic bands of the 16S rDNA amplified region among the isolates tested. These two enzymes were able to distinguish a group of N<sub>2</sub>-fixing *Burkholderia* strains isolated from the rhizosphere and root interior of sugarcane, maize and teosinte plants grown in Brazil, Mexico and South Africa (20).

In the case of the RISA, the formation of two groups was also observed, but at a lower similarity level (56%) when compared with the ARDRA analysis (Fig. 3). The RISA-RFLP analyses allowed the detection of a higher variation among the isolates when compared to the 16S rDNA, confirming other studies carried out with nitrogen-fixing bacteria (2,19,25).

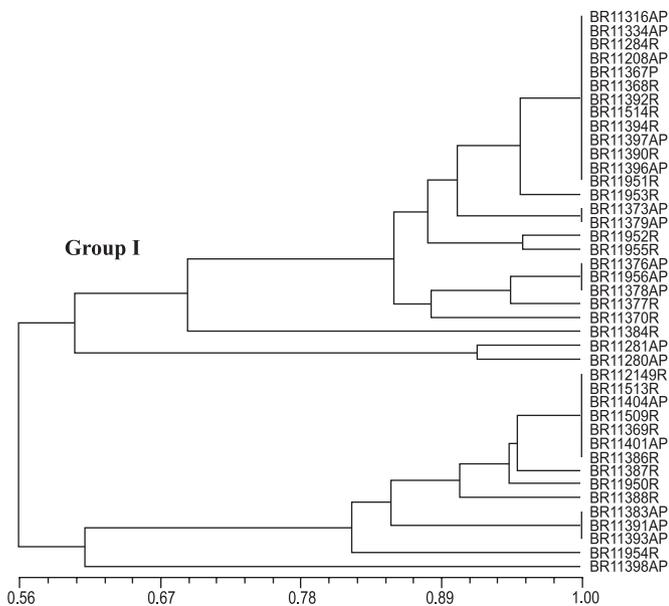
The polyphasic approach based on the consensus analysis of the phenotypic and genotypic (ARDRA and RISA) characteristics showed that the isolates formed two great groups with 68% of similarity (Fig. 4). The formation of two groups, with 70 % similarity, was also observed by Loganathan *et al.*



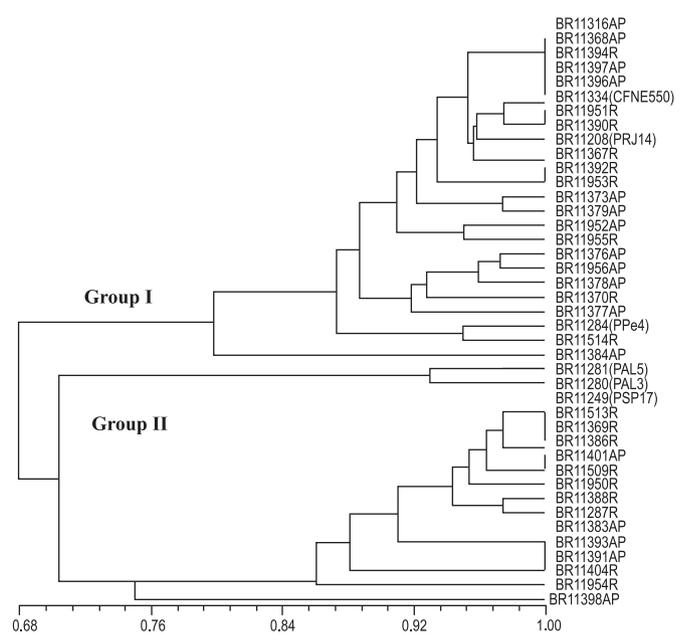
**Figure 1.** Utilization of carbon sources by different isolates and reference strains of *G. diazotrophicus* obtained from roots and aerial part of different sugarcane varieties maintained in the germoplasm bank.



**Figure 2.** ARDRA-RFLP similarity cluster of 35 isolates, obtained from sugarcane varieties originated from different countries and maintained in the germoplasm bank, and 6 reference strains of *G. diazotrophicus*. Dendrogram was generated by the algorithm UPGMA and similarity matrix obtained from the use of Jaccard coefficient. AP: aerial part, R: roots.



**Figure 3.** RISA-RFLP similarity cluster among 35 isolates obtained from sugarcane varieties originated from different countries and 6 reference strains of *G. diazotrophicus*. AP: aerial part, R: roots.

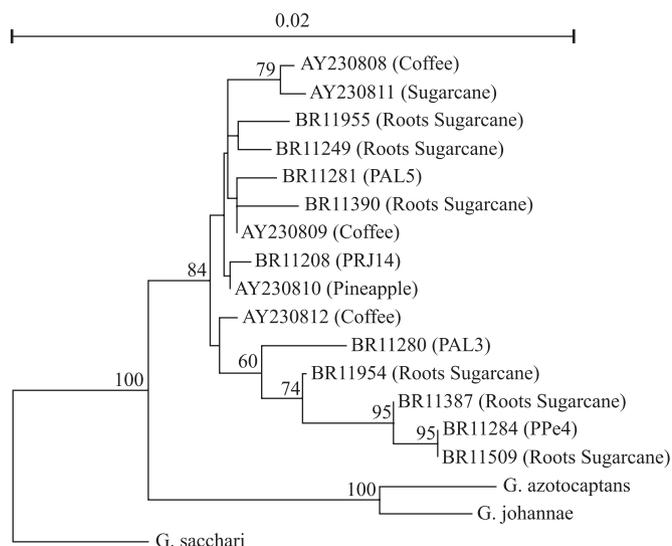


**Figure 4.** Consensus dendrogram obtained by combining the data of utilization of carbon sources, ARDRA and RISA-RFLP among 35 isolates from sugarcane and 6 reference strains of *G. diazotrophicus*. Dendrogram was generated by the algorithm UPGMA and similarity matrix obtained from the use of Jaccard coefficient. AP: aerial part, R: roots.

(13) when analyzing nine new strains of *G. diazotrophicus* isolated from *Eleusine coracana* and compared to the type strain PAL5 of *G. diazotrophicus* using the RAPD technique. Perin *et al.* (17) analyzed the diversity of 123 isolates of *G. diazotrophicus* originated from sugarcane using the Enzyme-Linked Immunosorbent Assay (ELISA) technique and also observed the formation of two groups with distance of 35% between them.

The cluster analysis based on polyphasic approach showed a random distribution of the isolates in relation to the part of the plant, sugarcane variety and country origin and therefore suggests a certain independence of genetic evolution of this bacterium in relation to its origin. Similar results were also observed by Rosado *et al.* (23) for isolates of *Paenibacillus azotofixans* (nowadays *P. durus*).

Sequencing of 16S rDNA unit of eight isolates, representatives of different branches within the ARDRA and RISA-RFLP clusters, showed that all of them belongs to the *G. diazotrophicus* species according to the phylogenetic analysis (Fig. 5). These isolates clustered together with the reference strains obtained from sugarcane, pineapple and coffee plants but separated from the species *G. azotocaptans* and *G. johannae*. Recently, a new nitrogen-fixing and cellulosic species of the



**Figure 5.** Phylogenetic analysis of the 16S rDNA sequences of different nitrogen-fixing isolates obtained from several sugarcane varieties maintained in the germoplasm bank. The tree was constructed by Neighbour-joining. The bootstrap values (1.000 replicas), program Treecon are indicated in the grouping base. The scale bar indicates the distance in substitutions by nucleotide. Reference sequences used in this analysis were obtained from the Genebank: BR11281 (PAL5 – ATCC49037); AF127413: *Gluconacetobacter sacchari*; AF111841: *G. johannae*; AF192761: *G. azotocaptans*; AY230814: PAL3, sugarcane strain; AY230810: UAP-AC10, pineapple strains; AY230809: UAP-AC7, pineapple strains; AY230811: CFNE550, sugarcane strains; AY230808: UAP-cf51, coffee strains.

genus *Gluconacetobacter*, named *G. kombuchae*, was isolated from a Kombucha tea plants grown in India and clustered quite distant from the already known diazotrophic *Gluconacetobacter* species (24).

In conclusion, these results showed that data from the phenotypic and genotypic techniques show here were efficient to reveal intra-specific variability among the *G. diazotrophicus* isolates at different levels and can be applied in a selection of strains for sugarcane inoculation studies. The absence of effect from origin of isolation, as sugarcane variety or part of the plant, on the genetic diversity suggest that this nitrogen-fixing species has maintained its genetic structure along the sugarcane breeding programs carried out by different countries in the world.

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#### RESUMO

##### Caracterização polifásica de isolados de *Gluconacetobacter diazotrophicus* obtidos de diferentes variedades de cana-de-açúcar.

Foi realizado a caracterização polifásica de 35 isolados obtidos de variedades de cana-de-açúcar cultivadas no Brasil, através de testes fenotípicos (uso de fontes diferentes de carbono) e genotípicos (técnicas de ARDRA e RISA-RFLP). Houve variação entre os isolados com relação à utilização de fontes de carbono. Glicose e sacarose foram usadas por todos isolados, diferentemente de mio-inositol, galactose e ribose que não foram metabolizados. Os resultados da análise polifásica dos dados confirmam a formação de dois grupos, que apresentaram 68% de similaridade. Observou-se maior distância genética entre os isolados quando a técnica de RISA-RFLP foi aplicada. O sequenciamento da região 16S do rDNA mostrou que todos os isolados pertencem à espécie *G. diazotrophicus*. Não foi observado efeito da parte da planta ou variedade de cana-de-açúcar no agrupamento dos isolados. Em conjunto, esses resultados poderão auxiliar no estudo de seleção de estirpes para inoculação em cana-de-açúcar, orientando programas de melhoramento vegetal.

**Palavras chaves:** fixação biológica de nitrogênio, diversidade genética, interação planta-bactéria

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