ABSTRACT. A macrorestriction map of the *Gluconacetobacter diazotrophicus* PAL5, an important nitrogen-fixing endophyte of several plants such as sugarcane, coffee, sweet potato and pineapple, was constructed using pulsed-field gel electrophoresis (PFGE) and DNA hybridization. For PFGE analysis, single and double digests of the chromosome were carried out with *I-Ceu*, *Swa* and *Pvu* restriction enzymes. The resulting DNA fragments were positioned into physical map, using combinatorial analysis of hybridization results, restriction enzymes sites position and DNA fragments length. *G. diazotrophicus* has 4 *rrn* operons into a circular chromosome, with estimated length of 4,240 ± 41.6 Kb. The hybridization results allowed the positioning of 42 genetic markers on chromosome (39 single copy and 3 repeated elements), as well as the detection of 1 *rrn* operon with inverted orientation. The results contained in this paper were important to finish the genome assembly of the *G. diazotrophicus* PAL5, which was deposited and is available in the EMBL database.

Key words: *Gluconacetobacter diazotrophicus*, PFGE, physical map, genetic map, *rrn* operons.

INTRODUCTION

*Gluconacetobacter diazotrophicus* is α nitrogen-fixing acetic acid α-proteobacterium, found in endophytic association with several sugarcane cultivars, especially in agricultural areas where nitrogen fertilizer input is low (Gillis et al. 1989; Reis et al. 1994; Fuentes-Ramírez et al. 1999; Fischer and Newton 2005). Besides sugarcane, *G. diazotrophicus* has been detected into other plant species such as Cameroon grass (*Pennisetum purpureum*), sweet potato (*Ipomoea batatas*) (Döbereiner et al. 1988), coffee (*Coffea arabica*), the grass *Eleusine coracana* and pineapple (*Ananas comosus*) (Estrada-de los Santos et al. 2001; Muñoz-Rojas et al. 2005). The ability to colonize a number of families such as *Poaceae*, *Convulvulaceae*, *Rubiaceae* and *Bromeliaceae* suggests that *G. diazotrophicus* possibly has the potential to benefit different plant hosts (Döbereiner et al. 1988; Reis et al. 1994; Estrada-de los Santos et al. 2001; Muñoz-Rojas et al. 2005).

The *G. diazotrophicus* biotechnological potential is emphasized by the demonstration that up to 60% of the nitrogen can be acquired through biological nitrogen fixation in some sugarcane varieties (Urquiaga et al. 1992; Baldani et al. 1997; Baldani and Baldani 2005), and the ability to promote plant growth by means of phyto-hormone production, such as auxins and gibberellin (Muthukumarasamy et al. 2002; Muñoz-Rojas and Caballero-Mellado 2003; Lee et al. 2004; Muthukumarasamy et al. 2006). Furthermore, a potential biological control role has been attributed to *G. diazotrophicus*, due to its antagonistic activity against *Xanthomonas albilineans*, as a result of the production of a bacteriocin (Piñon et al. 2002; Blanco et al. 2005), and against *Colletotrichum falcatum*, possi-
ably because of its ability to ferment sugars and reduce medium at pH below 3.0 (Muthukumarasamy et al. 2000).

*G. diazotrophicus* is able to fix nitrogen and grow at high sucrose concentration (30%) and low pH (3.0). However, the nitrogenase activity is partially inhibited by $\text{NH}_4^+$ at high sucrose concentrations, though is not affected by high concentration of $\text{NO}_3^-$ (25mM) (Baldani et al. 1997; Reis and Döbereiner 1998; Fischer and Newton 2005). The persistence of nitrogen fixation in the presence of nitrate is an additional benefit, because *G. diazotrophicus* could be used as agro-stimulant even in cultivars previously treated with nitrogen fertilizers.

Physical maps are considered powerful tools for localization and isolation of genes, studying the organization of genomes, and as a preliminary step for efficient sequencing (Heber et al. 2000). Additionally, physical maps generated by pulsed field gel electrophoresis (PFGE) of large DNA fragments, followed by hybridization and fingerprinting methods, is an alternative to assess the genome organization in organisms for which the complete genome sequence is not available (Majunder et al. 1996; Marcone and Seemüller 2001). Macromechanism mapping has been applied to the examination of bacterial genome structures and topology for many years (Weinel et al. 2001). Currently, physical mapping strategies have been further developed to verify and accelerate the assembly, in the gap closure phase of microbial genome shotgun-sequencing projects, acting as quality control of sequence and contig assemblies (Chan and Goodwin 1999; Shimizu et al. 2001; Weinel et al. 2001).

Here, we describe the PFGE-based analysis of the *G. diazotrophicus* PAL5 chromosome, and subsequent construction of a physical and genetic map, reporting the genome size, number and orientation of the rRNA (*rrn*) operons. Moreover, hybridization results of DNA probes against macromechanism DNA fragments enabled the location of 42 genetic markers on this chromosome, representing a first demonstration of the genomic organization of this important endophyte.

**MATERIAL AND METHODS**

**Bacterial strains and growth conditions**

*G. diazotrophicus* PAL5 strain (ATCC49037; BR11281) was cultured in Dygs broth (glucose 2.0 g, tryptone 1.5 g, yeast extract 2.0 g, $K_2\text{HPO}_4$ 0.5 g, $\text{MgSO}_4\cdot7\text{H}_2\text{O}$ 0.5 g, glutamic acid 1.5 g per liter) pH 6.0, in a gyratory shaker at 30 °C and 200 rpm during approximately 40 h, to obtain a culture with 1.3 optical density (OD$_{600}$). Additionally, we performed control cultures in Agar Dygs plates (Dygs broth supplemented with 1.5% bacteriologic agar) at 30 °C during 48 h, from each culture used for DNA extraction, to confirm the culture purity. The stock strain is maintained frozen at −70 °C in Dygs broth supplemented with 20% glycerol.

*Escherichia coli* clones from shotgun library were grown in Luria-Bertani (LB) broth (yeast extract 5 g, tryptone 10 g, NaCl 10 g per liter) pH 7.0, supplemented with 100 µg/ml of ampicillin, in a gyratory shaker at 37 °C and 200 rpm during 24 h. The shotgun library are maintained frozen at −70 °C, in 96 well plates with 150 µl of LB broth supplemented with 100 µg/ml of ampicillin and 20% glycerol per well.

**Preparation of high molecular weight genomic DNA**

Genomic DNA was prepared from 1 ml of *G. diazotrophicus* PAL5 cultures, in logarithmic phase of growth (OD$_{600}$ 1.3). Briefly, cells were suspended in 25 µl of EDTA 50 mM (pH 8.0) buffer, and 10 µl of lysozyme (10 mg/ml), 190 µl of lysis solution (6 mM Tris-Cl – pH 7.5, 100 mM EDTA – pH 7.5, 1 M NaCl, 0.5% Brij58, 0.2% Deoxycholate, 0.5% Sarkosyl), and 225 µl of 2% low-melting-point agarose at 50 °C were added. This mix was gently homogenized and molten in blocks form. The blocks were suspended in 5 ml of lysis solution supplemented with 20 µg/ml of Rnase A, and incubated at 37 °C for 16 h with gentle shaking. After the incubation period, the lysis solution was replaced by 5 ml of a solution containing 0.4 M EDTA (pH 9.3), 1% sarcosyl, and 1 mg/ml of proteinase K, and incubation continued for 24 h at 50 °C. The agarose blocks were treated with 1 mM phenylmethylsulfonyl fluoride (PMSF), and washed four times for 30 min each in 5 ml of CHEF-TE (100 mM Tris-Cl pH 7.5; 100 mM EDTA pH 7.5). The blocks were stored in 5 ml of 70% ethanol at 4 °C for several months.

**Restriction digestions**

The rare cut restriction enzymes used in this study were *I-CeuI*, *PacI* and *SwaI* (New England Biolabs, Ontario, Canada). The agarose blocks containing the genomic DNA were sliced in 4 pieces, and immersed into 1× restriction buffer supplied by the manufacturer and incubated at 4 °C. After 1 hour, the buffer was removed and 100 µl of fresh 1× buffer, containing 10 µg of bovine serum albumin (BSA) and 10 U of the enzyme were added, and incubated in the appropriate temperatures, according to the manufacturer’s recommendations during 6 h. In double digestions, after the incubation period, the restriction buffers were discarded, and the sliced blocks were washed once with CHEF-TE during 1 h at 4 °C, before of the second digestions in the proper buffers.
**Pulsed field gel electrophoresis (PFGE) and Southern blotting**

Electrophoresis was carried out in the Gene Navigator PFGE system (GE-Amersham Biosciences, Baie d’Urfe, Canada) with contour-clamped homogeneous field (CHEF). The electrophoresis parameters such as agarose concentration, buffers, pulse times, running voltage and buffer temperature varied, depending on the required experimental conditions, for detection of the DNA fragments in different length ranges (see legends of the figure 1A-C). Yeast chromosome PFGE marker (225 to 1,900 Kb), Lambda ladder PFGE marker (50 to 1,000 Kb) and Lambda low range marker (0.13 to 194 Kb) from New England Biolabs, were used as size standards. The gels were stained with ethidium bromide and photographed. The DNA fragments length was estimated by the mean of length values, attributed to the fragments in 10 different gels, using the results generated by the Fragment Analysis Software (GE-Amersham Biosciences). For southern blot, the gels were depurinated, denatured, neutralized and blotted to nylon membranes (Hybond+ from GE-Amersham Biosciences) (Sambrook and Russell 2001).

**DNA library and sequencing**

G. diazotrophicus PAL5 was cultivated as described above, and genomic DNA was isolated according to standard protocol (Sambrook and Russell 2001). One DNA library with average insert sizes of 1 Kb were generated from sonified genomic DNA. These DNA fragments were gel excised, concentrated, end-repaired, cloned into pUC18 vector at the Smal I site and electroporated into Escherichia coli DH10B (Invitrogen, California, USA). Out of this DNA library, a total of 3,456 clones were double-end sequenced twice in MegaBACE 4000 automated DNA sequencer (GE-Amersham Biosciences).

**Selection of clones containing rRNA and single copy genes**

The double-end sequencing reactions generated a total of 6,501 high quality reads which were aligned against non-redundant NCBI database, using the basic local alignment search tool (BLAST) for selection of clones used for DNA probes construction. Clones with inserts containing potential single copy genes of G. diazotrophicus PAL5 were chosen for hybridization: 1) their sequences should be unique in the library; 2) their sequences should not show high identity with sequences from others proteins of the same family; 3) their sequences should show homology to published single copy genes. Clones with inserts containing rRNA genes were selected. The sequences of the used clones for DNA probes construction are available under GenBank accession numbers EF508271 to EF508312.

**DNA probe construction**

Selected clones from the shotgun library were cultured in LB broth as described above, and plasmid DNA was prepared by the rapid alkaline lysis method (Sambrook and Russell 2001). These plasmids were submitted to PCR amplification, in 50 µl reaction volume containing 50 to 100 ng of DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl2, 50 pmol of each primer (universal M13 forward and reverse primers) and 2.5 U Taq polymerase (Promega, Madison, USA), using the following cycling conditions: an initial denaturation at 95 ºC for 5 min, followed by 35 cycles with a profile of 1 min at 95 ºC (denaturation), 30 s at 50 ºC (annealing) and 2 min at 72 ºC (extension), followed by a final extension for 5 min at 72 ºC. The PCR products were purified by gel excision, and resequenced to confirm the identity. PCR products were labeled by random prime reactions with [α-32P]dCTP, except the 5S rRNA probe that was labeled with [γ-32P]dATP through radioactive PCR (Sambrook and Russell 2001).

**Southern hybridization**

Hybridizations were carried out at 42 ºC in the following solution: 50% formamide, 0.5% SDS, 6x SSC (0.9 M NaCl, 9 mM sodium citrate; pH 7.0), 5x Denhardt’s solution (1 mg/ml of BSA, 1 mg/ml of Ficoll 400, 1 mg/ml of Polyvinylpyrrolidone; pH 7.0) and 100 µg of DNA calf thymus during 24 h. The membranes were then washed under variable conditions, depending on the desired stringency, dried and exposed into a cassette with a storage phosphor screen during 48 h. After exposure, the screen was scanned with Storm Software (GE-Amersham Biosciences) for documentation of the hybridization results. Following, the processed images were compared with the pictures of the gels used in each experiment. All hybridization experiments were realized at least twice to verify the reproducibility of the results.

**RESULTS**

The DNA fragments generated in each restriction profile were named according to the restriction enzymes that created them (C, P, S, CP, CS or PS), followed by a sequential number according to the decreasing size. In the double digestions, when a given fragment was not digested by a second restriction enzyme, this fragment received
the same nomenclature of the single digestion (Table 1). Under different electrophoresis conditions, the undigested genomic DNA of *G. diazotrophicus* PAL5 did not enter into the gels, suggesting that is a circular structure (data not shown).

Genomic DNA was digested by single and double digestions, which generated 6 different fingerprinting profiles. The single digestions consisted of digestions with the restriction enzymes *I*-CeuI, *Pac*I and *Swa*I, generating profiles identified as C, P and S, respectively. The double digestions consisted of digestions with the following restriction enzymes combinations: *I*-CeuI plus *Pac*I, *I*-CeuI plus *Swa*I and *Pac*I plus *Swa*I, generating profiles identified as CP, CS and PS, respectively (Table 1).

The PFGE profiles generated by single digestions revealed 4 to 6 different DNA fragments, with length ranging from 130 to 1,634 Kb, while those of the double digestions showed 9 to 11 DNA fragments, with length varying from 20 to 1,357 Kb. The *G. diazotrophicus* PAL5 chromosome size was estimated in 4,240 Kb ± 41.6 Kb, through the mean from the estimated genome sizes of all PFGE profiles, generated with single and double digestions, and these values ranged from 4,197 Kb to 4,300 Kb (Table 1).

For separation of all detectable DNA fragments, the PFGE gels were run at 3 different electrophoresis programs (Figs. 1A-C). However, the gels produced under electrophoresis condition of the Figure 1A, were considered the most representative, because the PFGE profiles exhibit all detectable DNA fragments. In the two other electrophoresis conditions used (Figs. 1B and C), DNA fragments with length between 2 and 70 Kb were not detected. In addition, under the PFGE conditions employed in Figure 1B, some bands classified in this work as doublets, i.e. bands P4 and P5, bands S5 and S6 were separated, confirming their existence. Electrophoresis procedures performed for detection of larger DNA fragments did not detect any additional bands (data not shown).

45 DNA fragments generated from single and double digestions of the *G. diazotrophicus* PAL5 chromosome were related (Table 1). However, 2 fragments designated CP6 and PS6 were not detected in any experiment conducted in this study. By other hand, considering the number of DNA fragments observed in single digestions, the expected number of DNA fragments in double digestions, the length sum of the detected DNA fragments in PFGE profiles CP and PS, and the estimated length of *G. diazotrophicus* PAL5 chromosome, our results suggested the existence of these DNA fragments and estimate that its present length smaller than 20 Kb.

The intron-encoded restriction endonuclease *I*-CeuI, which recognizes a highly conserved 26 bp sequence into the large 23S rRNA subunit, cleaved *G. diazotrophicus* chromosome in four fragments, suggesting the existence of

| Table 1. Number and estimated length of the DNA fragments generated by single and double digestions of the *Gluconacetobacter diazotrophicus* PAL5 chromosome. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| I-CeuI | Single Digestions | Double Digestions | I-CeuI / PacI | I-CeuI / Swal | PacI / Swal |
| Fragment | Size (Kb) | P1 | 1,577 | S1 | 1,634 | CP1 | 1,357 | CS1 | 1,052 | PS1 | 1,094 |
| C1 | 1,400 | P1 | 1,577 | S1 | 1,634 | CP1 | 1,357 | CS1 | 1,052 | PS1 | 1,094 |
| C2 | 1,235 | P2 | 927 | S2 | 1,152 | C4 | 625 | S3 | 680 | PS2 | 1,066 |
| C3 | 965 | P3 | 927 | S3 | 680 | C4 | 625 | S3 | 680 | PS2 | 1,066 |
| C4 | 625 | P4 | 200 | S4 | 467 | CP3 | 508 | C5 | 469 | S4 | 469 |
| C5 | 200 | P5 | ≈200 | S5 | 142 | CP4 | 393 | C6 | 142 | S4 | 469 |
| C6 | 142 | P6 | <20 | S6 | 130 | CP5 | 352 | S6 | 130 | PS6 | 285 |
| C7 | 130 | P7 | <20 | S6 | 130 | CP6 | <20 | S6 | 130 | PS6 | 285 |
| C8 | <20 | P8 | <20 | S6 | 130 | CP7 | <20 | S6 | 130 | PS6 | 285 |
| Total | 4,225 | ≈4,300 | 4,205 | ≈4,231 | ≈4197 | ≈4281 |

1 These bands are doublets.
2 Predicted fragment by chromosome digestion and alignment of larger DNA fragments, but not observed in PFGE gels.
3 The mean of the sums of the restriction fragments generated from single and double digestions of the *G. diazotrophicus* PAL5 chromosome was 4,240 Kb with standard deviation of 41.6 Kb.
4 rrn operons (PFGE profile C in Fig. 1A; Table 1). To confirm this hypothesis, we made hybridizations with DNA probes of the 16S (rrs), 23S (rrl) and 5S (rrf) rRNA genes (Table 2), that generally are arranged in this sequential orientation on bacterial genomes. Therefore, we expected 4 DNA fragments hybridized in the PFGE profile C, for each radioactive probe used. However, our results showed that only 3 DNA fragments hybridized in this profile for these probes (Fig. 2A; Table 3).

The rrs and rrl probes hybridized with the fragments C2, C3 and C4, while the rrf probe hybridized with the fragments C1, C2 and C3 (Fig. 2A; Table 3), confirming that the G. diazotrophicus PAL5 chromosome was circular and had an inverted orientation of 1 rrn operon, located between the fragments C4 and C1, based on the strong signal of the C4 fragment obtained in hybridization with rrs and rrl probes, and absence of signal for the C1 fragment, while with the rrf probe occurred the opposite.

Based on the hybridization results (Table 3) generated with single copy gene probes (Table 2), we verified overlaps between individual DNA fragments from the six different PFGE profiles of the chromosomal DNA, because these probes showed unequivocal hybridizations with one fragment into each profile. An example of this approach is shown in Figure 2B, where the nifA probe was hybridized and showed overlap between the DNA fragments C3, P4 and S3 contained in these profiles (Table 3). Likewise, all hybridization results were analyzed and annotated, making possible the detection of 12 gene clusters that showed different hybridization profiles (Table 3), indicating proximity of genes located on the same cluster. Furthermore, these data were used to determine the molecular markers positioning on this bacterial chromosome.

All restriction fragments conferred hybridization signals, with at least one of the 42 DNA probes, except fragments CP6, PS6, P5 and S6 because we could not selected from our DNA library probes that gave hybridization signals with these fragments. However, these 4 DNA fragments that were not detected by hybridization were assembled into the physical map by combinatorial analysis of DNA fragment length and restriction site position of the restriction enzyme which generated them.

The DNA fragments P1 and P2 (PFGE profile P from Fig. 1A; Table 1), CS2 and S3 (PFGE profile CS from Fig. 1A; Table 1), PS1 and PS2, PS4 and PS5 (PFGE profile PS from Fig. 1A; Table 1) were undistinguishable one from other on PFGE gels. However, the hybridization results with single copy genes probes showed that the bands P2,

**Figure 1.** PFGE profiles of Gluconacetobacter diazotrophicus PAL5 genomic DNA observed in 3 different electrophoresis conditions. PFGE profiles generated from single or double digestions: C (I-Ceu), P (PacI), S (SwaI), CP (I-Ceu plus PacI), CS (I-Ceu plus SwaI), PS (PacI plus SwaI); Y - yeast chromosome PFGE marker; λ - Lambda ladder PFGE marker (50–1000 Kb); γ - Lambda Low Range PFGE marker (2.03–194 Kb); A - Electrophoresis procedure carried out in 1% agarose gel and running buffer containing 0.5× TBE at 6V/cm at 13 °C in a sequence of three ramps (ramp A consisted of an initial switch time of 80 s, a final switch time of 100 s during 16 h; ramp B consisted of an initial switch time of 100 s, a final switch time of 120 s during 8 h; and ramp C consisted of an initial switch time of 120 s, a final switch time of 140 s during 16 h; B - Electrophoresis procedure carried out in 1% agarose gel and running buffer containing 0.5× TBE at 6V/cm at 13 °C with initial switch time of 1s and final switch time of 50s during 23h. C - Electrophoresis procedure carried out in 1% agarose gel and running buffer containing 1× TAE at 4 V/cm at 13 °C with initial switch time of 0.1 s and final switch time of 10 s during 16 h.
CS2, PS2 and PS5 were doublets, that exhibited overlaps with different individual fragments included in others PFGE profiles, and consequently gave hybridization signals with genes probes located in different gene clusters (Table 3).

Considering the overlaps between the majority of DNA fragments from different PFGE profiles, rrn genes hybridization results (Table 3), DNA fragments length, existence of doublets in PFGE profiles (Table 1) and location of restriction enzymes sites, 45 DNA fragments from six different PFGE profiles, were ordered into physical map of the *G. diazotrophicus* PAL5 chromosome (Fig. 3), using a reduced number of radioactive probes, and avoiding the use of partial digestions of the chromosome, two-dimensional PFGE and linking clones. In total, the hybridizations allowed 42 genetic markers to be allocated into the *G. diazotrophicus* PAL5 physical map (Fig. 3).

**DISCUSSION**

Because the *G. diazotrophicus* PAL5 genome has a high GC content (66.4%), the restriction enzymes *PacI* and *SwaI* were chosen to obtain the DNA fragments used to construct the physical map described here. The aim of this strategy was to generate a low number of DNA fragments, easily manageable by PFGE methodology. Additionally, these rare-cutting restriction enzymes were related by other authors that mapped GC rich chromosomes as suitable enzymes to construct macrorestriction physical maps of bacterial chromosomes because they cleave these chromosomes into a small number of large DNA fragments (Ramos-Díaz and Ramos 1998; Widjaja *et al.* 1999; Llamas *et al.* 2002).

In addition, with the purpose of determinate the number, position and orientation of *rrn* operons, following the strategy previously suggested for precise location of the *rrl* genes on chromosomes (Ramos-Díaz and Ramos 1998; Widjaja *et al.* 1999; Melkerson-Watson *et al.* 2000; Zé-Zé *et al.* 2000; Dudez *et al.* 2002; El-Osta *et al.* 2002), we used an intron-encoded restriction endonuclease I-CeuI, that recognizes a highly conserved 26 bp restriction site into *rrl* gene.

Among the 45 DNA fragments generated in single and double digestions, only the fragments named as CP6 and PS6 (Table 1) were not detected in any experiment, but the results suggest that these predicted DNA fragments probably diffused of the blocks during preparations. This argument is supported by related results from other studies that observed diffusion of smaller DNA fragments from agarose blocks during preparations, or low fluorescence signals in staining procedures (Römling *et al.* 1989; Chan and Goodwin 1999; Melkerson-Watson *et al.* 2000).

Physical methods provided direct measurements of genome content for various microorganisms (Ramos-Díaz and Ramos 1998; Zé-Zé *et al.* 2000; Blank and Stemke 2000; Marcone and Seemüller 2001; Oana *et al.* 2002). In this

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**Figure 2.** Hybridization with rrn probes against PFGE profile generated with I-CeuI digestion of Gluconacetobacter diazotrophicus PAL5 chromosome, and an example of hybridization results generated with single copy genes against PFGE profiles. A - hybridization results with rrn probes; B - hybridization results with nIaA probe. The single and double digestions of the *G. diazotrophicus* PAL5 chromosome were C (I-CeuI), P (PacI), S (SwaI), CP (I-CeuI plus PacI), CS (I-CeuI plus SwaI), PS (PacI plus SwaI). Y - yeast chromosome PFGE marker. rrs - 16S ribosomal RNA gene; rrl - 23S ribosomal RNA gene; rrf - 5S ribosomal RNA gene. C1, C2, C3 and C4 are the DNA fragments generated from chromosome digestion with I-CeuI restriction enzyme (see Table 1).
### Table 2. Description of the *rrn* and single copy gene probes used for construction of the *Gluconacetobacter diazotrophicus* PAL5 chromosome physical map.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Product of gene</th>
<th>Organism</th>
<th>Identity</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>acoC</td>
<td>EF508280</td>
<td>Dihydrolipoamide acetyltransferase</td>
<td>Pseudomonas putida F1</td>
<td>67%</td>
<td>5e-48</td>
</tr>
<tr>
<td>araE</td>
<td>EF508310</td>
<td>Sugar-proton symporter</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>99%</td>
<td>0.0</td>
</tr>
<tr>
<td>araJ</td>
<td>EF508297</td>
<td>Arabinose efflux permease</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>100%</td>
<td>6e-110</td>
</tr>
<tr>
<td>clpP</td>
<td>EF508291</td>
<td>ATP dependent Clp protease proteolytic subunit 2</td>
<td>Acidiphilium cryptum JF-5</td>
<td>79%</td>
<td>3e-123</td>
</tr>
<tr>
<td>csp</td>
<td>EF508282</td>
<td>Cold shock protein</td>
<td>Methyllobacterium radiotolerans JCM 2831</td>
<td>82%</td>
<td>8e-37</td>
</tr>
<tr>
<td>dhpF</td>
<td>EF508285</td>
<td>Nonribosomal peptide synthetase DhpF</td>
<td>Burkholderia pseudomallei JCM 668</td>
<td>76%</td>
<td>4e-86</td>
</tr>
<tr>
<td>dnuE</td>
<td>EF508281</td>
<td>DNA polymerase III, alpha chain</td>
<td>Acidiphilium cryptum JF-5</td>
<td>72%</td>
<td>2e-142</td>
</tr>
<tr>
<td>fcuA</td>
<td>EF508288</td>
<td>Ferrichrome receptor FcuA</td>
<td>Gluconobacter oxydans 621H</td>
<td>83%</td>
<td>3e-115</td>
</tr>
<tr>
<td>hutC</td>
<td>EF508272</td>
<td>Fumarate hydratase class II</td>
<td>Rhodopseudomonas palustris CGA009</td>
<td>74%</td>
<td>3e-66</td>
</tr>
<tr>
<td>gntB</td>
<td>EF508300</td>
<td>Glutamate synthase (NADPH) large chain</td>
<td>Gluconobacter oxydans 621H</td>
<td>80%</td>
<td>3e-120</td>
</tr>
<tr>
<td>gntP</td>
<td>EF508296</td>
<td>Gluconate transporter permease</td>
<td>Raistonia solanacearum GI1000</td>
<td>69%</td>
<td>6e-104</td>
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<tr>
<td>hemE</td>
<td>EF508271</td>
<td>Uroporphyrinogen III decarboxylase</td>
<td>Gluconobacter oxydans 621H</td>
<td>71%</td>
<td>7e-115</td>
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<tr>
<td>hemH</td>
<td>EF508312</td>
<td>Ferrochelatase</td>
<td>Acidiphilium cryptum JF-5</td>
<td>77%</td>
<td>2e-106</td>
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<tr>
<td>hlyD</td>
<td>EF508311</td>
<td>Secretion protein HlyD</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>100%</td>
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<tr>
<td>hupH</td>
<td>EF508286</td>
<td>Histidine ammonia-lyase (Histidase) protein</td>
<td>Gluconobacter oxydans 621H</td>
<td>77%</td>
<td>7e-100</td>
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<tr>
<td>ilvD</td>
<td>EF508303</td>
<td>Dihydroxy-acid dehydratase</td>
<td>Gluconobacter oxydans 621H</td>
<td>74%</td>
<td>0.0</td>
</tr>
<tr>
<td>kdpC</td>
<td>EF508276</td>
<td>Potassium-transporting ATPase C chain</td>
<td>Mesorhizobium lot MAF303099</td>
<td>81%</td>
<td>7e-11</td>
</tr>
<tr>
<td>kup</td>
<td>EF508305</td>
<td>Potassium uptake protein</td>
<td>Gluconobacter oxydans 621H</td>
<td>65%</td>
<td>5e-09</td>
</tr>
<tr>
<td>lhr</td>
<td>EF508287</td>
<td>Superfamily II helicase and inactivated derivatives</td>
<td>Burkholderia pseudomallei 1106</td>
<td>80%</td>
<td>1e-09</td>
</tr>
<tr>
<td>lig3</td>
<td>EF508301</td>
<td>ATP-dependent DNA ligase</td>
<td>Rhizobium sp. NGR234</td>
<td>69%</td>
<td>4e-87</td>
</tr>
<tr>
<td>lsdA</td>
<td>EF508277</td>
<td>Levansucrase precursor</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>100%</td>
<td>0.0</td>
</tr>
<tr>
<td>mcpA</td>
<td>EF508306</td>
<td>Methyl-accepting chemotaxis protein</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>99%</td>
<td>0.0</td>
</tr>
<tr>
<td>metE</td>
<td>EF508309</td>
<td>5-methyltetrahydropteroylglutamate- homocysteine methyltransferase</td>
<td>Caulobacter crescentus CB15</td>
<td>80%</td>
<td>0.0</td>
</tr>
<tr>
<td>mexI</td>
<td>EF508304</td>
<td>Multidrug efflux RND transporter</td>
<td>Pseudomonas fluorescens PFO-1</td>
<td>70%</td>
<td>1e-86</td>
</tr>
<tr>
<td>mfs</td>
<td>EF508289</td>
<td>Multidrug efflux MFS membrane fusion protein</td>
<td>Methylobacterium radiotolerans JCM 2831</td>
<td>96%</td>
<td>6.8</td>
</tr>
<tr>
<td>mgtA</td>
<td>EF508283</td>
<td>Mg2+-importing ATPase</td>
<td>Bradyrhizobium japonicum USDA 110 DNA</td>
<td>79%</td>
<td>1e-105</td>
</tr>
<tr>
<td>nilA</td>
<td>EF508279</td>
<td>Mo/Fe nitrogenase specific transcriptional regulator</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>100%</td>
<td>0.0</td>
</tr>
<tr>
<td>nuoG</td>
<td>EF508308</td>
<td>NADH-dehydrogenase gamma subunit</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>100%</td>
<td>0.0</td>
</tr>
<tr>
<td>pdhA</td>
<td>EF508293</td>
<td>Pyruvate dehydrogenase E1 component alpha</td>
<td>Gluconobacter oxydans 621H</td>
<td>73%</td>
<td>9e-100</td>
</tr>
<tr>
<td>pepC</td>
<td>EF508302</td>
<td>Phosphoenolpyruvate carboxylyase</td>
<td>Granulibacter bethesdensis CGDNIH1</td>
<td>66%</td>
<td>2e-79</td>
</tr>
<tr>
<td>pilT</td>
<td>EF508290</td>
<td>PIIT protein-like, outer membrane heme receptor</td>
<td>Acetobacter pasteurianus</td>
<td>80%</td>
<td>5e-37</td>
</tr>
<tr>
<td>pyrB</td>
<td>EF508292</td>
<td>Aspartate carbamoyltransferase</td>
<td>Gluconobacter oxydans 621H</td>
<td>76%</td>
<td>1e-161</td>
</tr>
<tr>
<td>rtf</td>
<td>EF508284</td>
<td>SS ribosomal RNA</td>
<td>Bartonella henselae strain Houston-1</td>
<td>100%</td>
<td>0e-32</td>
</tr>
<tr>
<td>rtl</td>
<td>EF508307</td>
<td>23S ribosomal RNA</td>
<td>Acetobacter intermedius</td>
<td>97%</td>
<td>0.0</td>
</tr>
<tr>
<td>rrs</td>
<td>EF508273</td>
<td>16S ribosomal RNA</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>98%</td>
<td>0.0</td>
</tr>
<tr>
<td>sucD</td>
<td>EF508278</td>
<td>Succinyl-CoA synthetase, alpha subunit</td>
<td>Acidiphilium cryptum JF-5</td>
<td>77%</td>
<td>5e-84</td>
</tr>
<tr>
<td>trpA</td>
<td>EF508298</td>
<td>Inner membrane transport protein of toluene-induced toluene exclusion pump</td>
<td>Gluconacetobacter diazotrophicus</td>
<td>100%</td>
<td>0.0</td>
</tr>
<tr>
<td>ublE</td>
<td>EF508299</td>
<td>SAM-dependent methyltransferase</td>
<td>Pseudomonas putida G8-1</td>
<td>72%</td>
<td>3e-48</td>
</tr>
<tr>
<td>xdhA</td>
<td>EF508274</td>
<td>Xanthine dehydrogenase XdhA protein</td>
<td>Gluconobacter oxydans 621H</td>
<td>73%</td>
<td>1e-177</td>
</tr>
<tr>
<td>xseA</td>
<td>EF508295</td>
<td>Exodeoxyribonuclease VII large subunit</td>
<td>Silicibacter pomeroyi DDS-3</td>
<td>67%</td>
<td>1e-17</td>
</tr>
<tr>
<td>ylfF</td>
<td>EF508275</td>
<td>D-xylene ABC transporter, periplasmic-D</td>
<td>xylose binding protein</td>
<td>74%</td>
<td>2e-88</td>
</tr>
<tr>
<td>zntA</td>
<td>EF508294</td>
<td>Cation transport P-type ATPase</td>
<td>Erythrobacter litoralis HTCC2594</td>
<td>65%</td>
<td>9e-17</td>
</tr>
</tbody>
</table>

1. Names of the genes that were used for probes construction.
2. GenBank accession numbers of the nucleotide sequences from used clones for DNA probes construction.
3. Products of genes from used clones for DNA probes construction.
4. Microorganisms that present best alignment score of its sequence against NCBI data bank using blastn tool.
5. Identities percentage observed in alignment of the probe sequence against NCBI data bank using blastn tool.
6. e-value score observed in alignment of the probe sequence against NCBI data bank using blastn tool.
Table 3. Hybridization experiments results with \( rm \) and single copy gene probes.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Genes (^2)</th>
<th>( I)-Ceu (^3)</th>
<th>PacI (^3)</th>
<th>Swal (^3)</th>
<th>( I)-Ceu / PacI (^3)</th>
<th>( I)-Ceu / Swal (^3)</th>
<th>PacI / Swal (^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hemE, hemH, kdpC, mfs and pyrB</td>
<td>C4</td>
<td>P2</td>
<td>S2</td>
<td>C4</td>
<td>C4</td>
<td>PS1</td>
</tr>
<tr>
<td>2</td>
<td>dhhF</td>
<td>C3</td>
<td>P2</td>
<td>S2</td>
<td>CP4</td>
<td>CS4</td>
<td>PS1</td>
</tr>
<tr>
<td>3</td>
<td>lig3, mexl and mgtA</td>
<td>C3</td>
<td>P2</td>
<td>S3</td>
<td>CP4</td>
<td>S3</td>
<td>PS5</td>
</tr>
<tr>
<td>4</td>
<td>nifA, lsdA and pilT</td>
<td>C3</td>
<td>P4</td>
<td>S3</td>
<td>P4</td>
<td>S3</td>
<td>P4</td>
</tr>
<tr>
<td>5</td>
<td>fumC, hutH, kup, and ubiE</td>
<td>C3</td>
<td>P3</td>
<td>S3</td>
<td>CP5</td>
<td>S3</td>
<td>PS4</td>
</tr>
<tr>
<td>6</td>
<td>araE, araJ, clpP, csp, fcuA, hlyD, lhr, ilvD, and xseA</td>
<td>C2</td>
<td>P3</td>
<td>S1</td>
<td>CP3</td>
<td>CS1</td>
<td>PS3</td>
</tr>
<tr>
<td>7</td>
<td>dnaE, gntP, mcpA, pepC and sucD</td>
<td>C2</td>
<td>P1</td>
<td>S1</td>
<td>CP2</td>
<td>CS1</td>
<td>PS2</td>
</tr>
<tr>
<td>8</td>
<td>acoC, pdhA, trpA, xdhA and zntA</td>
<td>C1</td>
<td>P1</td>
<td>S1</td>
<td>CP1</td>
<td>CS2</td>
<td>PS2</td>
</tr>
<tr>
<td>9</td>
<td>glnB and nuoG</td>
<td>C1</td>
<td>P1</td>
<td>S4</td>
<td>CP1</td>
<td>S4</td>
<td>S4</td>
</tr>
<tr>
<td>10</td>
<td>xylF and metE</td>
<td>C1</td>
<td>P1</td>
<td>S2</td>
<td>CP1</td>
<td>CS3</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>rss and rrl</td>
<td>C2/C3</td>
<td>P1/P2/P3</td>
<td>S1/S2/S5</td>
<td>CP2/CP5</td>
<td>CS1/C4/CS5</td>
<td>PS1/PS2/PS5</td>
</tr>
<tr>
<td>12</td>
<td>rrl</td>
<td>C1/C2/C3</td>
<td>P1/P2/P3</td>
<td>S1/S2/S5</td>
<td>CP1/CP3/CP4</td>
<td>CS2/CS3/CS4/CS6</td>
<td>PS1/PS2/PS5</td>
</tr>
</tbody>
</table>

\(^1\) Gene clusters that present the same hybridization profile.

\(^2\) Name of the genes used for probes construction (see Table 2).

\(^3\) Single and double digestions generated from G. diazotrophicus PAL5 chromosome (see Table 1).

---

**Figure 3.** Physical map of Gluconacetobacter diazotrophicus PAL5 chromosome using the \( I\)-Ceu, PacI and Swal restriction enzymes. C, P and S – restriction sites of the \( I\)-Ceu, PacI and Swal restriction enzymes, respectively. Restriction fragments were named according to established criteria in materials and methods (see Table 1). The DNA fragments CP6 and PS6 were predicted from chromosome digestion results and aligned on this physical map, but were not observed in PFGE gels. The genetic markers are described in Table 2. The exact positioning of the molecular markers in DNA fragments, were not determined in this physical map. The innermost circle represents the scale in megabases of the G. diazotrophicus chromosome. 1 This value represent the length mean of the G. diazotrophicus chromosome.
study, the *G. diazotrophicus* PAL5 genome size was estimated in 4,240 Kb ± 41.6 Kb. In addition, results based on hybridization using the *rrn* genes, demonstrated that *G. diazotrophicus* has four *rrn* operons in a circular chromosome, and one *rrn* operon is an inverted orientation in relation to others. These results are in agreement with several authors that used this strategy to confirm the number, location and inversions of *rrn* operons (Ramos-Díaz and Ramos 1998; Blank and Stemke 2000; Padovan et al. 2000; Zé-Zé et al. 2000; El-Osta et al. 2002; Oana et al. 2002).

The use of single copy gene probes (Table 2), permitted the detection of 12 gene clusters, that showed different hybridization profiles and revealed hybridization signals with the majority of DNA fragments (except fragments P5, S6, CP6 and PS6) (Table 3), and together with the *rrn* genes hybridization data, DNA fragments length and restriction sites position, allowed the assembly of the *G. diazotrophicus* PAL5 chromosome physical map, as well as the positioning of molecular markers on its structure. Similar strategy was used for construction of a physical and genetic map of the *Cowdria ruminantium* chromosome (Villiers et al. 2000).

Among 42 genetic markers allocated into the *G. diazotrophicus* PAL5 physical map (Fig. 3), some genes such as *mcpA*, *nifA* and *lsdA*, are present in several genomes as part of operons, suggesting the presence of related genes in their neighborhood. Additionally, the *hemE* and *hemH* genes, which are considered strategic markers, because in some bacterial genomes are described as flanking genes of the replication origin (oriC) (Capela et al. 2001; Kaneko et al. 2002), were mapped. Consequently, in the *G. diazotrophicus* PAL5 physical map (Fig. 3), the fragment C4 was allocated in position zero, because this is the smaller DNA fragment that hybridized with the *hemE* and *hemH* probes.

The hybridization results described here in this paper were used by the Riogene Genomic Sequencing Consortium (http://www.riogene.lncc.br), to help to obtain the complete genomic sequence of *G. diazotrophicus* PAL5, that is now completely sequenced and available in EMBL database under accession numbers AM889285, AM889286 and AM889287. This approach is in agreement with previous studies (Lai et al. 1999; Blank and Stemke 2000; Weinel et al. 2001), which considered PFGE-based physical maps as an efficient tool, for quality control of sequence assembly generated from shotgun sequencing data, once this strategy allows the detection of possible misassemblies within sequence contigs or contig scaffolds, and helps the re-ordering of an assembly, especially in the flanking regions of *rrn* genes. However, in this study the chromosome size was estimated in 4,240 Kb, but the real size detected in sequencing results was 3,944 Kb, representing an acceptable discrepancy of 7% (296 Kb) between estimated and real size of *G. diazotrophicus* chromosome.

The main contribution of this work resides on the fact that little was known on genetic organization of *G. diazotrophicus* PAL5, an important endophytic nitrogen-fixing microorganism, isolated from crops of tropical and subtropical regions, in association with different plant hosts. Furthermore, the data presented here was a first step to understand its genomic organization and contributed for elucidation of the complete genome sequence of this microorganism.

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