ORIGINAL ARTICLE

Physical and genetic map of the *Gluconacetobacter diazotrophicus* PAL5 chromosome

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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 digestions of the chromosome were carried out with *I-Ceu*I, *Swa*I and *Pac*I restriction enzymes. The resulting DNA fragments were positioned into physical map, using combinatory 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 Kb ± 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.

RESUMEN. Un mapa de macrorrestricción de la bacteria *Gluconacetobacter diazotrophicus* PAL5, una importante endófita fijadora de nitrógeno de diversos vegetales como la caña de azúcar, el café, la batata y la piña, fue construido utilizándose electroforesis en gel de campo pulsado (PFGE) e hibridación de ADN. Para el análisis por PFGE, el cromosoma fue digerido por digestiones simples y dobles, con endonucleasas de restricción *I-Ceu*I, *Swa*I y *Pac*I. Los fragmentos del ADN que resultaron fueron posicionados en el mapa físico, utilizándose un análisis combinatorio entre los resultados de hibridación, la posición de los sitios de cortes de enzimas de restricción y el tamaño de los fragmentos. *G. diazotrophicus* tiene 4 operones *rrn* en un cromosoma circular, con tamaño estimado de 4,240 Kb ± 41.6 Kb. Los resultados de hibridación permitieron el posicionamiento de 42 marcadores genéticos en el cromosoma (39 copias simples y 3 elementos repetidos), así como la detección de 1 operón *rrn* con la orientación invertida. Los resultados contenidos en este manuscrito fueron importantes para finalizar el montaje del genoma de *G. diazotrophicus* PAL5, que fue depositado y está disponible en la base de datos EMBL.

Palabras clave: *Gluconacetobacter diazotrophicus*, PFGE, mapa físico, mapa genético, operones rrn.

INTRODUCTION

Gluconacetobacter diazotrophicus is α nitrogen-fixing acetic acid a-proteobacterium, found in endophytic association with several sugarcane cultivars, especially in agricultural areas where nitroen 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; Munõz-Rojas *et al*. 2005). The ability to colonize a number of families such as *Poaceae*, *Convolvulaceae*, *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; Munõz-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; Munõz-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-

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bly 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 NH_4^+ at high sucrose concentrations, though is not affected by high concentration of 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 nitrate 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). Macrorestriction 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 macrorestriction 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, peptone 1.5 g, yeast extract 2.0 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 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-HCl – 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% sarkosyl, 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-HCl 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-Ceu*I, *Pac*I and *Swa*I (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.

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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-Amershan Biosciences). For southern blot, the gels were depurined, denatured, neutralized and blotted to nylon membranes (Hybond+ from GE-Amershan 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 *Sma*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-Amershan 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 $MgCl_2$, 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 °C 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-^{32}P]dCTP, except the 5S rRNA probe that was labeled with [γ-^{32}P]dATP through radioactive PCR (Sambrook and Russell 2001).

Southern hybridization

Hybridizations were carried out at 42 °C in the following solution: 50% formamide, 0.5% SDS, 6× SSC (0,9 M NaCl, 9 mM sodium citrate; pH 7,0), 5× Dendhardt'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-Amershan 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

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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-Ceu*I, *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-Ceu*I plus *Pac*I, *I-Ceu*I 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-Ceu*I, 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.

| | Single Digestions | | | | | | Double Digestions | | | | | |
| | *I-Ceu*I | | *Pac*I | | *Swa*I | | *I-Ceu*I / *Pac*I | | *I-Ceu*I / *Swa*I | | *Pac*I / *Swa*I | |
Fragment	Size (Kb)	Fragment	Size (Kb)	Fragment	Size (Kb)	Fragment	Size (Kb)	Fragment	Size (Kb)	Fragment	Size (Kb)
C1	1,400	P1	1,577	S1	1,634	CP1	1,357	CS1	1,052	PS1	1,094
C2	1,235	P2	≈1,400 [1]	S2	1,152	C4	625	S3	680	PS2	1,066 [1]
C3	965	P3	927	S3	680	CP2	576	CS2	≈680 [1]	S4	469
C4	625	P4	200	S4	467	CP3	508	C4	625	PS3	390
-	-	P5	≈200 [1]	S5	142	CP4	393	S4	468	PS4	285
-	-	-	-	S6	130	CP5	352	CS3	260	PS5	≈285 [1]
-	-	-	-	-	-	P4	200	CS4	152	P4	200
-	-	-	-	-	-	P5	≈200 [1]	S6	130	P5	≈200 [1]
-	-	-	-	-	-	CP6	< 20 [2]	CS5	80	S5	142
-	-	-	-	-	-	-	-	CS6	70	S6	130
-	-	-	-	-	-	-	-	-	-	PS6	< 20 [2]
Total [3]	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 combinatory 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,
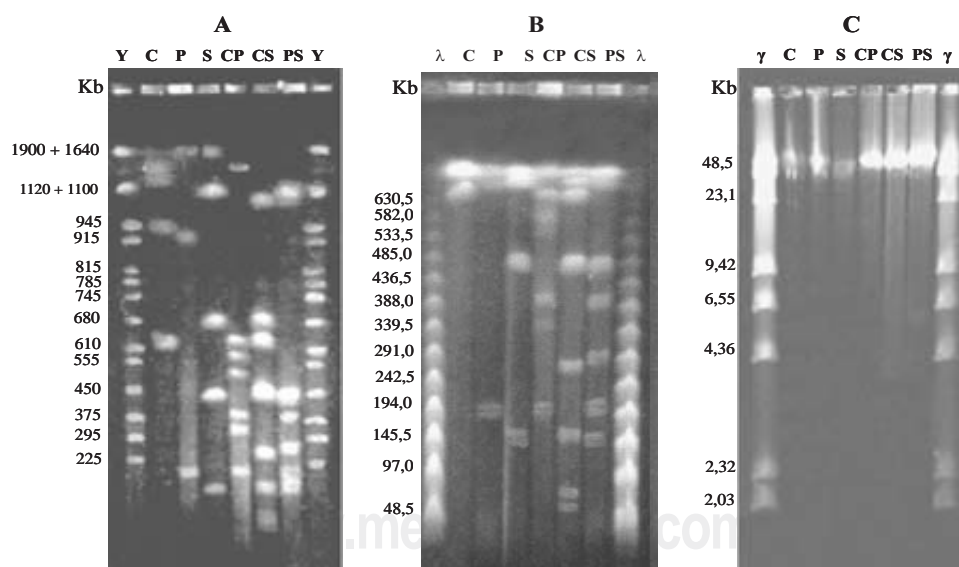

Figure 1. *PFGE profiles of* Gluconacetobacter diazotrophicus *PAL5 genomic DNA observed in 3 different electrophoresis conditions. PFGE profiles generated from single or double digestions: C (*I-Ceu*I*), P (*Pac*I*), S (*Swa*I*), CP (*I-Ceu*I plus *Pac*I*),CS (*I-Ceu*I plus *Swa*I*), PS (*Pac*I plus *Swa*I*); Y- yeast chromosome PFGE marker; λ - Lambda ladder PFGE marker (50–1000 Kb); λ- Lambda Low Range PFGE marker (2.03–194 Kb); **A** - Electrophoresis procedure carried out in 1% agarose gel and running buffer containing 0.5x 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 0.5x 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 1x 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.*

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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 *Pac*I and *Swa*I 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-Ceu*I, 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
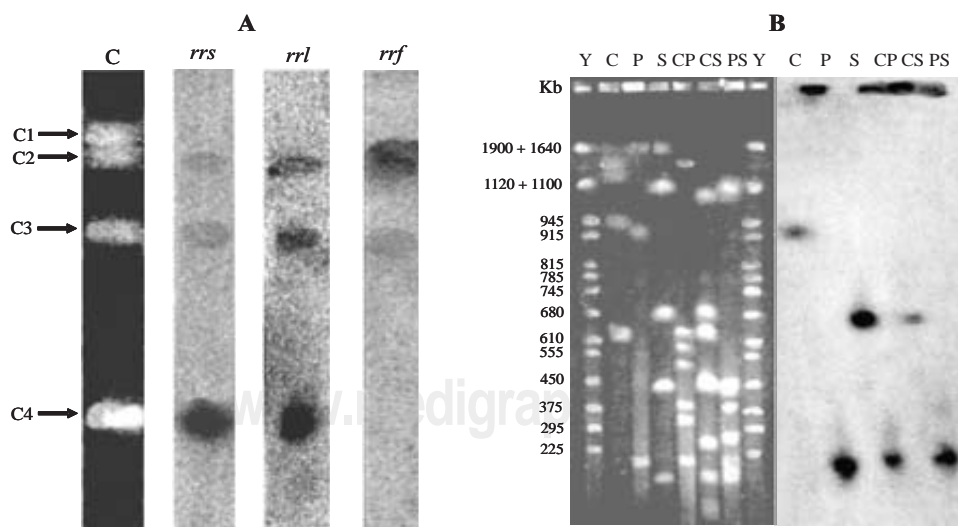

Figure 2. *Hybridization with* rrn *probes against PFGE profile generated with* I-Ceu*I digestion of* Gluconacetobacter diazotrophicus *PAL5 chromosome, and an example of hybridization results generated with single copy genes against PFGE profiles. **A** - hybridization results with* rrn *probes; **B**- hybridization results with* nifA *probe. The single and double digestions of the* G. diazotrophicus *PAL5 chromosome were C (*I-Ceu*I), P (*Pac*I), S (*Swa*I), CP (*I-Ceu*I plus* Pac*I), CS (*I-Ceu*I plus* Swa*I), PS (*Pac*I plus* Swa*I). 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-Ceu*I 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.

Gene [1]	Accession number [2]	Product of gene [3]	Organism [4]	Identity [5]	Probability [6]
acoC	EF508280	Dihydrolipoamide acetyltransferase	*Pseudomonas putida* F1	67%	5e-48
araE	EF508310	Sugar-proton symporter	*Gluconacetobacter diazotrophicus*	99%	0.0
araJ	EF508297	Arabinose efflux permease	*Gluconacetobacter diazotrophicus*	100%	6e-110
clpP	EF508291	ATP dependent Clp protease proteolytic subunit 2	*Acidiphilium cryptum* JF-5	79%	3e-123
csp	EF508282	Cold shock protein	*Methylobacterium radiotolerans* JCM 2831	82%	8e-37
dhbF	EF508285	Nonribosomal peptide synthetase DhbF	*Burkholderia pseudomallei* 668	76%	4e-86
dnaE	EF508281	DNA polymerase III, alpha chain	*Acidiphilium cryptum* JF-5	72%	2e-142
fcuA	EF508288	Ferrichrome receptor FcuA	*Gluconobacter oxydans* 621H	83%	3e-115
fumC	EF508272	Fumarate hydratase class II	*Rhodopseudomonas palustris* CGA009	74%	3e-66
gltB	EF508300	Glutamate synthase (NADPH) large chain	*Gluconobacter oxydans* 621H	80%	3e-120
gntP	EF508296	Gluconate transporter permease	*Ralstonia solanacearum* GMI1000	69%	6e-104
hemE	EF508271	Uroporphyrinogen III decarboxylase	*Gluconobacter oxydans* 621H	71%	7e-115
hemH	EF508312	Ferrochelatase	*Acidiphilium cryptum* JF-5	77%	2e-106
hlyD	EF508311	Secretion protein HlyD	*Gluconacetobacter diazotrophicus*	100%	0.0
hutH	EF508286	Histidine ammonia-lyase (Histidase) protein	*Gluconobacter oxydans* 621H	77%	7e-100
ilvD	EF508303	Dihydroxy-acid dehydratase	*Gluconobacter oxydans* 621H	74%	0.0
kdpC	EF508276	Potassium-transporting ATPase C chain	*Mesorhizobium loti* MAFF303099	81%	7e-11
kup	EF508305	Potassium uptake protein	*Gluconobacter oxydans* 621H	65%	5e-09
lhr	EF508287	Superfamily II helicase and inactivated derivatives	*Burkholderia pseudomallei* 1106	80%	1e-09
lig3	EF508301	ATP-dependent DNA ligase	*Rhizobium sp.* NGR234	69%	4e-87
lsdA	EF508277	Levansucrase precursor	*Gluconacetobacter diazotrophicus*	100%	0.0
mcpA	EF508306	Methyl-accepting chemotaxis protein	*Gluconacetobacter diazotrophicus*	99%	0.0
metE	EF508309	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	*Caulobacter crescentus* CB15	80%	0.0
mexI	EF508304	Multidrug efflux RND transporter	*Pseudomonas fluorescens* PfO-1	70%	1e-86
mfs	EF508289	Multidrug efflux MFS membrane fusion protein	*Methylobacterium radiotolerans* JCM 2831	96%	6.8
mgtA	EF508283	Mg2+-importing ATPase	*Bradyrhizobium japonicum* USDA 110 DNA	79%	1e-105
nifA	EF508279	Mo/Fe nitrogenase specific transcriptional regulator	*Gluconacetobacter diazotrophicus*	100%	0.0
nuoG	EF508308	NADH dehydrogenase gamma subunit	*Gluconacetobacter diazotrophicus*	100%	0.0
pdhA	EF508293	Pyruvate dehydrogenase E1 component alpha	*Gluconobacter oxydans* 621H	73%	9e-100
pepC	EF508302	Phosphoenolpyruvate carboxylase	*Granulibacter bethesdensis* CGDNIH1	66%	2e-79
pilT	EF508290	PilT protein-like, outer membrane heme receptor	*Acetobacter pasteurianus*	80%	5e-37
pyrB	EF508292	Aspartate carbamoyltransferase	*Gluconobacter oxydans* 621H	76%	1e-161
rrf	EF508284	5S ribosomal RNA	*Bartonella henselae* strain Houston-1	100%	2e-32
rrl	EF508307	23S ribosomal RNA	*Acetobacter intermedius*	97%	0.0
rrs	EF508273	16S ribosomal RNA	*Gluconacetobacter diazotrophicus*	98%	0.0
sucD	EF508278	Succinyl-CoA synthetase, alpha subunit	*Acidiphilium cryptum* JF-5	77%	5e-84
trpA	EF508298	Inner membrane transport protein of toluene-induced toluene exclusion pump	*Gluconacetobacter diazotrophicus*	100%	0.0
ubiE	EF508299	SAM-dependent methyltransferase	*Pseudomonas putida* GB-1	72%	3e-48
xdhA	EF508274	Xanthine dehydrogenase XdhA protein	*Gluconobacter oxydans* 621H	73%	1e-177
xseA	EF508295	Exodeoxyribonuclease VII large subunit	*Silicibacter pomeroyi* DSS-3	67%	1e-17
xylF	EF508275	D-xylose ABC transporter, periplasmic-D xylose binding protein	*Pseudomonas syringae* pv. tomato str.DC3000	74%	2e-88
zntA	EF508294	Cation transport P-type ATPase	*Erythrobacter litoralis* HTCC2594	65%	9e-17

[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 *rrn* and single copy gene probes.

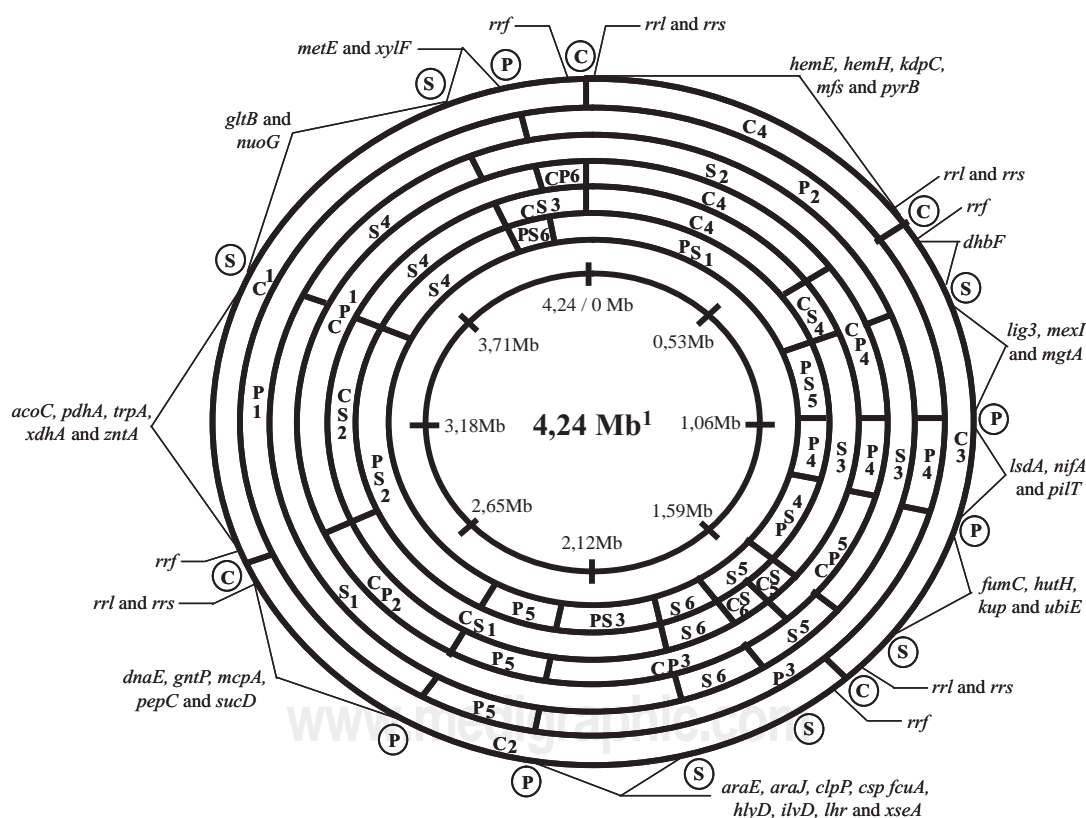| Cluster [1]	Genes [2]	I-CeuI [3]	PacI [3]	SwaI [3]	I-CeuI / PacI [3]	I-CeuI / SwaI [3]	PacI / SwaI [3]
1	*hemE, hemH, kdpC, mfs* and *pyrB*	C4	P2	S2	C4	C4	PS1
2	*dhbF*	C3	P2	S2	CP4	CS4	PS1
3	*lig3, mexI* and *mgtA*	C3	P2	S3	CP4	S3	PS5
4	*nifA, lsdA* and *pilT*	C3	P4	S3	P4	S3	P4
5	*fumC, hutH, kup,* and *ubiE*	C3	P3	S3	CP5	S3	PS4
6	*araE, araJ, clpP, csp, fcuA, hlyD, lhr,ilvD,* and *xseA*	C2	P3	S1	CP3	CS1	PS3
7	*dnaE, gntP, mcpA, pepC* and *sucD*	C2	P1	S1	CP2	CS1	PS2
8	*acoC, pdhA, trpA, xdhA* and *zntA*	C1	P1	S1	CP1	CS2	PS2
9	*gltB* and *nuoG*	C1	P1	S4	CP1	S4	S4
10	*xylF* and *metE*	C1	P1	S2	CP1	CS3	-
11	*rrs* and *rrl*	C2/C3/C4	P1/P2/P3	S1/S2/S5	C4/CP2/CP5	CS1/C4/CS5	PS1/PS2/S5
12	*rrf*	C1/C2/C3	P1/P2/P3	S1/S2/S5	CP1/CP3/CP4	CS2/CS3/CS4/CS6	PS1/PS2/S5

[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-CeuI*,* PacI *and* SwaI *restriction enzymes. C, P and S – restriction sites of the* I-CeuI*,* PacI *and* SwaI *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.*

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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 misassembles 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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