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ORIGINAL PAPER

Isolation and characterization of active promoters from *Gluconacetobacter diazotrophicus* strain PAL5 using a promoter-trapping plasmid

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Abstract *Gluconacetobacter diazotrophicus* is a nitrogen-fixing, endophytic bacterium that has the potential to promote plant growth and increase yield. Genetically modified strains might get more benefits to host plants, including through expression of useful proteins, such as Cry toxins from *B. thuringiensis*, or enzymes involved in phytohormone production, proteins with antagonistic activity for phytopathogens, or that improve nutrient utilization by the plant. For that, expression systems for *G. diazotrophicus* are needed, which requires active promoters fused to foreign (or innate) genes. This article describes the construction of a *G. diazotrophicus* PAL5 promoter library using a promoter-less *lacZ*-bearing vector, and the identification of six active promoters through β-galactosidase activity assays, sequencing and localization in the bacterial genome. The characterized promoters, which are located on distinct regions of the bacterial genome and encoding either sense or antisense transcripts, present variable expression strengths and might be used in the future for expressing useful proteins.

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Kátia Regina dos Santos Teixeira: In memoriam.

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Introduction

Gluconacetobacter diazotrophicus is a nitrogen-fixing, endophytic bacterium that was initially isolated from sugarcane (Cavalcante and Döbereiner 1988; Gillis et al. 1989; Dong et al. 1995), and later from other agronomically important plants, such as wheat, rice, coffee, sweet potato, elephant grass, banana and pineapple (Baldani and Baldani 2005). This bacterium has been considered as one of the possible candidates responsible for the high levels of N_2 fixation detected in sugarcane (Boddey et al. 2003). It has been shown that inoculation of *G. diazotrophicus* strain PAL5 in a consortium with four additional endophytic nitrogen-fixing bacterial strains has the potential to promote sugarcane growth and increase yield (Oliveira et al. 2002, 2006). In addition, there is evidence that, beyond nitrogen fixation, other bacterial benefits might be acting for plant growth promotion (Sevilla et al. 2001), such as phytohormone production (Bastián et al. 1998), antagonistic activity against phytopathogens (Blanco et al. 2005; Saravanan et al. 2007), and improvement of nutrient utilization by the plant (Maheshkumar et al. 1999; Saravanan et al. 2007; Logeshwaran et al. 2009).

If wild-type strains of *G. diazotrophicus* present potential to be used in inoculants for agronomically important crops, genetically modified *G. diazotrophicus* strains may get even more beneficial features, exemplified by modifications for expressing Cry proteins from *Bacillus thuringiensis* with lethality against plague insects (Subashini et al. 2011; Rapulana and Bouwer 2013). In order to generate new recombinant strains for expressing useful proteins,

expression systems for *G. diazotrophicus* are needed, which requires active promoters fused to foreign (or innate) genes.

Strong promoters have been isolated from *Leifsonia xyli* (basonym *Clavibacter xyli*) subsp. *cynodontis*, the causative pathogen of ratoon stunting disease in sugarcane, with the aim to express useful proteins inside plants without having to transform the plant genome (Haapalainen et al. 1996). In *Rhizobium etli*, which is a nodule-forming, nitrogen-fixing symbiont of bean roots, a collection of active promoters has been isolated from a genomic DNA library constructed in a suited promoter-trap vector, which permitted to characterize the regulon of the main sigma factor σ^{70} (SigA) in that bacterial species (Ramírez-Romero et al. 2006). Also, in *Azospirillum brasilense* strain Sp245, a plant growth-promoting rhizobacterium (PGPR) of cereal crops, the promoter-trapping approach of differential fluorescence induction (DFI) has been used to identify promoters that are induced in the presence of wheat seed extracts, revealing genes involved in the interaction with the plant (Pothier et al. 2007). Works like those illustrate the importance of isolating and characterizing promoters from plant-associated bacteria for both knowledge gain and potential agrobiotechnological application.

This article describes the construction of a *G. diazotrophicus* PAL5 promoter library using a promoter-less *lacZ*-bearing vector, and the identification of active promoters through β-galactosidase activity assays, sequencing and localization in the bacterial genome. This approach permitted the identification of six *G. diazotrophicus* active promoters with variable expression strengths, which are located on distinct regions of the bacterial genome, and might be used in the future for expressing useful proteins.

Materials and methods

Determination of minimal inhibitory concentration (MIC) of antibiotic and relative vector stability

In order to determine the tetracycline MIC for *G. diazotrophicus* PAL5, this strain was grown in a 500-ml erlenmeyer flask containing 150 µl of the culture media DYGS (Rodrigues Neto et al. 1986) (2.0 g l^{-1} glucose, 1.5 g l^{-1} bacterial peptone, 2.0 g l^{-1} yeast extract, 0.5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1.5 g l^{-1} glutamic acid, 0.1 mg l^{-1} biotin, 0.2 mg l^{-1} pyridoxal-HCl, pH 6.0, 15 g l^{-1} agar) or LZGD (simplified DYGS medium in which modification was exclusion of both bacterial peptone and yeast extract) in the presence of serial dilutions of tetracycline, in an ELISA plate, for up to 2 day, at 30 °C, 250 rpm. The optical density of the cell culture suspensions was determined using an iEMS microplate reader (Labsystems) at 600 nm.

Vector stability assays comprised in culturing *G. diazotrophicus* PAL5 harboring pPW452 in DYGS medium under the absence of selective pressure for more than forty generations (estimated following optical density at 600 nm), plating on both the presence and absence of tetracycline, and counting the colony-forming units (CFUs), which revealed the number of resistant (vector-bearing) and total CFUs, respectively.

Construction of a lacZ promoter-trap library

Vector pPW452 is a derivative of pMP220 (Spaink et al. 1987), in which the multicloning site (flanked by two *Hind*III cleavage sites) was inverted (P. Woodley, unpublished). Those are promoter probe vectors containing a multicloning site located upstream to a promoter-less *lacZ* reporter gene. To construct the promoter library in pPW452, total *G. diazotrophicus* PAL5 DNA was prepared using the CTAB method (Doyle 1987), and partially digested with *Sau*3AI. Resulting fragments with 0.5–1.0-kb were excised from an agarose gel, purified using Wizard SV Gel and PCR Clean-Up System (Promega), cloned into the unique *Bgl*II site of pPW452, and the resultant recombinant plasmids were transferred into *E. coli* DH10B cells. Transformants were selected on 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal)-containing LB medium supplemented with 10 mg l^{-1} tetracycline. All colonies of the promoter library were pooled, the plasmid DNA was prepared from the mixed clone cells, and used to transform cells of *G. diazotrophicus* PAL5 through electroporation, essentially as described (Teixeira et al. 1999). Transformants were selected on X-Gal-containing DYGS medium in the presence of 100 mg l^{-1} tetracycline. *G. diazotrophicus* clones were picked using sterilized toothpicks, transferred to 96-well plates, cultured in liquid DYGS medium, and stored in 50 % glycerol at −70 °C.

Clone culture conditions for LacZ activity evaluations

2.5 µl of each clone suspension were transferred from the stock plates at −70 °C onto solid DYGS medium containing 100 mg l^{-1} tetracycline, and cultured for 5–7 days at 30 °C. The resulting colonies were then picked, using a 96-well format picker, for inoculating into 1 ml of liquid DYGS medium containing 100 mg l^{-1} tetracycline in 96-deep-well plates. Clones were cultured for 3–4 days at 30 °C, 250 rpm. Then, clones were reinoculated 1:400 into 1 ml of LZGD medium (with glucose or another carbon source when specified) containing 10 mg l^{-1} tetracycline, in 96-deep-well plates, and then cultured for 3-5 days at 30 °C, 250 rpm. Alternatively, clones were inoculated on one of the following solid media in the presence of X-Gal: DYGS, LGI (Baldani et al. 2005) (pH 6.0), acetic LGI

(Sievers and Swings 2005), or BMS (Baldani et al. 2005) (supplemented with 10 % sucrose).

Chromogenic assays of beta-galactosidase activity

Beta-galactosidase activity assays using ortho-nitrophenyl-β-D-galactopyranoside (ONPG) were performed as initially described (Miller 1972), but adapted to the 96-well format (Griffith and Wolf 2002; Schwab et al. 2007). Briefly, 40 µl of the cell suspension were transferred to a well of a 96-deep-well plate containing 360 µl of Z buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $Na_2H_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 0.0027 % SDS, 0.39 % β-mercaptethanol, pH 7.0). For cell permeabilization, 25 µl of chloroform were added in each well. Plates were vortexed, briefly centrifuged using a microplate rotor, and 80 µl of ONPG solution (4.0 $g{\cdot}l^{-1}$ in Z buffer) were added into each well. When the reaction solution stained yellow, it was stopped by adding 200 µl of 1 M Na_2CO_3, and the time required for the reaction was registered. The 96-deep-well plate was centrifuged for 5 min, at 3600×*g*, and 200 µl of the supernatant were transferred into a well of an ELISA microplate. Absorbance at 414 nm was determined using an iEMS microplate reader (Labsystems). The optical density of the cell culture suspensions (200 µl) was determined using the same microplate reader at 600 nm.

Fluorogenic assays of beta-galactosidase activity

The fluorogenic substrate 5-acetylaminofluorescein di-β-D-galactopyranoside (C2FDG) was used for assaying beta-galactosidase activity of the *G. diazotrophicus* clones essentially as described for other bacterial species (Rowland et al. 1999). In brief, the cell suspension was centrifuged for 7 min at 3600×*g*, the supernatant was discarded, and cells were suspended in the same volume of phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). 50 µl of the cell suspension were transferred to a well of an ELISA microplate containing 50 µl of 66 µM C2FDG. The microplate was incubated at 30 °C for 3 h, and then scanned using a Typhoon FLA 9000 manifold (GE Healthcare) with LPB filter. Fluorescence intensity values were obtained through pixel quantification from the image files using ImageQuant TL 7.0 (GE Healthcare). Fluorescence arbitrary units were given as a function of the optical density of the cell culture suspensions, determined as described for chromogenic assays.

Genetic characterization of beta-galactosidase highly active clones

Cell suspensions of the *G. diazotrophicus* clones were diluted 1:10 in PCR-grade water, boiled for 10 min, and then used as PCR template with primers PPWleft (5′-TTGAGCAACTGACTGAAATGC-3′) and PPWrightV3 (5′-GCTCACCCCAAAAATGGCA-3′), which anneal to the flanks of the multiple cloning site of pPW452. PCR products were precipitated by the addition of 10 % volume of 3 M sodium acetate and 2.5 volumes of ethanol, and dissolved in PCR-grade water.

Alternatively, the cell suspensions in DYGS medium were centrifuged, and the supernatant was discarded. Cells were resuspended in 150µl of GET buffer (50 mM glucose, 10 mM EDTA, 25 mM TRIS–HCl, pH 8.0), and lysed by adding 150 µl of lysis solution (0.4 M NaOH, 2 % SDS). The cell lysate was directly used to transform competent *E. coli* DH10B cells. Plasmids from the resulting transformant clones were prepared through the alkaline lysis technique (Ish-Horowicz and Burke 1981).

Clones were sequenced with primer PPWleft or PPWrightV3 using a 3500 Genetic Analyzer (Applied Biosystems), or by Macrogen Inc. (South Korea).

Results

Representativeness of the *G. diazotrophicus* PAL5 promoter-trap library

MIC determination results of tetracycline for *G. diazotrophicus* strain PAL5 (Fig. 1a) suggested that 100 $g{\cdot}l^{-1}$ tetracycline was efficient to select transformants with vector pPW452 and derivatives carrying the promoter library. Also, vector stability assays suggested that under the absence of selective pressure, the vector was rapidly lost (Fig. 1b). Therefore, the use of tetracycline was important to guarantee maintenance of the vector and derivatives in PAL5 cells.

Near 8100 colonies of *E. coli* DH10B cells were obtained presenting variable degrees of blue color intensity on X-Gal-containing medium after transformation with the PAL5 promoter library. A *Hind*III restriction analysis involving a random sample of 44 clones revealed the presence of inserts with the expected size range (0.5–1.0 kb) in 33 clones, suggesting the presence of desired inserts in approximately 75 % of the promoter library. Extrapolating that percentage to the whole library, the success-rate was estimated to be approximately 8100 × 75 % = 6100 valid clones. Therefore, the genomic relative coverage of the promoter library was calculated as previously described (Clarke and Carbon 1976), but considering both DNA strands for transcriptional orientation reasons relating to *lacZ*, which would double the genome size (factor "2"):

$$P_1 = 1 - (1-f)^{\mathrm{N}} = 1 - [1 - 750/(3,999,591 \times 2)]^{6100} \cong 44\,\%,$$

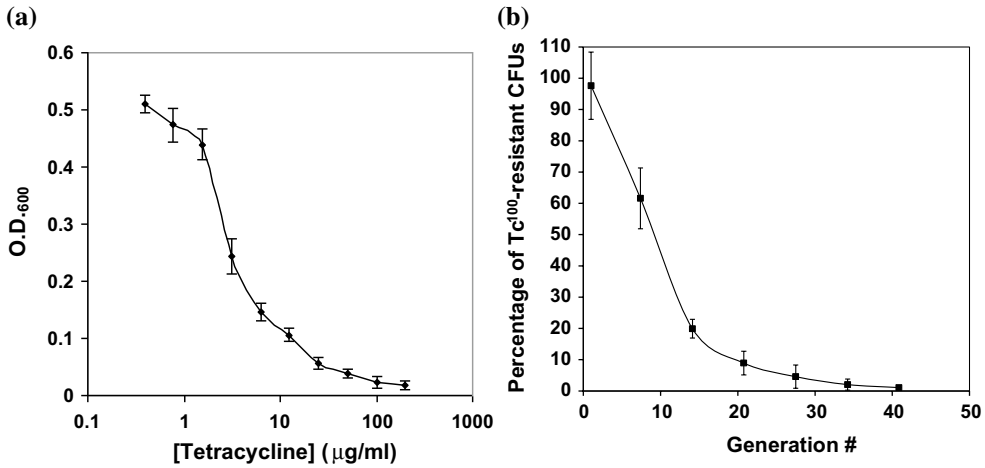

Fig. 1 Evaluations for using vector pPW452 to construct the *G. diazotrophicus* PAL5 promoter library. **a** Minimal inhibitory concentration (MIC) of tetracycline, the selection marker of vector pPW452, in DYGS medium. **b** Vector pPW452 stability in *G. diazotrophicus* PAL5 cells under non-selective pressure

in which f is the fraction of the total genome represented by a fragment with a given average size, and N is the number of positive clones in the library.

The promoter library was then extracted from the pooled *E. coli* clones, and transferred into *G. diazotrophicus* cells by electroporation, a procedure that resulted in roughly 7400 tetracycline-resistant colonies of clones. The probability P_2 of a given cloned plasmid in *E. coli* has been included in the ~7400 colonies of the PAL5 transformed cells, considering that all had the same chance, follows the same statistics as above:

$$P_2 = 1 - [1 - (1/8100)]^{7400} = 60\,\%.$$

Considering the relative genomic coverage of the promoter library calculated in *E. coli* (44 %), in *G. diazotrophicus* the relative genomic coverage of the promoter library would be reduced to 44 % × 60 % ≅ 26 %.

To validate those estimations, a random sample of 71 clones in *G. diazotrophicus* was analyzed through DNA sequencing of their inserts. Of those, 30 clones presented empty vector sequence, while 41 showed *G. diazotrophicus* PAL5 genome sequences (Table 1). Thus, the percentage of positive clones was 41/71 = 58 %, in contrast to 75 % in *E. coli*, as shown above. Reduction of positive clones after transferring the promoter library to another bacterial strain might result from the greater probability of smaller plasmids (empty or small insert-containing vector) to enter host cells (Hanahan 1983). In accordance with this, among the positive *G. diazotrophicus* clones, most had fragment sizes near the minimal limit of the selected length to construct the promoter library (~500 bp).

The 41 insert-containing clones showed some redundancy, actually representing 21 distinct constructions involving the *G. diazotrophicus* PAL5 genome (Table 1). Although this redundancy was statistically expected, one might suppose that the five distinct constructions that appeared more than once among clones would be "hot spots" for cloning. Nevertheless, one of those constructions, appearing ten times, is a chimera of three regions of the *G. diazotrophicus* PAL5 genome, and that combination with a determined configuration should be a rare event for a random cloning process. Thus, probably that redundancy is associated to the transfer process of the promoter library between host bacteria, and maybe in that case its high frequency should be a coincidence within the sample of clones analyzed.

Another observation was the presence of chimeras, which appeared six times among 21 distinct constructions (~29 %). That was acceptable considering that it could not damp our goal of isolating active promoters, although eventually it might difficult to precisely identify and localize them on the right place of the PAL5 genome.

Optimal conditions for β-galactosidase activity assays

Initial evaluations of β-galactosidase expression involving clones on DYGS medium supplemented with 100 $mg \cdot l^{-1}$ tetracycline and X-Gal resulted in faintly blue colonies, suggesting that other growth conditions, including other media and/or lower concentration of antibiotic, might favour reporter enzyme expression. Hence, the following tests aimed to choose appropriate culture conditions for the promoter expression/β-galactosidase activity assays. Two random clones previously presenting β-galactosidase activity on X-Gal-containing DYGS medium, arbitrarily named p1 and p2, plus the clone of the empty vector pPW452, and

Table 1 Sequencing data of the *G. diazotrophicus* PAL5 promoter library according to analysis of 41 random clones

Frequency	Cloned region[a]	Insert length (sum of chimeras) (bp)
10	182,997–183,922::3,299,287–3,298,658:: 916,248–915,867	1935 (925 + 629 + 381)
1	486,251–485,644::2,250,604–2,250,063	1148 (607 + 541)
1	574,699–575,224	526
1	620,848–621,409::2,246,524–2,246,734	773 (562 + 211)
1	874,761–875,335	575
4	1,061,128–1,061,683	556
2	1,168,841–1,168,187::939,454–940,248	1450 (655 + 795)
1	1,266,435–1,266,015::1,634,620–1,635,254	1056 (421 + 635)
1	1,444,673–1,444,943	271
1	[b]	486
1	2,034,193–2,034,735::570,166–570,533	911 (543 + 368)
1	2,203,224–2,202,748	476
1	2,307,785–2,308,437	653
1	2,411,823–2,412,800	978
1	2,443,444–2,443,953	510
1	2,450,573–2,450,032	542
1	2,699,414–2,698,470	945
7	3,035,034–3,034,519	515
1	3,734,092–3,734,333	242
1	3,839,369–3,839,573	205
2	3,894,464–3,895,144	680

[a] Nucleotide numbers according to *G. diazotrophicus* PAL5 genome project (Bertalan et al. 2009), GenBank access number AM889285

[b] The cloned fragment corresponds to a repetitive genomic region present at 1,593,013–1,592,528; 2,146,446–2,146,931; 2,511,970–2,512,455; 2,697,050–2,696,565; and 2,803,597–2,804,082

also non-transformed PAL5, were all plated on other media for *G. diazotrophicus* in the presence of two different concentrations of tetracycline (Fig. 2). On DYGS medium and under the lower concentration (10 $mg \cdot l^{-1}$) of tetracycline, β-galactosidase expression (based on blue stain intensity) seemed to be improved (Fig. 2a), when compared to the higher concentration of antibiotic (100 $mg \cdot l^{-1}$). Non-transformed PAL5 was unable to grow neither on DYGS nor BMS at 10 $mg \cdot l^{-1}$ tetracycline, in contrast to LGI, and acetic LGI, in which growth was observed under that antibiotic concentration. On the latter media, little or no blue staining at all was observed, and the same was true on BMS, probably due to the high levels of gum produced by the bacteria in this medium.

It is well known that MIC is not constant for a given antibiotic, as it can be affected by the growth medium composition, among other factors. Considering that DYGS containing 10 $mg \cdot l^{-1}$ tetracycline seemed attractive for β-galactosidase activity assays, the finding that non-transformed PAL5 was sensitive to that antibiotic concentration would guarantee the exclusive presence of transformed PAL5 cells in the bacterial culture under those conditions. Nevertheless, DYGS medium limits comparative studies of gene expression levels, as it is a complex medium. Thus, complex ingredients were removed from DYGS composition, and a simpler medium, LZGD (for assaying LacZ activity in *G. diazotrophicus*), was developed and tested. First, MIC of tetracycline for *G. diazotrophicus* strain PAL5 was evaluated in liquid LZGD medium, and results suggested that 100 or even 10 $g \cdot l^{-1}$ tetracycline was more than enough to select transformants with vector pPW452 and derivatives carrying the promoter library (Fig. 3a). Then, the same four strains of Fig. 3a were plated on LZGD in the presence of two different concentrations of tetracycline and three different N sources (Fig. 3b–g). Similarly to DYGS medium, in LZGD under the lower concentration (10 $mg \cdot l^{-1}$) of tetracycline, β-galactosidase expression seemed to be improved, when compared to the higher concentration of the antibiotic (100 $mg \cdot l^{-1}$), especially with sodium glutamate as N source (which is also present in DYGS composition). However, in contrast to DYGS, in LZGD non-transformed PAL5 was capable to grow at 10 $mg \cdot l^{-1}$ tetracycline on solid medium, suggesting that a higher antibiotic concentration, such as 100 $mg \cdot l^{-1}$, was necessary to guarantee the exclusive presence of plasmid-bearing bacteria, a condition that avoids underestimation of β-galactosidase activity of the library clones. The contrasting results of tetracycline sensitivity between solid and

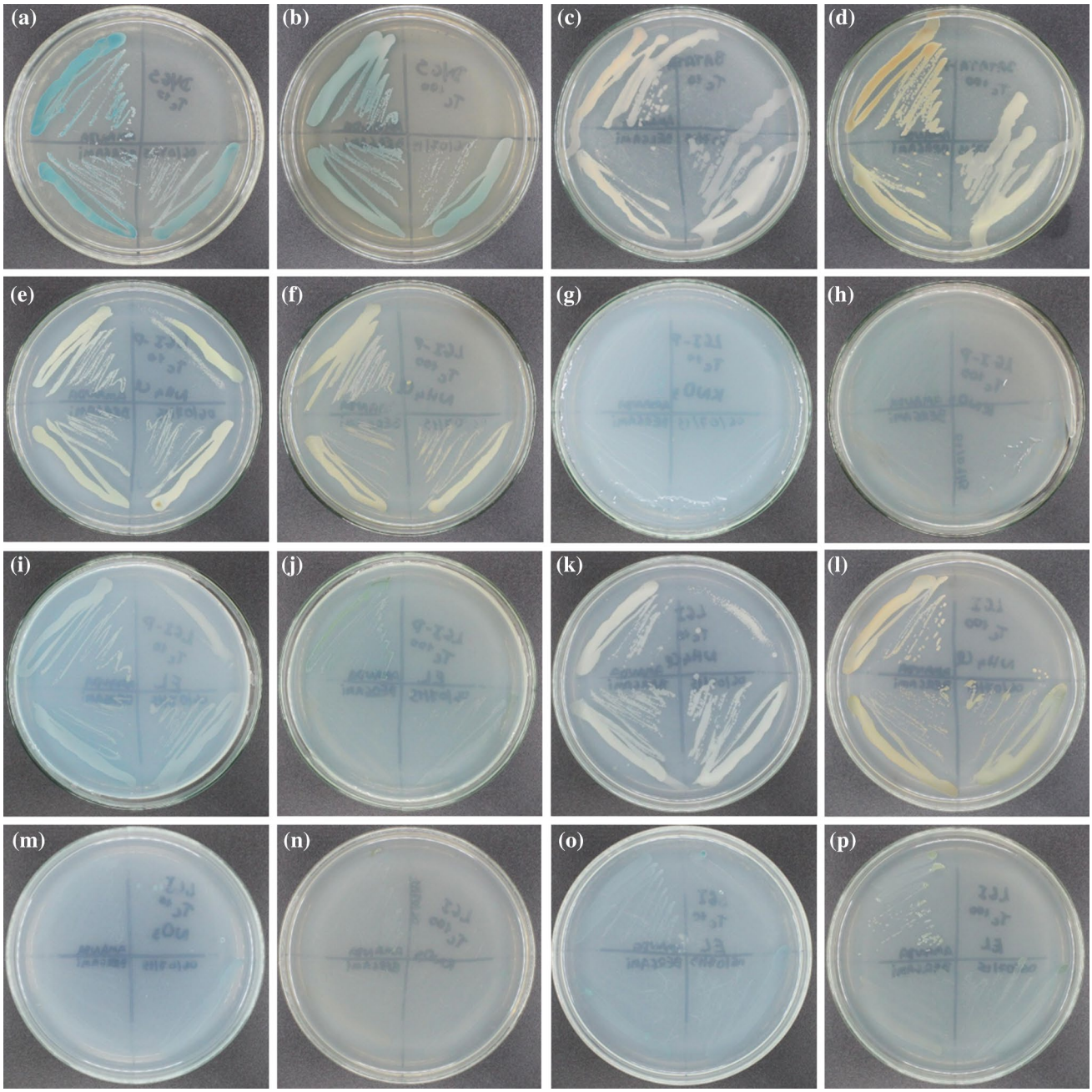

Fig. 2 Evaluation of the adequate growth conditions to assay for β-galactosidase activity. Within each Petri plate, clockwise, non-transformed PAL5 (*upper right*), PAL5 (pPW452) (empty vector), PAL5 (p1), and PAL5 (p2) were plated on: **a**, **b** DYGS medium amended with 10 or 100 mg·l^{-1} tetracycline (Tc^{10} or Tc^{100}), respectively; **c**, **d** BMS Tc^{10} or Tc^{100}; **e**–**j** Acetic LGI with 20 mmol·l^{-1} NH_4Cl, or 20 mmol·l^{-1} KNO_3, or 20 mg·l^{-1} yeast extract as N source amended with Tc^{10} or Tc^{100}, alternately; and **k**–**p** LGI with 20 mmol·l^{-1} NH_4Cl, or 20 mmol·l^{-1} KNO_3, or 20 mg·l^{-1} yeast extract as N source amended with Tc^{10} or Tc^{100}, alternately

liquid LZGD media (Fig. 3a, f) led to further investigation, which confirmed that non-transformed PAL5 is sensitive to Tc^{10} in liquid LZGD, when compared to the transformed strain (Fig. 3h). This was important for the subsequent β-galactosidase activity assays.

To verify if the general tendencies observed for β-galactosidase activity among different solid media would be also detected among their liquid forms, 96 clones were grown in four liquid media for three days, and assayed for β-galactosidase activity using either the chromogenic or the fluorogenic substrates. Table 2 shows the results for an active clone only, suggesting quite the same tendencies observed on solid media. Therefore, for practical purposes, LZGD, a simplified form of DYGS medium, amended with

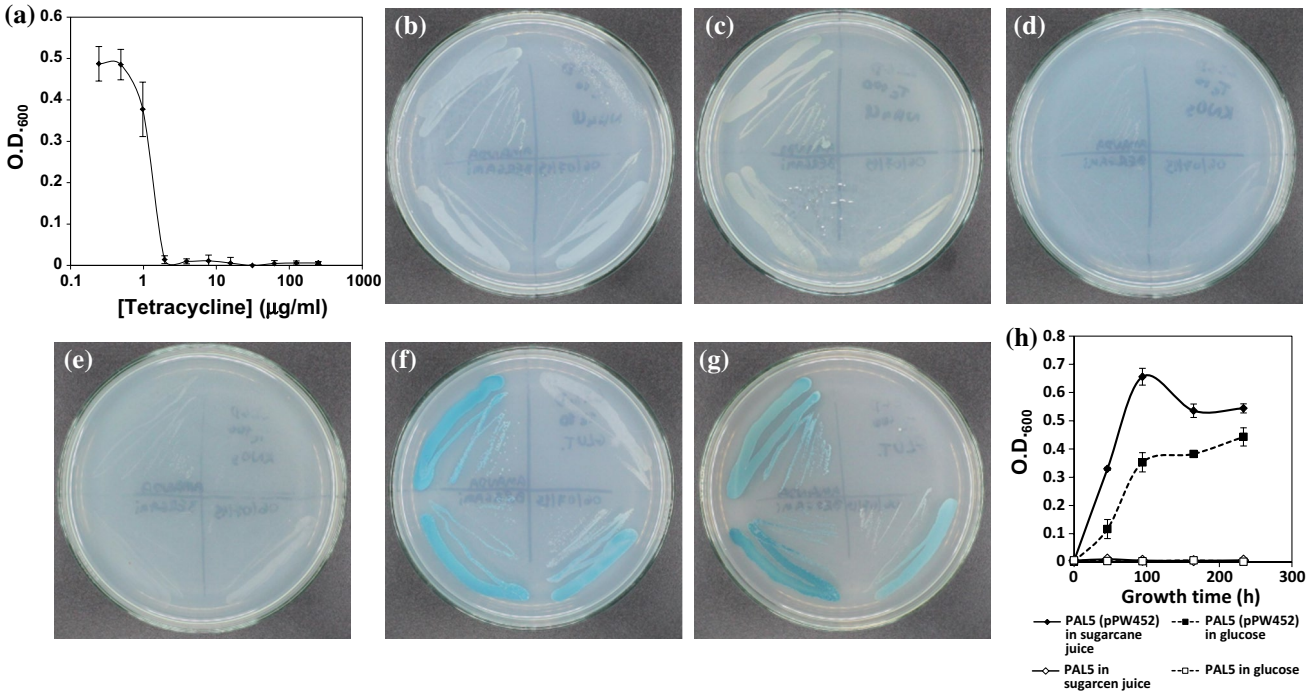

Fig. 3 Viability of using LZGD medium for screening clones of the *G. diazotrophicus* PAL5 promoter library. **a** Minimal inhibitory concentration (MIC) of tetracycline (with 20 mmol·l^{-1} sodium glutamate as nitrogenous source). **b–g** Adequate growth conditions to assay for β-galactosidase activity with 20 mmol·l^{-1} NH_4Cl, or 20 mmol·l^{-1} KNO_3, or 20 mmol·l^{-1} sodium glutamate as N source amended with Tc^{10} or Tc^{100}, alternately [within each Petri plate, clockwise: non-transformed PAL5 (*upper right*), PAL5 (pPW452) (empty vector), PAL5 (p1), and PAL5 (p2)]. **h** Growth profiles of PAL5 (pPW452) and PAL5 in LZGD medium with glucose or sugarcane juice as C source supplemented with 20 mmol·l^{-1} sodium glutamate as N source in the presence of 10 mg·l^{-1} tetracycline

Table 2 Evaluation of growth and assay conditions for β-galactosidase activity in *G. diazotrophicus*

	pPW59-B09				PPW452			
	β-galactosidase activity with ONPG[a]	β-galactosidase activity with C2FDG[a]	D.O.$_{600}$	pH	β-galactosidase activity with ONPG[a]	β-galactosidase activity with C2FDG[a]	D.O.$_{600}$	pH
DYGS Tc^{10}	100 %	100 %	0.609	7.0	9.7 %	3.0 %	0.647	7.0
Tc^{100}	75 %	35 %	0.716	7.0	7.7 %	3.8 %	0.821	7.0
LZGD Tc^{10}	79 %	3.7 %	0.457	8.0	14 %	2.9 %	0.381	8.0
Tc^{100}	33 %	14 %	0.265	4.0	3.4 %	0.5 %	0.246	4.0
LGI Tc^{10}	67 %	~0 %	0.184	3.5	7.5 %	~0 %	0.185	3.5
Tc^{100}	8.9 %	~0 %	0.099	3.0	1.7 %	~0 %	0.105	3.0
LGI-P[b] Tc^{10}	38 %	10 %	0.248	3.0	1.3 %	0.5 %	0.411	3.0
Tc^{100}	45 %	5.8 %	0.130	3.0	5.0 %	1.5 %	0.157	3.0

[a] Percentage of β-galactosidase activity (in arbitrary units) relative to pPW59-B09 in DYGS Tc^{10} using this substrate

[b] Acetic LGI (Sievers and Swings 2005)

10 mg·l^{-1} tetracycline, has been considered a good option for β-galactosidase activity assays with the cost-effective ONPG substrate.

β-Galactosidase active clones

Following the approach of a previous work involving a mutant library of *Herbaspirillum seropedicae* strain Z78 (Schwab et al. 2007), the initial strategy to screen the *G. diazotrophicus* promoter library for β-galactosidase active clones was plating them on solid LZGD medium in the presence of X-Gal and observing development of blue colour. Then, clones qualitatively selected would be quantitatively assayed for β-galactosidase activity using ONPG or C2FDG, in order to statistically validate the colour-based observations. Our goal in the current work was to isolate

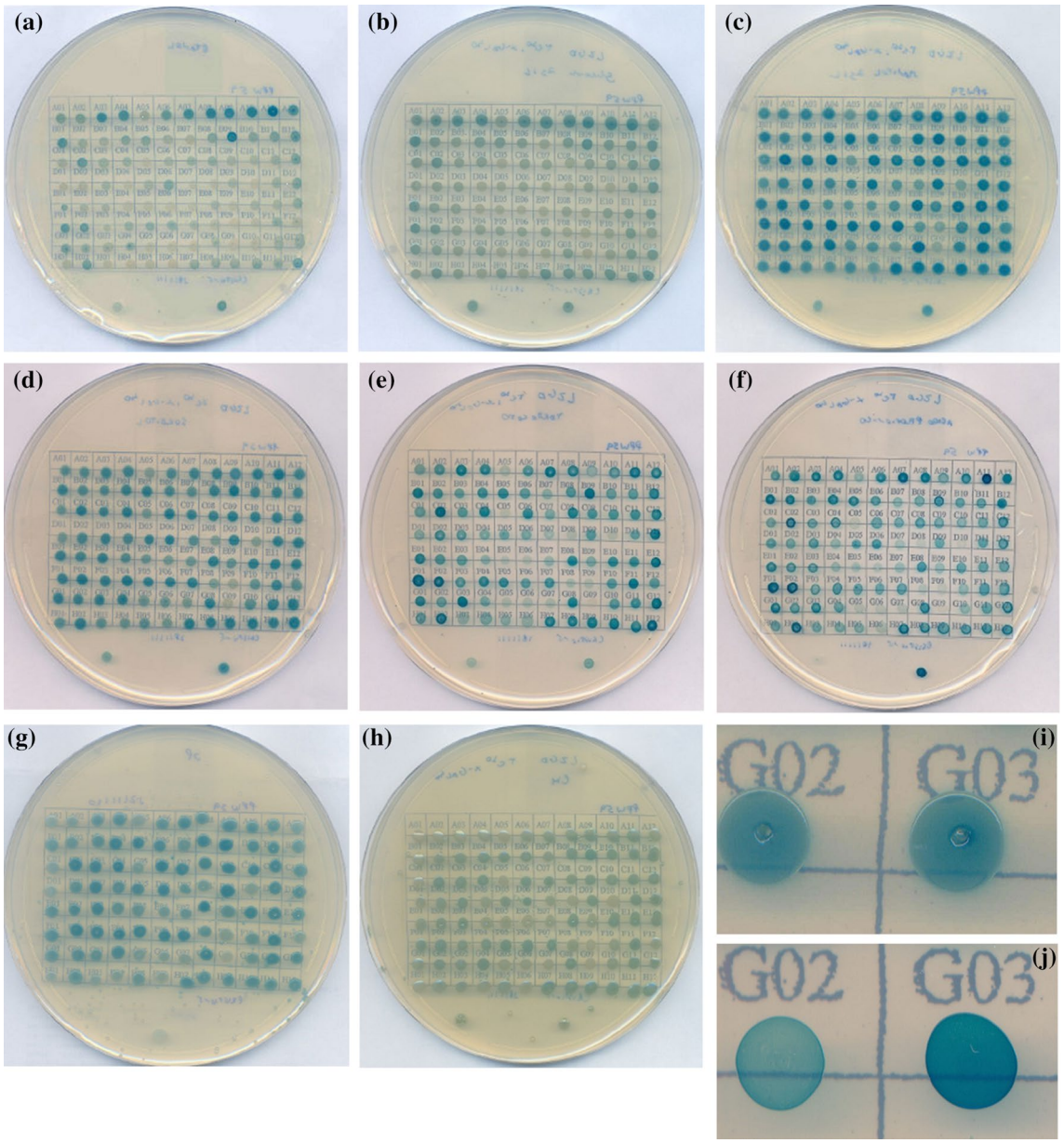

Fig. 4 β-Galactosidase activity of 96 clones (plate PPW59) from the *G. diazotrophicus* promoter library on diverse carbon sources: **a** ethanol, **b** glucose, **c** mannitol, **d** sorbitol, **e** potassium tartrate, **f** potassium propionate, **g** sugarcane juice, commercial variety SP701143, and **h** sugarcane juice, wild variety Chunee; **i** and **j**, a zoom in two clones on sorbitol and potassium tartrate, respectively, illustrating different aspects assumed by the colonies on solid media with X-Gal

promoters constitutively expressed and/or activated under a specific condition. A set of 480 clones (five 96-well plates from the promoter library) were plated on LZGD medium in the presence of X-Gal and supplemented with one of diverse carbon sources, all appropriate to support *G. diazotrophicus* growth (Li and MacRae 1991; Dong et al. 1995; Tejera et al. 2004; Barbosa et al. 2006). Alternatively, sterilized juice from two sugarcane varieties (either commercial or wild) was used as carbon source instead. Also, tetracycline was added at 10 $mg{\cdot}l^{-1}$, an antibiotic concentration that has resulted in better development of blue colour (Fig. 3f–g). There were only small differences in blue colour development among colonies grown under different carbon sources; apparently, this was due to gum production or non-uniform growth (Fig. 4). Nevertheless, 15 among 480 screened clones were selected for further characterization through β-galactosidase activity assays, because they seemed to present high enzymatic activity in the presence of at least one of the carbon sources tested. Results of those assays showed that two out of fifteen pre-selected

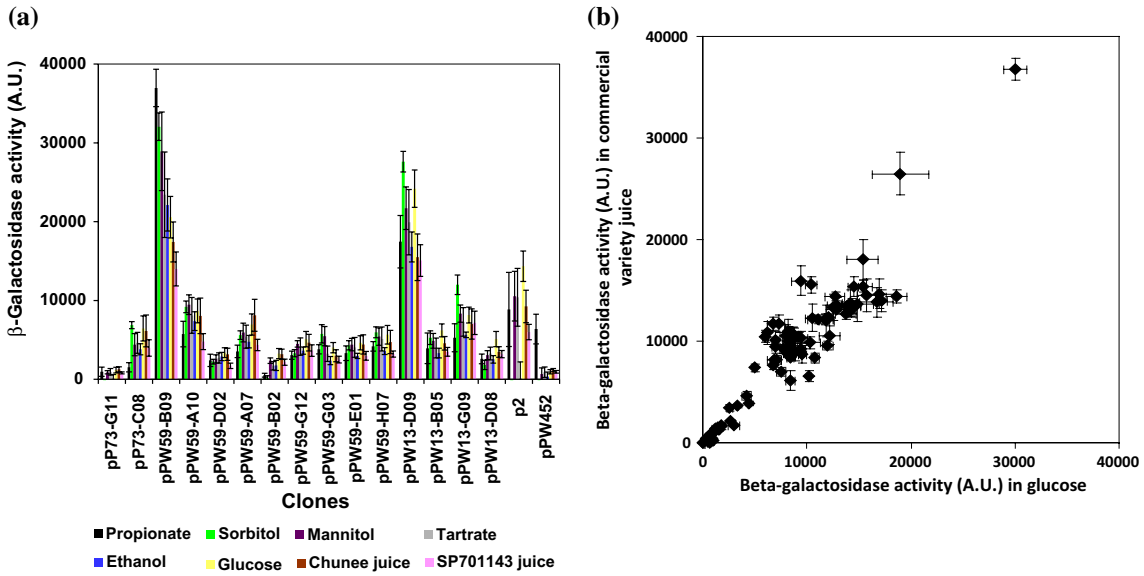

Fig. 5 β-Galactosidase activity of clones grown in liquid media with the carbon sources indicated. **a** Clones pre-selected from the qualitative tests on X-Gal-containing solid media; clone p2 is the same as in Fig. 3 (see details in text). **b** A random sample of 96 clones (or a 96-well plate) from the promoter library. *Error bars* are standard deviation of four replicates of the enzymatic assays

clones had significantly increased activity when compared to the empty vector pPW452 (Fig. 5a). Clone pPW59-B09 (position B09 of the 96-well plate number 59) presented increased levels of β-galactosidase activity in the presence of organic acids and polyalcohols, and lower levels of enzymatic activity with glucose and the sugarcane juices. In turn, clone pPW13-D09 presented a similar pattern of enzymatic activity to pPW59-B09, except that, in the presence of glucose, its activity was the second highest among the carbon sources tested. Clone p2, which has been selected for its LacZ activity on solid media (Figs. 2, 3) and was included in the assays, presented intermediate levels of enzymatic activity when compared to both pPW59-B09 and pPW13-D09 clones in liquid medium supplemented with the carbon sources tested.

Although the results of β-galactosidase activity assays permitted the identification of two highly active clones, they did not correspond well to the qualitative results on solid media: under this condition, most pre-selected clones seemed (through X-Gal-derived blue color) significantly active, but results with ONPG suggested that LacZ qualitative tests were quite elusive. Thus, screening of the promoter library for new highly active and/or regulated clones was performed through the more laborious β-galactosidase activity assays. To simplify the screening procedure, less growth conditions were tested. Once useful promoters would be those active when in contact with sugarcane compounds, as they might be used afterwards in planta for expression of target proteins, sterilized juice from a commercial variety was used in the growth medium to screen a 96-well plate of the promoter library, in comparison with glucose-containing medium. Using directly the quantitative procedure, two new clones showed high β-galactosidase activity under both conditions tested (Fig. 5b): pPW73-F07 and pPW73-B04.

Characterization of active promoters

Plasmids from clones presenting promoter activity, including both p1 and p2, were submitted to insert sequencing. All the six clones sequenced revealed distinct genomic regions (Fig. 6). Clone pPW73-B04 bears a fragment that spans from most of the 3′ region of ORF GDI_0566, which encodes a nitrate reductase, and part of its 3′ intergenic region, which would be the upstream region of the neighboring ORF GDI_0565, coding for a glyoxalase. A putative RpoD-dependent promoter was identified in that region, which probably is the glyoxalase gene promoter, and might be responsible for the LacZ activity detected in clone pPW73-B04.

Clone pPW59-B09 contains the 3′-terminal of ORF GDI_1624, coding for a glycosyl transferase, and the 5′-terminal of GDI_1623, which encodes a phytoene synthase, in the opposite transcription orientation of *lacZ*. In spite of this apparently incongruent finding, significant levels of promoter activity were detected through β-galactosidase activity assays (Fig. 5a). Also, two putative promoter sequences were identified in the 3′-terminal of GDI_1624, and specially one is very similar to the consensus sequence of RpoD-type promoters (Lisser and Margalit 1993). Thus,

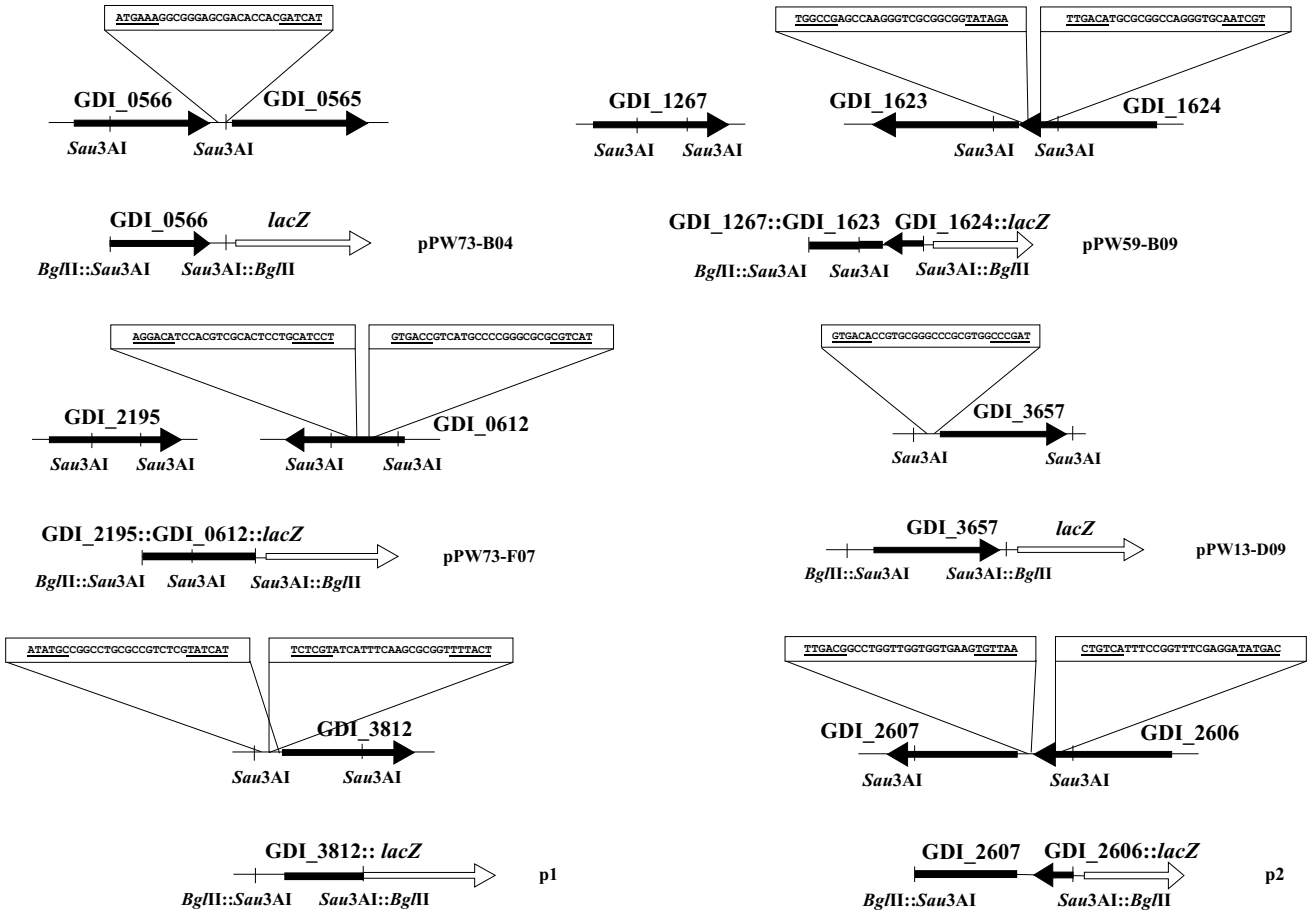

Fig. 6 *G. diazotrophicus* PAL5 genomic regions presenting promoter activity identified in this work. Transcriptional orientation of the ORFs (*black arrows*) in each representation of the genomic regions (*above*) is maintained in the respective representation of the transcriptional fusion with *lacZ* (*white arrow*) in the constructed plasmids (*below*). Restriction sites involved in cloning procedures are indicated. Annotated gene products (Bertalan et al. 2009) of the locus tags are: GDI_0565 = glyoxalase; GDI_0566 = nitrate reductase; GDI_1267 = putative coenzyme PQQ synthesis protein E; GDI_1623 = phytoene synthase; GDI_1624 = glycosyl transferase; GDI_2195 = hypothetical protein; GDI_0612 = AroD/3-dehydroquinate dehydratase; GDI_3657 = putative exported protein; GDI_3812 = putative signalling protein, GGDEF family; GDI_2606 = IstB-like ATP-binding protein; GDI_2607 = hypothetical protein. Sequences of putative RpoD-dependent promoters are shown in *boxes*, with characteristic −35/−10 regions underlined. Physical maps are not to scale

it is possible that the *lacZ* gene in clone pPW59-B09 is transcribed from an antisense RNA (asRNA) promoter located in the complementary strand, 3′-terminus, of a glycosyl transferase gene.

Sequencing of clone pPW73-F07 revealed a chimera of two distinct regions of the *G. diazotrophicus* PAL5 genome, in which the mid part of the ORF GDI_2195, encoding a hypothetical protein, was fused to the mid part also of GDI_0612 or *aroD*, which codes for a 3-dehydroquinate dehydratase. The former has the same transcription orientation as *lacZ*, while the latter is in the opposing direction in the middle of the construction. A search for putative promoter sequences revealed two probable RpoD-type promoters within the GDI_0612 sequence, in the complementary strand, which resembles the situation of clone pPW59-B09, mentioned above. Hence, the *lacZ* gene in clone pPW73-F07 might be transcribed from an antisense RNA (asRNA) promoter within the *aroD* gene.

Clone pPW13-D09 comprehends ORF GDI_3657, which encodes a putative exported protein, and its upstream intergenic region, in which a putative RpoD-like promoter was found. Thus, the high levels of β-galactosidase activity detected in that clone (Fig. 5a) could be ascribed to this probable promoter.

Clone p1, which was randomly selected for previously presenting β-galactosidase activity on X-Gal-containing DYGS medium, contains the upstream region plus the 5′-terminal of ORF GDI_3812, coding for a diguanylate cyclase. Two putative, overlapping RpoD-type promoters, and also a typical RpoE-like promoter (not shown), were

Table 3 Potential actors of pervasive transcription in *G. diazotrophicus* strain PAL5

Protein	Orthologue in PAL5 (locus tag)[a]
RpoD/σ^{70}	GDI_3335
RpoN/σ^{54}	GDI_2119; GDI_0424
RpoS/σ^{38}	N.H.
RpoH/σ^{32}	GDI_2254
FliA/σ^{28}	N.H.
RpoE/σ^{24}	GDI_1742; GDI_1743
FecI/σ^{19}	N.H.
N.H.	GDI_2765 (sigma subunit)
H-NS	N.H.
Rho	GDI_1257
NusG	GDI_0688
RNase III	GDI_2153

[a] Orthologues of *E. coli* in *G. diazotrophicus* were found using the reciprocal best hit (RBH) method with BLASTp and tBLASTn for search in both *G. diazotrophicus* PAL5 genomic sequences available (Bertalan et al. 2009; Giongo et al. 2010), and BLASTp for confirming back in the available *E. coli* genomes. N.H. = no BLAST hits

found upstream to that ORF, so probably *lacZ* gene in p1 clone would be transcribed from at least one of those promoters.

Finally, clone p2 bears most part of the 5′-terminus of ORF GDI_2607, which encodes a hypothetical protein, and a 3′ fragment of its neighbor ORF GDI_2606, coding for an IstB-like ATP-binding protein, including an intergenic region between both ORFs. Those ORFs are in the opposite transcription orientation to *lacZ*. Putative RpoD-type promoters were found in the complementary 3′-terminus of GDI_2606 and the intergenic region, both in the same transcription orientation as *lacZ*. That finding suggests another situation similar to clones pPW73-F07 and pPW59-B09, so the *lacZ* gene in clone p2 would be transcribed from an antisense RNA (asRNA) promoter located in the intergenic region and/or the 3′-terminus of ORF GDI_2606.

Discussion

Transcriptional promoters have been isolated and characterized from diverse bacteria for scientific and/or technological purposes. In this work we isolated and characterized six *G. diazotrophicus* PAL5 promoters with variable expression strengths that might be used for expressing useful proteins: while the promoter of clone pPW73-F07 showed the highest levels of LacZ expression through the enzymatic assays, the other five showed to be moderate to weak promoters. We used a promoter-less *lacZ*-bearing vector to construct a promoter library in PAL5 cells, and identified those active promoters through β-galactosidase activity assays, sequencing and localization in the bacterial genome. Interestingly, three out of the six clones presented promoters probably involved in transcription of antisense RNA, including that presenting the highest expression strength.

Characterization in this work of such a proportion of antisense promoters in PAL5 leads to infer that antisense RNA is omnipresent in *G. diazotrophicus*. In diverse bacteria, promoters located in the complementary strand of genes drive transcription of antisense RNAs with regulatory functions (Georg and Hess 2011; Sesto et al. 2012). Those molecules have important roles in nutrient metabolism, stress responses, toxin synthesis, and bacterial pathogenesis. In the plant pathogen *Agrobacterium tumefaciens*, a deep sequencing survey showed the presence of 76 cis-antisense sRNAs, suggesting considerable antisense transcription in that alpha-proteobacterium (Wilms et al. 2012). In another alpha-proteobacterium, the symbiotic nitrogen-fixing *Sinorhizobium meliloti*, a genome-wide survey revealed 117 cis-encoded antisense sRNAs (Schlüter et al. 2010). To our knowledge, there are no reports of the presence of those molecules in *G. diazotrophicus* or any other member of the *Acetobacteraceae*, an alpha-proteobacterial family. Also, recent reports have mapped transcription start sites (TSSs) to unexpected locations in bacterial genomes, including the non-coding strand, a phenomenon that has been termed as pervasive transcription (Wade and Grainger 2014). In *E. coli*, it has been demonstrated that there are more than 1000 intragenic TSSs that are positioned in the antisense orientation (Dornenburg et al. 2010). The genome of *G. diazotrophicus* strain PAL5 encodes diverse sigma subunits of RNA polymerase that might be involved in pervasive transcription (Table 3); also, proteins probably suppressive of transcription were annotated. A further investigation should be conducted in *G. diazotrophicus* in order to confirm the presence and function of antisense RNA, and to study possible regulatory mechanisms governing transcription from their promoters. For that purpose, transcriptional profiling through RNA-Seq could be a good choice, taking advantage of having the bacterial genome sequence available for mapping of sequencing reads.

Genome sequencing of *G. diazotrophicus* strain PAL5 by two independent groups (Bertalan et al. 2009; Giongo et al. 2010) has opened room to further genetic and functional genomics analysis of bacterial traits related to plant association and growth promotion. For instance, genome surveys has revealed the presence, and permitted genetic characterization, of: (1) a *gum* gene cluster, for which has been shown that its product, exopolysaccharide, is required for biofilm formation and plant colonization (Meneses et al. 2011); (2) homologs to genes from the alternative asparagine biosynthesis pathway, which affects nitrogenase activity and thus might be essential to bacterial survival during

interaction with plant (Alquéres et al. 2012); (3) *luxR* and *luxI* homologs composing a quorum sensing system, which has a role on the expression of a number of proteins including few probably involved in microbial interactions, host colonization and stress survival (Bertini et al. 2014); (4) homologs for reactive oxygen species (ROS)-detoxifying enzymes, including superoxide dismutase and glutathione reductase, which play an important role in the endophytic colonization of rice plants (Alquéres et al. 2013); and (5) several genes probably involved in iron metabolism, including *tonB*, which has a role in nitrogenase activity and biofilm formation capability, beyond siderophore accumulation (de Paula Soares et al. 2015). Those studies have been assisted by molecular tools, previously developed, such as the use of suite vectors for labelling PAL5 strain in plant association studies (Rouws et al. 2010), and/or a transposon-mediated mutagenesis system in genetic characterization studies (Rouws et al. 2008). However, there was a necessity of expression systems for availing on genetic characterization studies and/or developing useful genetically modified strains. By using one of the six promoters characterized here with a determined expression strength, a protein (gene) of interest might be cloned just downstream to one of those promoters already cloned in the pPW452 plasmid; alternatively, a certain promoter might be easily isolated through digestion with *Hind*III enzyme, which has two cleavage sites flanking the multicloning site of that vector, and cloned into another vector bearing the protein (gene) of interest.

Gene expression systems have been developed in more or less extent for acetic acid bacteria (*Acetobacteraceae*), the family in which *G. diazotrophicus* is included (Saichana et al. 2014). For the acetobacterium *Gluconobacter oxydans*, development of those systems is well advanced, thanks to active promoters that had been characterized (Kallnik et al. 2010; Shi et al. 2014). As concluded by those authors, the *G. oxydans* promoters could be used as a tool for overexpression of enzymes involved in industrial production processes, increasing even more the bacterial potential in industrial bioconversion. Similarly, the approach used in the current work permitted to characterize active promoters of *G. diazotrophicus* strain PAL5, with potential to be used in expression systems for useful proteins in agriculture. Among these, beyond proteins already mentioned above and previously characterized in PAL5 strain, we anticipate Cry toxins from *B. thuringiensis*, as already described with Cry1Ac (Subashini et al. 2011; Rapulana and Bouwer 2013). Also, induced homologous expression of enzymes involved in phytohormone production (Bastián et al. 1998), antagonistic activity for phytopathogens (Blanco et al. 2005; Saravanan et al. 2007), and improvement of nutrient utilization by the plant (Maheshkumar et al. 1999; Saravanan et al. 2007; Logeshwaran et al. 2009) might also be expressed using engineered genetic constructions.

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