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Original article

Gluconic acid produced by *Gluconacetobacter diazotrophicus* Pal5 possesses antimicrobial properties

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Abstract

Gluconic acid is produced in large quantities by the endophytic and diazotrophic bacterium *Gluconacetobacter diazotrophicus* Pal5. This organic acid derives from direct oxidation of glucose by a pyrroloquinoline-quinone-linked glucose dehydrogenase in this plant growth-promoting bacterium. In the present article, evidence is presented showing that gluconic acid is also responsible for the antimicrobial activity of *G. diazotrophicus* Pal5. The broad antagonistic spectrum includes Gram-positive and -negative bacteria. Eukaryotic microorganisms are more resistant to growth inhibition by this acid. Inhibition by gluconic acid can be modified through the presence of other organic acids. In contrast to other microorganisms, the Quorum Sensing system of *G. diazotrophicus* Pal5, a regulatory mechanism that plays a key role in several microbe–microbe interactions, is not related to gluconic acid production and the concomitant antagonistic activity.

Keywords: Organic acid; Glucose; Inhibition; Antimicrobial

1. Introduction

In nature, microorganisms interact with each other, with their environment and with a particular host through a wide range of physical as well as chemical factors from the primary and secondary metabolism. Among the latter, siderophores and antibiotics are among the most important. In the former group of compounds, metabolic intermediates can also play a role in microbe–microbe interactions [1,2].

Organic acids synthesized by taxonomically diverse microorganisms through different biochemical pathways possess well-known antimicrobial activities. For instance, lactic acid is a weak organic acid from the hexose fermentation pathway of lactic acid bacteria with inhibitory activities not only of industrial but also ecological relevance [1]. It has been shown that gluconic acid produced by *Pseudomonas* species and *Enterobacter intermedius* has inhibitory activities against *Gaeumannomyces graminis* var. *tritici* and the protozoan *Colpoda steinii*, *Vahlkampfia* sp. and *Neobodo designis*, respectively [3,4].

The endophytic and diazotrophic Gram-negative bacterium *Gluconacetobacter diazotrophicus* is a plant growth-promoting microorganism that lives in association with sucrose-rich crops such as sugarcane, sweet potato, sweet sorghum, pineapple, banana and coffee [5–7]. The production of gluconic acid by *G. diazotrophicus* Pal5 is related to the extracellular oxidation of glucose by a pyrroloquinoline quinone-linked glucose dehydrogenase (PQQ-GDH) [8]. This organic acid has been associated, not only in *G. diazotrophicus* but also in other bacteria, with solubilization of insoluble phosphate and zinc salts [9,10]. It has

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been postulated that increasing the solubility of mineral nutrients in this way for the plant *G. diazotrophicus* enhances growth of the host. In addition to its capacity to fix atmospheric nitrogen [11] *G. diazotrophicus* also promotes plant growth through production of auxin and gibberellins phytohormones [12], and inhibition of phytopathogens such us *Xanthomonas albilineans*, *Fusarium* sp. and *Helminthosporium carbonum* [7]. These features have increased the interest in utilization of *G. diazotrophicus* in development of biofertilizers. Although the chemical structure of antimicrobial substances produced by *G. diazotrophicus* has not been completely solved to a molecular level up to date, biochemical and genetic data suggest that this microorganism synthesizes bacteriocin-like molecules [13–15].

In this article, evidence is presented showing that gluconic acid, a weak organic acid produced by *G. diazotrophicus* Pal5, is also responsible for the microbial inhibition that this strain exerts. The spectrum of susceptible strains is broader than previously known, and this activity can be modified by the composition of the environment that surrounds the cells. In addition to the phosphate-solubilizing capacity of gluconic acid produced by this microorganism, this compound has the potential for antagonizing putative competitors. However, its production is not regulated by quorum sensing (QS), a regulatory system that influences oxidative fermentations in *Gluconacetobacter intermedius* [16] and plays a key role in control of microbial interactions in several microorganisms [17].

2. Materials and methods

2.1. Microorganisms and culture conditions

G. diazotrophicus Pal5 (ATCC 49037) was maintained in DYGS agar medium supplemented with glucose at a final concentration of 10 mM [18]. Overnight cultures of *G. diazotrophicus* PAL5 prepared in 10 mM glucose DYGS were utilized to inoculate flasks containing DYGS supplemented with different carbon sources, including glucose, sorbitol, sodium gluconate, fructose at final concentrations of 10 mM and 300 mM, and glycerol at final concentrations of 20 mM and 400 mM. When required, 300 mM glucose DYGS agar plates were supplemented separately with succinic acid, malic acid, fumaric acid, citric acid and alpha-ketoglutaric acid at a final concentration of 20 mM. Cultures were grown aerobically at 30 °C. Indicator strains were cultured in the corresponding medium indicated in Table 2.

2.2. Characterization of antimicrobial activity

G. diazotrophicus Pal5 was cultured in 300 mM glucose DYGS and cell-free supernatant was fractionated using a high performance liquid chromatography (HPLC) device equipped with a 7.8 × 300 mm Rezex Phenomenex column, a 7.8 × 50 mm Rezex Phenomenex guard column and a photo diode array detector (PDA) at 210 nm (Waters 2998). Peak separation was performed in a 20 mM H_2SO_4 mobile phase at 0.6 mL min^{-1} flow rate at a column temperature of 55 °C. Fractions collected every 30 s were concentrated by evaporation. After dissolving in 100 µLl of distilled water, antimicrobial activity of each fraction was assayed in casein-starch agar (CSA) plates with *Streptomyces coelicolor* as the indicator strain, as described in Section 2.3.

2.3. Detection of antimicrobial activity in supernatants of G. diazotrophicus *Pal5 cultures*

Antimicrobial activity in liquid samples was analyzed with the well diffusion method utilizing *X. albilineans* and *S. coelicolor* as the indicator strains [19]. Briefly, at late exponential growth phase, cultures of *G. diazotrophicus* Pal5 were centrifuged for 10 min at 8000 rpm and supernatants were stored at −20 °C until further analysis. Plates of NA and CSA medium were streaked with *X. albilineans* and *S. coelicolor*, respectively, and 5 mm wells punched into the solid medium were filled with *G. diazotrophicus* Pal5 supernatants. Inhibition zones were evaluated after incubating the plates at 30 °C for 24 h. Solutions of commercial gluconic acid or fractions from liquid chromatography were evaluated with the same procedure.

2.4. Isolation and identification of endophytic microorganisms from sugarcane

Roots, leaves and internode sections of LCP 85–384 variety of sugarcane cultured in Tucumán, Argentine, were surface-sterilized. Samples were then inoculated in KB and

Table 1
Influence of carbon source on the inhibitory activity of *G. diazotrophicus* PAL5.

Condition	pH	Inhibitory activity	Inhibitory activity after pH neutralization[a]	Inhibitory activity after pH acidification[a]
10 mM Glucose	3.20	−	−	−
300 mM Glucose	3.14	++	−	++
10 mM Sorbitol	6.34	−	−	−
300 mM Sorbitol	6.23	−	−	−
10 mM Sodium gluconate	6.91	−	−	−
300 mM Sodium gluconate	7.36	−	−	++
10 mM Fructose	6.30	−	−	−
300 mM Fructose	6.34	−	−	−
20 mM Glycerol	6.26	−	−	−
400 mM Glycerol	6.32	−	−	−

[a] Activities were determined in liquid samples assayed with *S. coelicolor* as indicator strain. − no inhibition; ++ growth inhibition.

Table 2
Inhibitory spectrum of *G. diazotrophicus* PAL5 cultured in 300 mM glucose DYGS agar plates.

Microorganisms	Strain and origin	Culture medium	Inhibitory halo [mm][a]
Gram−positive bacteria			
Bacillus sp.	38 kB s.i	KB	18
Bacillus sp.	30 kB s.i.	KB	23
Staphylococcus sp.	139X s.i.	NA	40
Staphylococcus aureus	ATCC 25923	LB	42
Bacillus subtilis	356 e.i.	LB	39
Staphylococcus epidermidis	PA17 c.i.	LB	44
Enterococcus faecalis	ATCC 29212	LB	48
Listeria monocytogenes	PA07 c.i.	LB	47
Streptomyces coelicolor	A3 e.i.	SCA	20[b]
Leuconostoc mesenteroides subsp. *dextranicum*	DSM 20484	MRS	–
Lactobacillus hilgardii	DSM 20176	MRS	–
Leuconostoc mesenteroides subsp. *mesenteroides*	DSM 20343	MRS	–
Lactobacillus casei subsp. *casei*	DSM 20011	MRS	–
Lactobacillus pentosus	DSM 20314	MRS	–
Lactobacillus paraplantarum	DSM 10667	MRS	–
Weissella paramesenteroides	DSM 20288	MRS	14
Lactobacillus plantarum subsp. *plantarum*	DSM 20174	MRS	–
Lactobacillus brevis	DSM 20054	MRS	–
Lactobacillus parabuchneri	DSM 5987	MRS	–
Gram−negative bacteria			
Novosphingobium sp.	121X s.i.	NA	34
Enterobacter sp.	46 kB s.i.	KB	13
Escherichia sp.	47 kB s.i.	KB	9
Acidovorax avenae subsp. *avenae*	s.i.	NA	2.2
Pseudomonas syringae pv. *syringae*	B728a	LB	45
Xanthomonas albilineans	s.i.	NA	20[b]
Xanthomonas campestris pv. *campestris*	e.i.	NA	12[b]
Escherichia coli	ATCC 35218	LB	36
Escherichia coli	ATCC 25932	LB	43
Enterobacter aerogenes	PA14 c.i.	LB	34
Shigella sonnei	PA10 c.i.	LB	36
Salmonella enterica serovar Typhimurium	PA01 c.i.	LB	48
Salmonella enterica serovar Enteritidis	PA03 c.i.	LB	35
Serratia proteamaculans	136_1 e.i.	LB	23
Proteus vulgaris	PA11 c.i.	LB	38
Gluconacetobacter diazotrophicus	185 e.i.	DYGS	24
Gluconacetobacter diazotrophicus	186 e.i.	DYGS	–
Gluconacetobacter diazotrophicus	187 e.i.	DYGS	24
Eucaryotic microorganisms			
Rhodotorula mucilaginosa	ACL4 s.i.	YM	–
Rhodotorula sp.	ACL5 s.i.	YM	–
Ustilago-Sporisorium-Macalpinomyces complex	ACL11 s.i.	YM	–
Ustilago-Sporisorium-Macalpinomyces complex	ACL9 s.i.	YM	–
Meyerozyma sp.	ACL1 s.i.	YM	–
Meyerozyma sp.	ACL2 s.i.	YM	–
Candida fukuyamaensis	RCL3 e.i.	PDA	–
Saccharomyces cerevisiae	ATCC 32051	PDA	–
Candida sp.	c.i.	PDA	–
Rhizopus sp.	e.i.	PDA	–
Penicillium sp.	e.i.	PDA	–
Sclerotium rolfsii	ATCC 201126	PDA	–
Kloeckera apiculata	MC1 e.i.	YM	–
Trichosporon akiyoshidainum	HP2023 e.i.	YM	18

e.i.: environmental isolate; c.i.: clinical isolate; s.i.: sugarcane isolate.
[a] lack of inhibition is denoted with [−].
[b] Inhibitory activity determined in liquid samples.

NA media for isolation of endophytic bacteria. YM agar medium supplemented with tetracycline was utilized for isolation of endophytic yeasts. Identification of isolated strains was performed after amplification and sequencing of the 16S rDNA gene with primers 27F and 1492R for bacteria; primers NL1 and NL4 for amplification and sequencing of the D1/D2 domain of the LSU rDNA gene were utilized for identification of endophytic yeasts.

2.5. Determination of the antimicrobial spectrum of G. diazotrophicus Pal5 against other microorganisms

Five microliters of a *G. diazotrophicus* Pal5 culture, prepared as described in Section 2.1, were spot-inoculated on 300 mM glucose DYGS agar plates. After incubation at 30 °C for 48 h, colonies were exposed to chloroform vapors for 10 min and the remaining solvent was eliminated by aseptic aeration in a laminar flow hood. Plates with *G. diazotrophicus* Pal5 were then covered with a thin layer of 0.75% agar medium seeded with an actively growing culture of indicator strains. Inhibition zones were evaluated after incubating the plates overnight at 30 °C. For *X. albilineans* and *S. coelicolor*, antimicrobial activity was determined with supernatants of *G. diazotrophicus* Pal5 cultures (see Section 2.3).

2.6. Inactivation of the G. diazotrophicus Pal5 QS system

A quorum quenching strategy was followed to inactivate the QS system of *G. diazotrophicus* Pal5. pME6863 harboring the *aiiA*-encoded lactonase from *Bacillus* sp. A24, under the constitutive P_{lac} promoter [23], and the pME6000 control plasmid were conjugated independently by tri-parental mating using pRK2013 into *G. diazotrophicus* Pal5. To control the effectiveness of the strategy, organic extracts were prepared with samples obtained from cultures at late exponential growth phase and analyzed with *Agrobacterium tumefaciens* NTL4 (pCF218) (pCF232) and *Pseudomonas putida* F117 (pKR-C12) as biosensor strains [24,25].

2.7. Analytical determinations

Pyrroquinoline quinine-linked glucose dehydrogenase (PQQ-GDH) and FAD-linked gluconic acid dehydrogenase (FAD-GaDH) activities were determined in whole cells spectrophotometrically at 600 nm using 2,6-dichlorophenolindophenol (DCIP) as the electron acceptor [20,21]. *G. diazotrophicus* Pal5 was cultured for 24 h in 300 mM glucose DYGS supplemented separately with succinic acid, malic acid, fumaric acid, citric acid and alpha-ketoglutaric acid at a final concentration of 20 mM and cells were utilized for enzymatic determinations. Molar extinction coefficients of DCIP were taken as 15.1 mM^{-1} and 10 mM^{-1} for PQQ-GDH and FAD-GaDH, respectively. Total protein concentration of whole cell suspensions was measured by the Bradford method using bovine serum albumin as standard [22]. One unit of specific enzyme activity was defined as the

amount of protein required to convert 1 nmol of substrate per minute per milligram of total protein. Glucose concentrations in cell-free supernatants were determined with a glucose oxidase enzymatic kit (Wiener, Argentina). Total acidity was determined as the microliters of 1 M NaOH necessary to neutralize 1 ml of *G. diazotrophicus* Pal5 supernatant. Bacterial growth was measured turbidimetrically at 600 nm (OD) with a Beckman DU 640 spectrophotometer. pH was determined with a Sartorius PB-11 pH meter.

3. Results

3.1. Influence of growth conditions on the inhibitory activity of G. diazotrophicus *Pal5*

Spent supernatants obtained after growing *G. diazotrophicus* Pal5 in DYGS medium supplemented with different carbon sources were assayed for inhibitory activity utilizing *S. coelicolor* as indicator strain. Growth inhibition could only be observed when *G. diazotrophicus* Pal5 was cultured in 300 mM glucose DYGS (Table 1). Interestingly, while the different carbon sources produced slight alkalinization of the medium, only glucose-supplemented media presented acidic pH values after 48 h of incubation at 30 °C. Antagonism assayed in supernatant samples with the pH adjusted to 3.20 showed a positive result with samples obtained from 300 mM gluconate DYGS (Table 1). The dependence of antagonistic activity on pH values was confirmed after neutralizing, with NaOH, a sample of 300 mM glucose DYGS supernatant: at pH 7, the sample showed a loss of inhibitory activity that could be completely re-established after re-acidification of the sample to pH 3.20 (Table 1). The loss of antagonistic activity under non-acidic conditions suggested that the presence of an organic acid was involved in growth inhibition by *G. diazotrophicus* Pal5.

3.2. Analysis of the antimicrobial activity of G. diazotrophicus *Pal5*

To characterize production of antimicrobial activity, *G. diazotrophicus* Pal5 was cultured in 300 mM glucose DYGS and the growth and antagonistic activity were analyzed at regular intervals. Antimicrobial activity could be detected after 2 days of incubation when the diameter of the inhibition zone was 10 mm (Fig. 1a), reaching a maximum of 17 mm at day 4. The activity then began to decrease and was almost undetectable after 7 days of culture (Fig. 1a). The strain grew exponentially for 8 days, after which the culture entered the stationary growth phase. Noteworthy, no glucose could be detected in the supernatant 6 days after the beginning of the experiment, suggesting that growth after that time was supported by a different carbon source (Fig. 1b). 24 h after inoculation, the pH showed a strong decrease from 6.00 to 3.25, continuing to slowly decline until a value of 3.00 was reached (Fig. 1b). At day 8, the pH rapidly arrived at 6.00, where it remained constant until the end of the experiment. The disappearance of antagonistic activity with glucose consumption and neutralization of the medium suggested that gluconic acid production might be involved in microbial inhibition by *G. diazotrophicus* Pal5.

3.3. Identification of the antimicrobial compound produced by G. diazotrophicus *Pal5*

Considering the dependence on the carbon source and the pH, the profile of antimicrobial activity production, consumption of glucose and modifications in pH throughout the culture, we hypothesized that the antagonistic compound could be related to oxidation of glucose with concomitant production of gluconic acid. The measurement of total acidity of the spent culture medium supported this hypothesis: total acidity, expressed as microliters of 1 M NaOH necessary for neutralizing 1 mL of *G. diazotrophicus* PAL5 supernatant, attained a maximum value of 96 µl at day 4, at which time the largest diameter for growth inhibition was also determined, followed by a strong decrease to 64 and 30 µl at days 5 and 6, respectively (Fig. 1a). To identify the antagonistic compound, supernatant of a *G. diazotrophicus* Pal5 culture grown in 300 mM glucose DYGS was chromatographed by HPLC. Only one fraction, which contained a peak with a retention time of 4.8 min, showed inhibitory activity (Fig. 1c). Pure gluconic acid showed a retention time of 4.8 min, coincident with the active fraction from spent supernatants (Fig. 1c, inset). A growth inhibition zone observed when the same solution of gluconic acid utilized for HPLC was assayed with *S. coelicolor* confirmed the antimicrobial activity of this organic acid (data not shown). In addition, no growth inhibition could be detected with sterile distilled water with the pH adjusted to 3.00 (data not shown). Similar results were found with the phytopathogens *Xanthomonas campestris* pv. *campestris* and *X. albilineans* (data not shown).

3.4. Antimicrobial spectrum of G. diazotrophicus *Pal5*

Different microorganisms were examined by the deferred antagonism procedure to evaluate the antimicrobial activity of *G. diazotrophicus* Pal5, including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. The growth of a wide spectrum of microorganisms could be inhibited (Table 2). Although differing susceptibilities were observed according to the diameter of inhibition zones, both Gram-positive and Gram-negative bacteria were affected by *G. diazotrophicus* Pal5 when this strain grew in 300 mM glucose DYGS. Among the endophytic bacteria from sugarcane, wide variability in growth inhibition could be observed. Isolate 47 KB was more resistant, showing an inhibition zone of 9 mm; isolate 139X was the most susceptible strain among the endophytic isolates assayed, with an inhibition zone of 40 mm. 46 KB, 38 KB, 30 KB and 121X presented intermediate values of 13 mm, 18, 23 and 34 mm. Among the clinical and environmental strains assayed, the largest diameters could be observed with *Listeria monocytogenes* (47 mm), *Salmonella enterica* serovar Typhimurium (48 mm), *Staphylococcus aureus* (47 mm) and *Enterococcus faecalis* (48 mm). *Serratia*

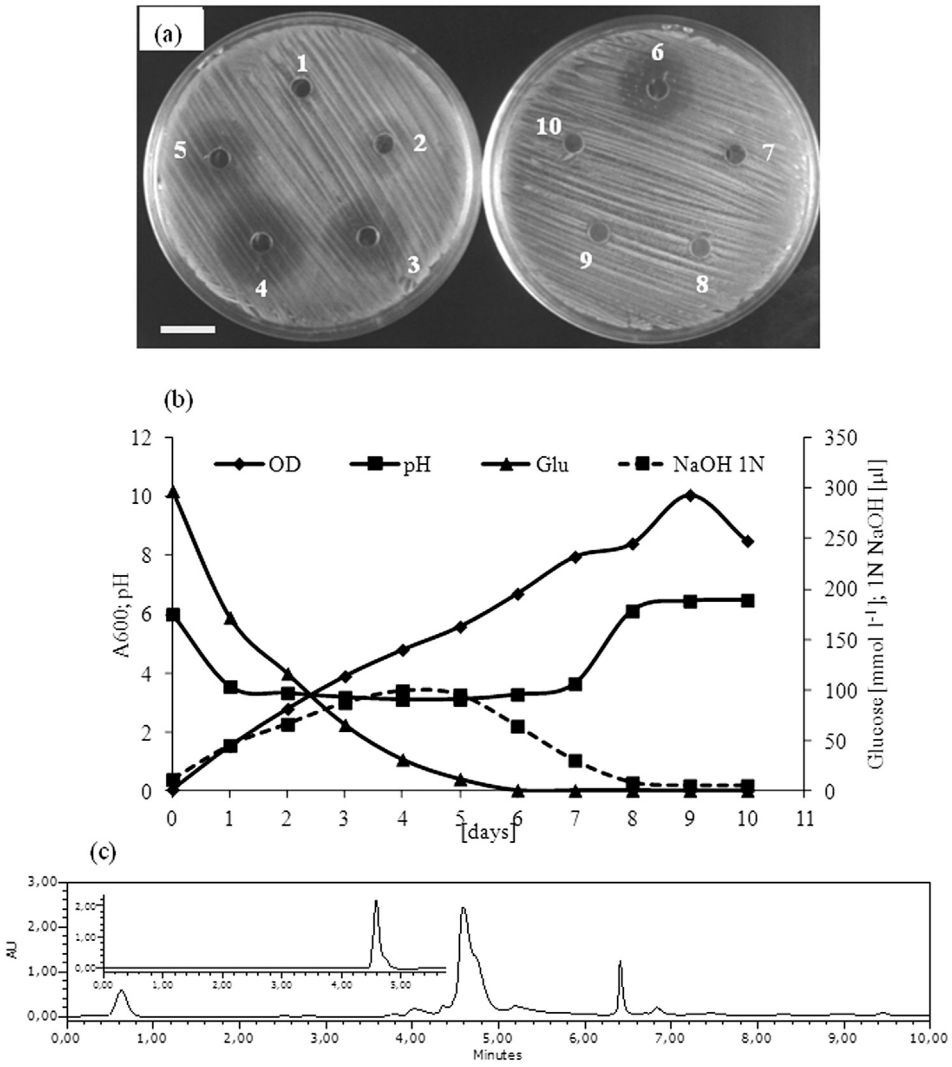

Fig. 1. Characterization of production of antimicrobial activity. *G. diazotrophicus* Pal5 was cultured in 300 mM glucose DYGS and after 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days samples were withdrawn for growth (OD), pH, residual glucose and total acidity measurement (a). Antimicrobial activity in collected samples was determined in assays developed with *S. coelicolor* as indicator strain. Determinations were performed at least in triplicate. Results are from a representative experiment. Bar is 10 mm (b). Identification of the antimicrobial compound produced by *G. diazotrophicus* Pal5. Cell-free supernatant was chromatographed by HPLC coupled with a PDA detector as described in Materials and methods. A main peak was eluted at 4.8 min. Pure gluconic acid analyzed under the same conditions showed identical retention time (inset). AU: absorbance unit (c).

proteamaculans proved to be more resistant to the inhibitory activity of *G. diazotrophicus* Pal5, presenting an inhibition zone of 23 mm. Phytopathogens from sugarcane were also susceptible to the inhibition by *G. diazotrophicus* Pal5: *Acidovorax avenae* subsp. *avenae* and *Pseudomonas syringae* pv. *syringae* presented inhibition zones of 22 mm and 45 mm. For unknown reasons, inhibition assays with *X. campestris* pv. *campestris* and *X. albilineans* could not be performed with the same procedure as that used with the majority of microorganisms described in this work. However, susceptibility could be determined with liquid samples of *G. diazotrophicus* Pal5 cultures, as described in section 2.3 showing zones of 12 mm and 20 mm for *X. campestris* pv. *campestris* and *X. albilineans*, respectively. Noteworthy, two environmental isolates of *G. diazotrophicus* were susceptible and one was resistant (Table 2). Among the bacterial species analyzed, a particular group of prokaryotic microorganisms resisted the antimicrobial activity produced by *G. diazotrophicus* Pal5: both homo- and hetero-fermentative lactic acid bacteria (LAB) could grow without showing an inhibition zone on top of *G. diazotrophicus*. In contrast, hetero-fermentative *Weissella paramesenteroides* was the only species of LAB affected. Although notably smaller compared to other susceptible strains, *W. paramesenteroides* showed an inhibition zone with a diameter of 14 mm. Because of the filamentous growth, inhibition of the Actinobacterium *S. coelicolor* could not be assayed under the described conditions utilized for other microorganisms and had to be analyzed with a particular protocol (see Section 2.3). However, this strain was a valuable tool for determinations in liquid samples. Under these conditions, *S. coelicolor* presented a diameter of 20 mm. On the other hand, the majority of the assayed eukaryotic microorganisms were not affected under

the described conditions (Table 2). The yeasts Candida fukuyamaensis, Candida sp., *Saccharomyces cerevisiae* and *Kloeckera apiculata*, as well as the filamentous fungi *Penicillium* sp., *Rhizopus* sp. and *Sclerotium rolfsii*, showed no inhibition when grown on top of *G. diazotrophicus* Pal5. In contrast, the yeast *Trichosporon akiyoshidainum* was the only eukaryotic microorganism affected by *G. diazotrophicus* Pal5, presenting a small but clearly visible zone of 18 mm diameter. Endophytic yeast belonging to the genera *Rhodotorula*, *Meyerozyma* and the *Ustilago-Sporisorium-Macalpinomyces* complex isolated from sugarcane were also resistant to inhibition by *G. diazotrophicus* Pal5.

3.5. Influence of organic acids on the antimicrobial activity of G. diazotrophicus Pal5

To test whether carboxylic acids could modulate the antimicrobial activity of *G. diazotrophicus* Pal5, the strain was cultured in 300 mM glucose DYGS agar supplemented independently with 20 mM of succinic, malic, fumaric, citric and alpha-ketoglutaric acid. Addition of any of the carboxylic acids assayed caused a decrease in the diameter of the inhibition zones (Fig. 2). The strongest reduction was determined when culture medium was supplemented with alpha-ketoglutaric acid, which caused a decrease of 45%. The lowest effect could be observed when 300 mM glucose DYGS was supplemented with fumaric acid, which caused a reduction of 25% in antimicrobial activity. The presence of succinic, malic and citric acid reduced the inhibitory activity to intermediate values between those obtained with alpha-ketoglutaric acid and fumaric acid, respectively (Fig. 2).

3.6. Modification of GDH and GaDH activities by organic acid

G. diazotrophicus Pal5 metabolizes glucose by means of a PQQ-GDH (pyrroquinoline quinine-linked glucose dehydrogenase) enzyme, with concomitant production of gluconic acid, which can be further oxidized to 2-ketogluconic acid in a reaction catalyzed by FAD-GaDH (FAD-linked gluconic acid dehydrogenase) (Fig. 3a). To study the decrease in the antimicrobial effect observed when carboxylic acids were present, PQQ-GDH and FAD-GaDH activities were determined. As shown in Fig. 4, the addition of 20 mM succinic, malic, citric, fumaric and alpha-ketoglutaric acids increased the activity of PQQ-GDH: from 196.63 mU μg^{-1} ± 35.92 when no organic acid was added, to a maximum of 398.63 mU μg^{-1} ± 83.28 when culture medium was supplemented with alpha-ketoglutaric acid. At the same time, in comparison, when no organic acid was added (71.38 mU μg^{-1} ± 12.89), a decrease in the FAD-GaDH activities could be determined when culture media were supplemented with succinic (49.49 mU μg^{-1} ± 11.06), malic (48.37 mU μg^{-1} ± 6.71), fumaric (39.42 mU μg^{-1} ± 12.55) and alpha-ketoglutaric acids (52.02 mU μg^{-1} ± 7.11) (Fig 4b). Tricarboxylic citric acid was the only compound producing an increase in FAD-GaDH activity, attaining a value of 91.17 ± mU μg^{-1} 15.25. As shown in Fig. 3b, pH of the culture media supplemented with organic acids presented higher values (between 0.5 and 0.75 pH units) in comparison when DYGS was amended with just 300 mM glucose.

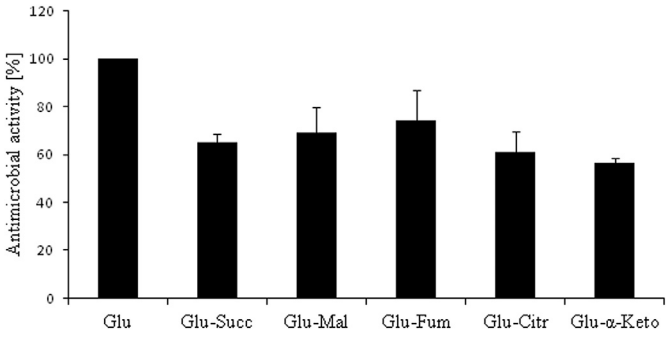

Fig. 2. Influence of organic acids on antimicrobial activity. 300 mM glucose DYGS medium was supplemented independently with succinic (Succ), malic (Mal), fumaric (Fum), citric (Citr) and α-ketoglutaric (α-Keto) acids at a final concentration of 20 mM. *G. diazotrophicus* Pal5 was spot-inoculated and after 48 h, plates were developed with *E. coli* ATCC 35218. Inhibitory activities were referred to that obtained without the addition of organic acids, which was considered to be 100%. Data are means of at least three replicates. Error bars represent standard deviations.

3.7. Influence of the QS system on the antimicrobial activity of G. diazotrophicus Pal5

Genome sequencing of *G. diazotrophicus* Pal5 showed that this endophytic strain harbors a complete QS system composed of a *luxI* and a *luxR* homolog [13]. Iida and col. showed that the QS system from the taxonomically related *G. intermedius* controls oxidative fermentation, including acetic and gluconic fermentations [16]. To analyze whether this regulatory mechanism can regulate gluconic acid production and the related antimicrobial activity, the QS system of *G. diazotrophicus* Pal5 was interrupted with a quorum quenching strategy. The success of this approach was confirmed analyzing organic extracts of *G. diazotrophicus* Pal5 (pME6000) and *G. diazotrophicus* Pal5 (pME6863) with bioassays developed with *A. tumefaciens* NTL4 (pCF218) (pCF232) and *P. putida* F117 (pKR-C12) (data not shown) [24,25]. As shown in Fig. 4, no growth differences could be observed between *G. diazotrophicus* Pal5 (pME6000) and *G. diazotrophicus* Pal5 (pME6863) with an active and an inactive QS system, respectively. Total acidity and pH of the samples also showed similar values. This was in agreement with the lack of a difference in the antagonistic activity measured under both conditions (data not shown).

4. Discussion

In nature, a broad range of physiological features from microorganisms have been shown to be related to interactions among them, and between them and their hosts. From *G. diazotrophicus* Pal5, non-symbiotic nitrogen fixation [11], production of phytohormones [18], solubilization of mineral nutrients [9,10] and production of antimicrobial substances

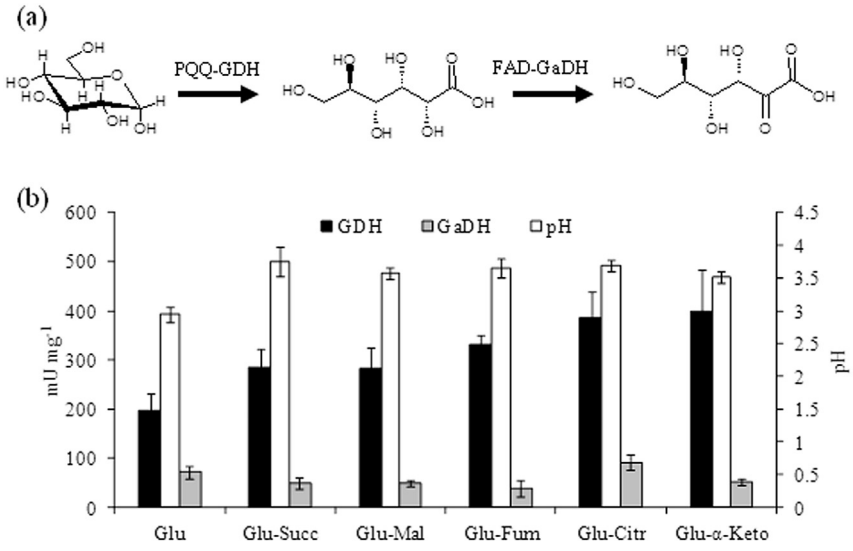

Fig. 3. Glucose metabolism in *G. diazotrophicus* PAL5 requires a PQQ-GDH enzyme with concomitant production of gluconic acid, which can be oxidized to 2-ketogluconic acid by FAD-GaDH (a). Modification of GDH, GaDH activities and final pH by weak organic acids. 300 mM glucose DYGS medium inoculated with *G. diazotrophicus* Pal5 was supplemented independently with succinic (Succ), malic (Mal), fumaric (Fum), citric (Citr) and α-ketoglutaric (α-Keto) acids at a final concentration of 20 mM. Data are means of at least three replicates. Error bars represent standard deviations (b).

have been described. Bacteriocin production has been related to antimicrobial activity among *G. diazotrophicus* strains and against the sugarcane pathogen *X. albilineans* [15]. In this report, evidence is presented indicating that production of gluconic acid by *G. diazotrophicus* Pal5 could also play a role in the microbial interaction through inhibition of other microorganisms. The antimicrobial activity of organic acids has been widely studied scientifically, and has been commercially exploited [26, and references therein]. The mechanism of these compounds is not fully understood, but it requires the presence of the undissociated form, and the potential target includes the cell wall, the cytoplasmic membrane and certain metabolic functions related to replication, synthesis and functions of proteins [27]. Noteworthy, the antagonistic properties of gluconic acid are not clear and remain controversial. While some authors have utilized gluconic acid for formulation of non-inhibitory buffers, others have shown the antagonistic activity of this compound against different phytopathogenic fungi and protozoa [3,4,28]. However, these differences could be due to the concentrations of gluconic acid, since gluconate buffers were used at a concentration of only 20 mM [28]. The effectiveness of organic acids as antimicrobial agents depends on the molarity, pH, concentration of the non-dissociated form and hydrophobicity [27]. As presented in this report, when glucose was utilized at 10 mM, it is plausible to hypothesize that the attained concentration of gluconic acid was not sufficient to antagonize microbial growth (see Table 1). At 300 mM, the production of gluconic acid was probably enough to inhibit growth of the microorganisms assayed. Interestingly, activity with sodium-gluconate-supplemented medium could only be observed when the pH was decreased to 3.20, a value at which the remaining gluconate could be protonated with the concomitant presence of the undissociated form of the organic acid. On the other hand, Alvarez and Martínez-Drets [8] showed that sorbitol, fructose or glycerol is used as a carbon source by *G. diazotrophicus* Pal5 with no acid production. In agreement, we show that the pH values remain close to neutrality, explaining the deficiency in inhibitory activity detected under these conditions even when samples are acidified. The profile of antagonistic activity, together with the growth, consumption of glucose and acidity, support the hypothesis that gluconic acid is responsible for the inhibition of other microorganisms (Fig. 1a and b). The decrease in activity until its disappearance is in agreement with the chemical nature of the responsible compound. Noteworthy, Luna [29] showed that when glucose is exhausted, *G. diazotrophicus* Pal5 utilizes the gluconic acid obtained previously from oxidation of the monosaccharide as the carbon and energy source with a concomitant increase in pH. The identity of the antagonistic molecule could be confirmed by comparison of the retention time of the active compound and pure gluconic acid by HPLC.

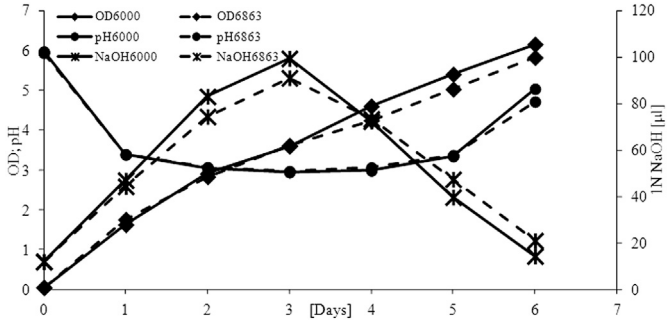

Fig. 4. Influence of a QS system on *G. diazotrophicus* Pal5. Plasmid pME6863 was conjugated in *G. diazotrophicus* Pal5 to inactivate the QS system through a quorum quenching strategy. As a control, plasmid pME600 was also independently conjugated. At regular intervals, samples were withdrawn for growth (OD), pH and total acidity measurement (NaOH).

G. diazotrophicus Pal5 presents a broader spectrum of antimicrobial activity than previously reported. A wide range of both Gram-positive and Gram-negative bacteria, including environmental isolates of *G. diazotrophicus* and endophytic bacteria from sugarcane, could be antagonized by the Pal5 strain (Table 2). We should also point out inhibition of bacterial phytopathogens, in particular, *X. albilineans* (responsible for the disease of sugarcane called leaf scald disease), *P. syringae* pv. *syringae* (responsible for bacterial red streak disease) and *A. avenae* subsp. *avenae* (responsible for red stripe disease) in terms of the agricultural importance of *G. diazotrophicus*. Inhibition of *X. campestris* pv. *campestris* is also remarkable, since this phytopathogen has not been related to sugarcane but to a model crucifer, *Arabidopsis thaliana*, which can also be colonized by *G. diazotrophicus*. Muñoz-Rojas et al. [14] have shown similar interactions among different *Gluconacetobacter* species and suggested that a bacteriocin-like molecule of low molecular mass was responsible for the inhibition. With the exception of *W. paramesenteroides* that presents a smaller but clearly visible inhibition zone, the resistance of LAB is remarkable. It has been postulated that resistance to organic acids can be mediated through degradation of these compounds. Noteworthy, the capability of organic acid fermentation, in particular gluconic acid, has been reported for several species of LAB [30]. At the same time, eukaryotic microorganisms including those isolated from sugarcane seem to be more resistant to the antagonistic effect of *G. diazotrophicus* Pal5. *T. akiyoshidainum* HP2023, a basidiomycetous isolated from the Las Yungas rainforest (Tucumán, Argentine) [31], was the exception to this rule among the assayed strains. The resistance of yeasts to weak organic acid has been related to an active acid extruding from the cell mediated by an ATP binding cassette (ABC) transporter and a limited initial diffusional entry of the acid into the cell [32]. It is thus plausible to hypothesize that production of gluconic acid by *G. diazotrophicus* Pal5 could play a role in interactions with other microorganisms that colonize the same ecological niche [33].

G. diazotrophicus Pal5 lives in close association with sugarcane colonizing the intercellular apoplastic stem spaces of the plant. In addition to its high sucrose concentration, apoplastic fluid also contains several carboxylic acids, including citric, malic and succinic acid, among others [34]. We show that the presence of other organic acids modifies the antimicrobial activity of *G. diazotrophicus* Pal5 (Fig. 3). The results presented here suggest that the existence of organic acids in the ecological niche of *G. diazotrophicus* Pal5 could play an indirect role in control of the antimicrobial activity of gluconic acid. The presence of the weak organic acids malic and succinic acid decreases production of gluconic acid by *Pseudomonas aeruginosa* M3 and SP1, two isolates from mung bean and sweet potato, respectively [35]. Similar behavior has been described for secretion of oxalic acid when *Klebsiella pneumonia* SM6 and SM11 are cultured in a succinate-supplemented medium [36]. The reduction in the production of gluconic and oxalic acids by *P. aeruginosa* M3 and SP1 and *K. pneumonia* SM6 and SM11 in the presence of dicarboxylic acids is due to carbon catabolite repression [35,36]. It is unlikely that this global control system is related to the effect determined for antimicrobial activity when carboxylic acids are present in DYGS cultures, since *G. diazotrophicus* Pal5 lacks a transport system for these acids [13]. According to GDH and GaDH activities, we suggest that the presence of these organic acids modifies the antimicrobial activity of gluconic acids through modification of the metabolism of *G. diazotrophicus* Pal5 (Fig. 4). In the presence of succinic, malic, fumaric, and alpha-ketoglutaric acid acids, more gluconic acid could be produced (according to the higher activities of PQQ-GDH) and, at the same time, less would be converted into 2-ketogluconic acid (according to the lower activities of FAD-GaDH). The weaker inhibition when carboxylic acids were present could then be attributed to major metabolism of gluconic acid by *G. diazotrophicus* Pal5. In contrast, it is plausible that, in citric-acid-supplemented medium, the gluconic acid produced is converted to 2-ketogluconic acid according to PQQ-GDH and FAD-GaDH activities (Fig. 4). The mechanism by which these carboxylic acids modify PQQ-GDH and FAD-GaDH activities requires further studies. Alvarez and Martínez-Drets [8] suggested that the catalytic site of PQQ-GDH faces the periplasmic space, where these organic acids might exert their effects. Another explanation is that, with higher pKa values than gluconic acid, these weak organic acids could buffer the extracellular medium at higher pH values, decreasing dissociation of gluconic acid with a concomitant reduction in inhibitory activity. From this point of view, the host plant could modulate the interactions between *G. diazotrophicus* and co-habiting microorganisms through secretion of weak organic acids.

Quorum sensing (QS) is a key regulatory mechanism of several physiological features of microorganisms [17]. In *G. intermedius* NCI1051, taxonomically related to *G. diazotrophicus* Pal5, QS is involved in control of oxidative fermentation, including acetic and gluconic fermentation by means of an OmpA family protein that is under the control of a GinI/GinR QS system [21]. *G. diazotrophicus* Pal5 possesses an active QS system that utilizes at least eight different chemical signals [13,37]. As presented in this report, under the assayed conditions the QS mechanism of *G. diazotrophicus* Pal5 is not involved in production of gluconic acid. In contrast, the disruption of the QS system of *Aeromonas hydrophila* AH-1N causes strong acidification of glucose-containing culture medium [38]. Similar behavior has been reported for *Serratia plymuthica* RVH1 and *Serratia marcescens* MG1 [39]. Noteworthy, the QS system of *G. diazotrophicus* Pal5 is located in a genome island, suggesting that this regulatory mechanism was acquired through horizontal gene transfer. This origin could be related to the lack of integration of QS into the main metabolic pathway for glucose oxidation with gluconic acid production in *G. diazotrophicus* Pal5. These results support the previous hypothesis that the regulatory mechanism of QS is not always related to control of a particular physiological feature in different microorganisms.

To the best of our knowledge this is the first report showing a direct link between the antagonistic activity of *G. diazotrophicus* Pal5 and gluconic acid production by this strain. Although these results were obtained under laboratory conditions, it can nonetheless be considered that gluconic acid provides *G. diazotrophicus* Pal5 an ecological advantage in nature. Furthermore, production of gluconic acid in particular, and organic acids in general, should be regarded as a plant growth-promoting characteristic of plant-associated microorganisms.

Conflict of interest

The authors declare that they have no competing interest.

Acknowledgments

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