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ORIGINAL PAPER

Gluconacetobacter diazotrophicus PAL5 possesses an active quorum sensing regulatory system

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Abstract The endophytic bacterium *Gluconacetobacter diazotrophicus* colonizes a broad range of host plants. Its plant growth-promoting capability is related to the capacity to perform biological nitrogen fixation, the biosynthesis of siderophores, antimicrobial substances and the solubilization of mineral nutrients. Colonization of and survival in these endophytic niche requires a complex regulatory network. Among these, quorum sensing systems (QS) are signaling mechanisms involved in the control of several genes related to microbial interactions, host colonization and stress survival. *G. diazotrophicus* PAL5 possesses a QS composed of a *luxR* and a *luxI* homolog, and produces eight molecules from the AHL family as QS signals. In this report data are provided showing that glucose concentration modifies the relative levels of these signal molecules. The activity of *G. diazotrophicus* PAL5 QS is also altered in presence of other carbon sources and under saline stress conditions. Inactivation of the QS system of *G. diazotrophicus* PAL5 by means of a quorum quenching strategy allowed the identification of extracellular and intracellular proteins under the control of this regulatory mechanism.

Keywords *Gluconacetobacter diazotrophicus* · Quorum sensing · Quorum quenching · Proteome · Activity · Bioassay

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Introduction

Gluconacetobacter diazotrophicus is an endophytic microorganism isolated from the inner tissues of a broad range of host plants, including sugarcane, sweet potato, coffee, tea, banana, pineapple, radish and rice, among others (Cavalcante 1988; Döbereiner 1993; Madhaiyan 2004; Tapia-Hernández 2000; Saravanan 2008). The plant growth-promoting capability of this Alphaproteobacterium has been related not only to its capacity to perform biological nitrogen fixation, but also the biosynthesis of siderophores, antimicrobial substances and the solubilization of mineral nutrients through the production of large quantities of gluconic acid (Saravanan 2008).

Colonization of and survival in an endophytic niche requires a complex regulatory network that

allows a fine-tuning in exopolysaccharide production, motility, chemotaxis, biosynthesis of secondary metabolites, etc. (Compant 2010; Downie 2010). Although the molecular bases for the endophytic lifestyle have not been completely elucidated in *G. diazotrophicus* PAL5, it has been reported that a wild type strain and a Nif^- mutant colonize equally the sugarcane (Sevilla 2001). In addition, the detoxifying enzymes superoxide dismutase and glutathione reductase play a key role in the colonization of rice roots by *G. diazotrophicus* (Alquéres 2013). The genome sequencing of *G. diazotrophicus* PAL5 revealed the presence of several signaling and regulatory genes that are part of the regulatory network, including 16 GGDEF family genes involved in cyclic di-GMP synthesis and 14 genes coding for membrane-bound histidine kinase signaling proteins (Bertalan 2009). In addition, the *G. diazotrophicus* PAL5 genome possesses a complete quorum sensing regulatory system (QS) composed of a *luxR* and a *luxI* homolog, located in one of its 21 genomic islands (GIs). QS are signaling mechanisms that play a key role in the control of the expression of several genes related to characteristics of importance for microbial interactions, host colonization and stress survival in both Gram positive and Gram negative microorganisms (Atkinson 2009; Fuqua 2001; Ng 2009). The most studied QS signals belong to the *N*-acylhomoserine lactone (AHL) family. In addition to an oxo- or hydroxyl-substitution, AHLs differ in the length of the acyl side chain. Short AHLs include substituted and unsubstituted C4-, C6-, C8 and C10-HSL; long AHLs include substituted and unsubstituted C12-, C14-, C16- and C18-HSL.

It has been reported that *G. diazotrophicus* PAL5 produces eight different molecules from the AHL family as QS signals. Five signals are unsubstitued at the third position of the acyl chain (C6-, C8-, C10-, C12-, and C14-HSL), and three are 3-oxo-AHLs (3-oxo-C10-, 3-oxo-C12, and 3-oxo-C14-HSL) (Nieto-Peñalver 2012). Despite the biological importance of this regulatory mechanism, the QS of *G. diazotrophicus* PAL5 has not been characterized.

The aim of this work was to study the influence of the environment, represented by modifications in the growth conditions, in the activity of the QS from *G. diazotrophicus* PAL5. In addition, we analyzed the proteome of *G. diazotrophicus* PAL5 regulated by QS through a quorum quenching approach.

Materials and methods

Bacteria and culture conditions

Gluconacetobacter diazotrophicus PAL5 (ATCC 49037) was cultured on a shaker with a gentle agitation (150 rpm) at 30 °C in DYGS medium (Reis 1994). DYGS was supplemented with glucose at final concentrations of 10, 30, 50 and 100 mM to analyze the influence of different concentrations of this carbon and energy source. The effect of other carbon sources was studied by supplementing DYGS with potassium gluconate, fructose and sorbitol at a final concentration of 10 mM; saccharose was utilized at a final concentration of 6 mM. 10 mM glucose DYGS was supplemented with 50 and 100 mM NaCl to stress osmotically *G. diazotrophicus* PAL5. 10 mM glucose DYGS + 50 mM NaCl was also evaluated with the osmoprotectants proline and trehalose at a final concentration of 5 and 1 mM, respectively. *Pseudomonas putida* F117 (pKR-C12) and *P. putida* F117 (pAS-C8) were grown aerobically in LB broth at 30 °C (Riedel 2001). *Agrobacterium tumefaciens* NTL4 (pCF218) (pCF232) was grown in AT medium supplemented at 30 °C (Zhu 1998). When required, the following antibiotics were utilized (μg ml^{-1}): gentamicin 25; spectinomycin 50; and tetracycline 5 (for *A. tumefaciens*) or 15 (for *G. diazotrophicus*). Growth was measured turbidimetrically at 600 nm (OD_{600}).

Relative quantification of AHLs

Gluconacetobacter diazotrophicus PAL5 was cultured in DYGS broth as described above until early stationary growth phase. Cultures were centrifuged at room temperature and cell-free supernatants were stored at −20 °C until quantification assays. Determination of the relative concentration of AHLs in cell-free supernatants was performed essentially as described elsewhere (Nieto-Peñalver 2012). Briefly, actively growing cultures of the biosensors *P. putida* F117 (pKR-C12) and *P. putida* F117 (pAS-C8) were supplemented with cell-free supernatants of PAL5 and grown aerobically at 30 °C for 6 h. Induction of fluorescence was measured using a Perkin-Elmer fluorescence spectrometer LS 55 with an excitation wave length of 474 nm and emission detection at 515 nm. The relative fluorescence units (RFU) were

calculated as the fluorescence at 515 nm divided by OD_{600}. Background fluorescence of the biosensor was measured by incubation of the strains in LB supplemented with sterile DYGS. Specific fluorescence units were calculated as the RFU minus the background fluorescence per unit of OD_{600} of the corresponding PAL5 culture. Quantifications were performed at least three times.

Inactivation of *G. diazotrophicus* PAL5 quorum sensing system

A quorum quenching strategy was followed for the inactivation of the QS of *G. diazotrophicus* PAL5. pME6863 harboring the *aiiA*-encoded lactonase from *Bacillus* sp. A24 under the constitutive P_{lac} promoter (Reimmann 2002), and the pME6000 control plasmid were conjugated independently by triparental mating using pRK2013 into *G. diazotrophicus* PAL5. To control the effectiveness of the strategy, organic extracts were prepared with acidified ethyl acetate from whole cultures collected at late exponential growth phase (Shaw 1997). Samples were analyzed by reverse phase-thin layer chromatography (RP-TLC) and plate bioassays developed with *A. tumefaciens* NTL4 (pCF218) (pCF232) and *P. putida* F117 (pKR-C12) as biosensor strains (Riedel 2001; Zhu 1998). For comparison, 1 nmol of C6-HSL and 0.1 nmol of C8-HSL were also spotted in the RP-TLC; 10 nmol of C10-HSL were also spotted in the plate bioassay.

Preparation of protein extracts

Gluconacetobacter diazotrophicus PAL5 (pME6000) and *G. diazotrophicus* PAL5 (pME6863) were cultured in 30 mM glucose DYGS supplemented with tetracycline as described above until late exponential growth phase. After culture centrifugation at 4 °C, supernatants and cell pellets were stored separately at −20 °C until further processing. For the preparation of intracellular protein extracts, cells were washed and resuspended in 50 mM Tris–HCl buffer (pH 8.00) with protease inhibitor Cocktail I (Calbiochem). Cells were broken with glass beads in 15 cycles of vigorous agitation in vortex with intervals on ice (Irazusta 2012). Cell debris was eliminated by centrifugation and protein extracts were stored at −20 °C. For the preparation of extracellular extracts, cell-free supernatants were concentrated by precipitation with ammonium sulfate until complete saturation. Precipitates were dissolved in 8.3 mM sodium phosphate buffer (pH 7.20) and dialyzed utilizing membranes with a molecular cut off of 12,000 Da. Samples were then precipitated with acetone at −20 °C and redissolved in 50 mM Tris–HCl buffer (pH 8.00) with 1 % SDS (sodium dodecyl sulphate). Protein concentration was determined with Bradford and Lowry assays for intracellular and extracellular fractions, respectively.

Proteome analysis

Extracellular proteins were analyzed by mono-dimensional SDS-PAGE in 12 % polyacrylamide denaturing gels and stained with Coomassie Brilliant Blue R-250. Intracellular proteins were analyzed by two-dimensional SDS-PAGE essentially as described elsewhere (Irazusta 2012). For first dimension, isoelectric focusing was performed in 11 cm immobilized pH gradient strips (3-11NL, GE Healthcare Life Science). Second dimension was performed in 12 % polyacrylamide denaturing gels and stained with Coomassie Brilliant Blue R-250. Images were obtained with an Image Scanner III and analyzed with ImageMaster 2D Platinum v 7.0 (GE Healthcare Life Sciences). For both mono- and two-dimensional SDS-PAGE only proteins differentially expressed in three independent samples were selected for identification. After treatment with trypsin, protein digestions were analyzed with a MALDI-TOF-TOF Ultraflex II and identified by peptide mass fingerprinting with MASCOT. The functional categories of the identified proteins were evaluated with KEGG (Kyoto Encyclopedia of genes and Genomes) at http://www.kegg.jp/kegg/.

Results

Influence of glucose on quorum sensing activity of *G. diazotrophicus* PAL5

The biosensors *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12) respond actively to short- and long-chain AHLs provided exogenously, respectively, through the production of green fluorescent protein (GFP) (Riedel 2001). These two strains were utilized to monitor the activity of the QS system of *G. diazotrophicus* PAL5 under different culture conditions. As

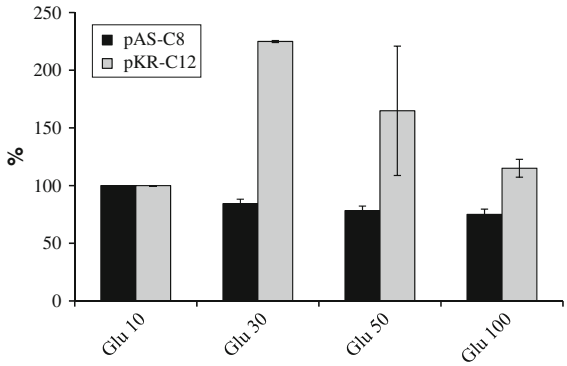

Fig. 1 Influence of glucose on quorum sensing activity. *G. diazotrophicus* PAL5 was cultured in DYGS supplemented with glucose at final concentrations of 10, 30, 50 and 100 mM. Levels of short- and long-AHLs in cell-free supernatants were indirectly measured with bioassays developed with *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12), respectively. For comparative purposes, values obtained in 10 mM DYGS were considered as 100 %. *Bars* represent standard deviations

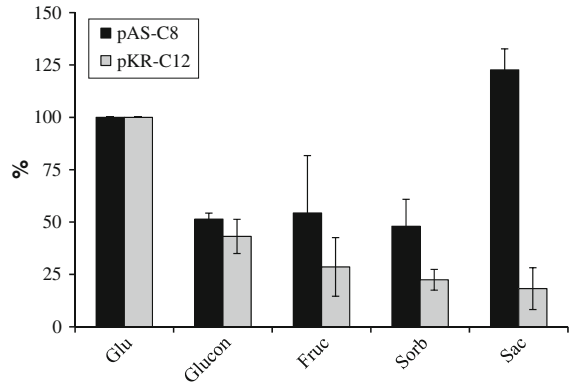

Fig. 2 Influence of carbon sources on quorum sensing activity. *G. diazotrophicus* PAL5 was cultured in DYGS supplemented with 10 mM of fructose, sorbitol and potassium gluconate. Saccharose was utilized at 6 mM. Levels of short- and long-AHLs were indirectly measured in bioassays with *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12), respectively. For comparison, values obtained in 10 mM glucose DYGS were considered as 100 %. *Bars* represent standard deviations

shown in Fig. 1, modifications in glucose concentration from 10 to 100 mM altered the activity of the QS system of *G. diazotrophicus* PAL5. Increases in carbohydrate concentration reduced 20–30 % the production of short-chain AHLs, as evidenced in bioassays with *P. putida* F117 (pAS-C8). In contrast, bioassays with *P. putida* F117 (pKR-C12) showed that the level of long-chain AHLs augmented more than twofold when glucose concentration changed from 10 to 30 mM (Fig. 1). However, these signal molecules decline with larger quantities of the carbohydrate attaining values similar to the control condition when the concentration of glucose was 100 mM. Unfortunately, concentrations above 100 mM could not be analyzed since the large quantity of gluconic acid produced by *G. diazotrophicus* PAL5 from glucose inhibited the biosensor growth (Nieto-Peñalver 2014).

Influence of the carbon source on quorum sensing activity of *G. diazotrophicus* PAL5

DYGS was independently supplemented with fructose, gluconate, sorbitol, and saccharose to analyze the influence of different carbon sources on the QS activity of *G. diazotrophicus* PAL5. For comparison, values obtained with 10 mM glucose were considered as 100 %. As shown in Fig. 2, with the exception of saccharose that increased 20 % the levels of short

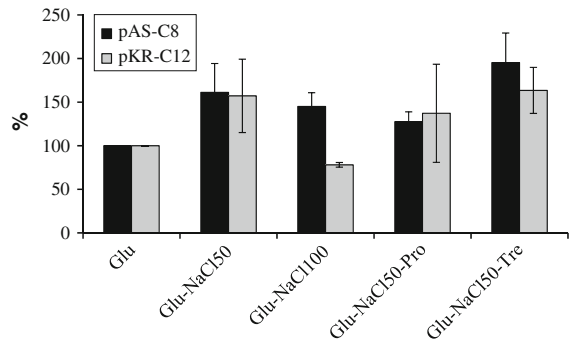

Fig. 3 Influence of saline stress on quorum sensing activity. *G. diazotrophicus* PAL5 was cultured in 10 mM DYGS supplemented with 50 and 100 mM of NaCl. DYGS + 50 mM NaCl was also supplemented with 5 mM proline or 1 mM trehalose. Levels of short- and long-AHLs were indirectly measured in bioassays with *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12), respectively. For comparison, values obtained in 10 mM glucose DYGS were considered as 100 %. *Bars* represent standard deviations

signals, the tested compounds decreased these signals by 50 %. In contrast, long chain-AHLs dimished under all the assayed conditions. While the lowest reduction with respect to the control condition (55 %) was determined with gluconate, the highest decrease was measured with saccharose, which caused a drop of more than 80 %. Values obtained for these signals with fructose and sorbitol were between those obtained with gluconate and with saccharose.

Modification of the activity of the *G. diazotrophicus* PAL5 quorum sensing system by the saline stress

The activity of the *G. diazotrophicus* PAL5 QS was analyzed under saline stress conditions. NaCl was utilized to supplement 10 mM glucose DYGS at final concentrations of 50 and 100 mM. For comparison, values obtained with 10 mM glucose were considered as 100 %. As shown in Fig. 3, the *G. diazotrophicus* PAL5 QS responded in a different way to the two assayed concentrations of salt. At 50 mM, NaCl increased 50 % the production of both short- and long-AHLs. However, when salt concentration was doubled to 100 mM, values of short-AHLs remained virtually the same to those determined when 50 mM was utilized, while long-AHLs decreased 25 % with respect to the control condition.

Bacteria respond to osmotic stress through the uptake and synthesis of compatible solutes or osmoprotectants. To study the response of the QS of *G. diazotrophicus* PAL5 under a saline stress, 5 mM proline was utilized as an osmoprotectant in 10 mM glucose DYGS amended with 50 mM NaCl. When proline was present, these two groups of signal molecules slightly decreased their values and, interestingly, at the same proportion (Fig. 3). Trehalose is another well-known osmoprotectant for microorganisms. However, in contrast to proline, when DYGS + 50 mM NaCl was supplemented with this disaccharide, short- and long-AHLs did not reduce to the control values. While long-chain signal molecules remained virtually at the same level than in 10 mM glucose DYGS + 50 mM NaCl without osmoprotectant, short signals increased even more achieving almost two fold the control value (Fig. 3).

Inactivation of quorum sensing system of *G. diazotrophicus* PAL5 by quorum quenching

One strategy for the identification of the genes regulated by QS is the inactivation of this regulatory system. The pME6863 plasmid conjugated in *G. diazotrophicus* PAL5 permits the expression of *aiiA*, which codes for AiiA, an AHL lactonase from the soil bacterium *Bacillus* sp. A24 that hydrolyze inactivating these signal molecules (Reimmann 2002). As control, the pME6000 vector was conjugated in parallel in the same host. This approach allowed the degradation of the AHLs produced by *G. diazotrophicus* PAL5 and the concomitant inactivation of the system. As shown in Fig. 4, the presence of pME6863 produced a strong decrease in the level of short-AHLs. The small spot present in extracts from *G. diazotrophicus* PAL5 (pME6863), which could be assigned to C8-HSL according to the migration in the RP-TLC, may be attributed to residual quantities of this signal molecule. Noteworthy, *A. tumefaciens* NTL4 (pCF218) (pCF232) has a very high sensitivity for C8-HSL related to the overproduction of the AHL receptor TraR (Zhu 1998). On the other hand, the absence of GFP production in the bioassays developed with *P. putida* F117 (pKR-C12) showed that the long chain-AHLs produced by *G. diazotrophicus* PAL5 were inactivated by the AiiA lactonase (Fig. 4).

Identification of proteins regulated by quorum sensing in *G. diazotrophicus* PAL5

To determine whether the QS system of *G. diazotrophicus* PAL5 is integrated to the regulatory network of this microorganism, and to study the QS regulon, a proteomic approach was utilized after its disruption by the quorum quenching strategy. Characteristics of selected spots, including molecular weight, MASCOT identification score, expression and KEGG categories are indicated in Table 1. Six extracellular proteins were found regulated by QS in *G. diazotrophicus* PAL5. Unfortunately, only three spots could be identified by MALDI-TOF-TOF. Superoxide dismutase coded in locus GDI2168 (according to *G. diazotrophicus* PAL5 genome; GenBank Accession No. AM889285) was found attenuated under the assayed conditions when the QS system was inactivated, according to the intensity of the bands in SDS-PAGE. A surface antigen protein (GDI0777) and the enzyme 6-phosphogluconate dehydrogenase (GDI0287) presented the same behavior when the signal molecules were hydrolyzed.

On the other hand, five proteins were found differentially expressed when the intracellular proteomes of *G. diazotrophicus* PAL5 (pME6000) and *G. diazotrophicus* PAL5 (pME6863) were compared (Table 1). While the chaperone DnaK (GDI1262) and the acetate kinase AckA (GDI1618) were attenuated in *G. diazotrophicus* PAL5 (pME6863), the metallopeptidase (GDI2948) and the inositol-1-monophosphatase (GDI2938) were augmented under this condition in

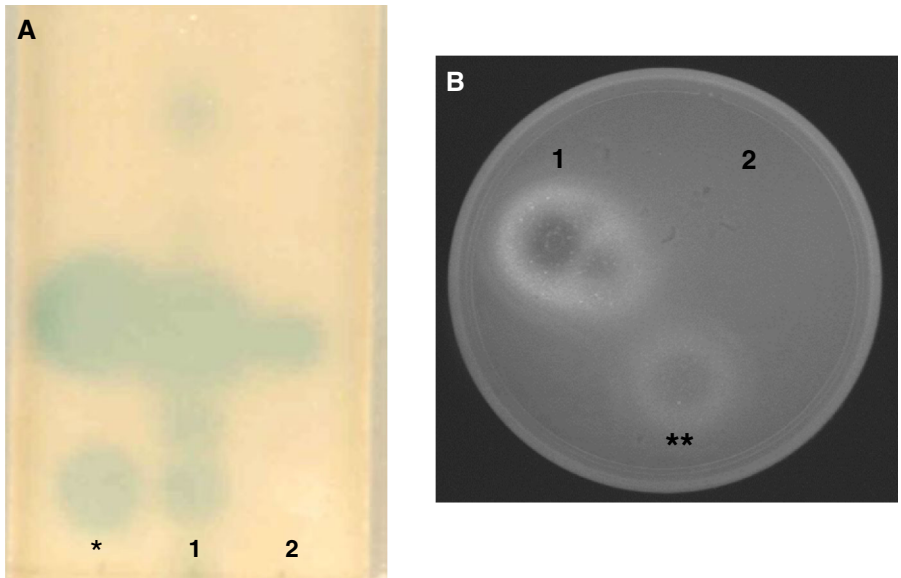

Fig. 4 Inactivation of quorum sensing system by quorum quenching. Organic extracts of (1) *G. diazotrophicus* PAL5 (pME6000) and (2) *G. diazotrophicus* PAL5 (pME6863) were analyzed by RP-TLC (**a**) and plate bioassays (**b**) developed with *A. tumefaciens* NTL4 (pCF218) (pCF232) and *P. putida* F117 (pKR-C12), respectively. For comparison, (*) C6-HSL and C8-HSL were also spotted together in the RP-TLC; (**) C10-HSL was spotted in the plate bioassay. Note that the AiiA lactonase coded in pME6863 inactivated the AHLs produced by *G. diazotrophicus* PAL5

comparison with *G. diazotrophicus* PAL5 (pME6000). Finally, the preprotein translocase SecA subunit (GDI0797) was absent when the QS system was inactivated. A sixth protein found strongly expressed in *G. diazotrophicus* PAL5 (pME6863) and absent in *G. diazotrophicus* PAL5 (pME6000) was identified as the pME6863-encoded AiiA lactonase.

Discussion

The QS system of *G. diazotrophicus* PAL5 responded actively to the modifications of the culture conditions. The modifications in the profile of short- and long-AHLs suggest that a group of molecules are required by *G. diazotrophicus* PAL5 for the regulation of specific functions at a given environmental condition. However, up to date it is not clear why some microorganisms produce several signals if they possess only one AHL receptor, i.e. one LuxR homolog. It has been proposed that while one particular molecule plays the main regulatory function, the remaining ones are involved in the fine-tuning through the competition for the ligand-binding domain located in the N-terminus of the LuxR homologs (Vannini 2002). It is then plausible that *G. diazotrophicus* PAL5 modifies the balance between the signal molecules to control the expression of particular genes under a given condition. A similar conclusion could be drawn when DYGS is supplemented with fructose, sorbitol or saccharose, where also particular changes in the proportions between short- and long-chain-AHLs could be determined (Fig. 2). Noteworthy, saccharose, the glucose source for this microorganism in the apoplastic fluid of sugarcane, was the only assayed compound producing an increase in signal molecules with short acyl chain and a strong decline in those with long acyl chain. Alterations in the relative proportions of AHLs have also been observed in *Rhizobium leguminosarum*, another plant-associated nitrogen fixing bacterium when cultured in complex or synthetic medium (Lithgow 2001). On the other hand, the metabolic cost for the production of a short-chain AHLs such as C4-HSL has been estimated in 8 ATP (Keller 2006). Luna et al. (2000) showed that when glucose is utilized as the carbon source, the respiratory chain of *G. diazotrophicus* PAL5 presents higher energetic efficiency in comparison with gluconate. Probably the lower levels of AHLs in gluconate-

Table 1 *G. diazotrophicus* PAL5 (pME6000) proteins differentially expressed in comparison to *G. diazotrophicus* PAL5 (pME6863)

Protein	Locus	MW	Pid score	Expression[a]	KEGG
Intracellular fraction					
Inositol-1-monophosphatase	GDI 2938	27597	184	Upregulated	Metabolism environmental information processing
DnaK chaperone	GDI 1262	67253	99	Downregulated	Genetic information processing
Metallopeptidase	GDI 2948	79251	141	Upregulated	Environmental information processing
AckA acetate kinase	GDI 1618	42322	77	Downregulated	Metabolism
Preprotein translocase SecA subunit	GDI 0797	101922	216	Absent	Genetic information processing
Extracellular fraction					
Superoxide dismutase	GDI 2168	25882	37	Downregulated	Environmental information processing cellular processes
Surface antigen protein	GDI 0777	24442	120	Downregulated	
6-phosphogluconate dehydrogenase	GDI 0287	35991	408	Downregulated	Metabolism

Locus in *G. diazotrophicus* PAL5 genome; MW, protein theoretical molecular weight; Pid score, MASCOT protein identification score

[a] Expression, upregulation or downregulation in *G. diazotrophicus* PAL5 (pME6000) in comparison to *G. diazotrophicus* PAL5 (pME6863); KEGG pathway as retrieved from http://www.kegg.jp/kegg/

supplemented DYGS are related to the different energetic state of the cell.

Saline stress also changed the relative proportions of both group of AHLs (Fig. 3). Nevertheless, long AHLs showed to be more dynamic as NaCl changed from 50 to 100 mM. It is likely that these signal molecules, or the relative proportions with respect to the short-AHLs as discussed above, are involved in the regulation of specific genes required for saline stress. At the same time it has to be considered that under stress conditions microorganisms modify the membrane lipids in order to adapt the membrane fluidity (Baysse 2005). Therefore, it cannot be stated that the alterations in the AHL profile are due only to a modification in the production of these molecules. Membrane lipids of *G. diazotrophicus* PAL5 at 100 mM NaCl could also allow a stronger interaction with long chain-AHLs, which could conduct to lower concentrations of these signals in supernatants. Proline, under the utilized conditions, functioned partially as an osmoprotector for *G. diazotrophicus* PAL5, since both short and long AHLs decreased between 10 and 20 % (Fig. 3). (Haudecoeur 2009) reported that proline also modulates the QS from the phytopathogen *A. tumefaciens* through the antagonism of γ-aminobutyric acid (GABA), a signal molecule produced by the host plant. In contrast, proline leads to a higher accumulation of 3-oxo-C8-HSL by *A. tumefaciens* through the GABA-induced enzymatic degradation of the AHL. On the other hand, the results found with trehalose are in concordance to those reported for *Burkholderia* sp. 033, where the disaccharide augmented the production of C6-, C8- and C10-HSL (Keum 2009). Even if AHL production is typically maximal at late exponential growth phase (Fekete 2010), a modification in the profile of AHLs of *G. diazotrophicus* PAL5 throughout the growth caused by the osmotic stress or other stress conditions cannot be ruled out. Duan and Surette (2007) showed that several environmental conditions, in particular increasing concentrations of NaCl, modify the *P. aeruginosa* PAO1 the expressions of *lasI* and *rhlI*, which encodes the two AHL synthases of this bacterium. Although the results presented in this work show that *G. diazotrophicus* PAL5 possesses a functional QS, which responds actively to the environmental conditions, further experiments are required to analyze the role of this regulatory mechanisms in the osmotic stress response in this bacterium. In addition, a different response of the QS from *G. diazotrophicus* PAL5 under osmotic stress conditions when osmoprotectants others than proline or trehalose, e.g., glicine betaine, carnitine or mannitol, are present cannot be ruled out.

The utilization of the quorum quenching strategy was successful for the inactivation of the QS system

and the study of the proteome of *G. diazotrophicus* PAL5 regulated by QS. Analysis of differentially expressed proteins showed that, even if the QS system is located in a GI (Bertalan 2009), this mechanism is integrated in the regulation of the physiology of *G. diazotrophicus* PAL5. The repressed and over-expressed proteins in *G. diazotrophicus* PAL5 (pME6863) in comparison with *G. diazotrophicus* PAL5 (pME6000) shows that this QS system, similar to QS of other microorganisms, acts both positively and negatively on the regulation of gene expression.

The identification of the selected proteins allows the hypothesis that the QS of *G. diazotrophicus* PAL5 plays a role for the stress resistance and host colonization. Inositol-1-monophosphatase has been related to several biological processes including inositol phosphate metabolism and phytate degradation. The mutation of *suhB* gene that codes the inositol-1-monophosphatase from *Burkholderia cenocepacia* causes pleiotropic effects, including alterations in protein secretion and biofilm formation (Rosales-Reyes 2012). The molecular chaperone DnaK is a heat shock protein related to protein folding that permits a broad range of organisms to survive under different stress conditions. Lack of motility and sensitivity to nutrient starvation, H_2O_2 and UV-irradiation have been related to mutations of *dnaK* in different microorganisms (Genevaux 2007). Interestingly, (Lery et al. 2011) reported that *G. diazotrophicus* PAL5 produces largest quantities of DnaK when the strain is grown in the presence of sugarcane plantlets in comparison with pure cultures. The metallopeptidase is a zinc-metallopeptidase that belongs to the M13.009 subfamily of peptidases, according to MEROPS Peptidase Database (http://merops.sanger.ac.uk/) (Rawlings 2012). This enzyme could have a nutritional function, as in *Photorhabdus luminescens* (Marokházi 2004), or be involved in the colonization of the host, as in *Porphyromonas gingivalis* (Ansai 2003). The AckA acetate kinase catalyzes the transfer of a phosphate from ATP to acetate for the production of acetyl phosphate. AckA is a key enzyme in the "acetate switch", a change in microbial life-style for the scavenging for acetate in nutrient depleted conditions. Noteworthy, (Studer et al. 2008) reported that the acetate switch of *Vibrio fischeri* is also under the control of the QS system of this bacterium. SecA is the ATPase subunit of the Sec system, an ubiquitous bacterial mechanism for the translocation of proteins across membranes. This process is required for the membrane and cell wall formation, nutrient uptake and the relationships with high organisms, i.e. pathogenesis and symbiosis. It is interesting to mention that Qi et al. (2002) have shown that DnaK can promote the protein transport in SecA deficient *E. coli* mutants. It is then plausible that the lower levels of SecA in *G. diazotrophicus* PAL5 (pME6863) is related to the higher production of DnaK observed in 2D SDS-PAGE.

The superoxide dismutase (SOD) enzymes catalyze the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide protecting the cell from the damage caused by the reactive oxygen species. Up to date, few SOD enzymes have been described as extracellular proteins. It has been suggested that the secretion of SOD is important for the pathogenesis of *Mycobacterium tuberculosis* and *M. bovis* (Kang 1998; Kusunose 1976). Noteworthy, the QS system of *P. aeruginosa* is also involved in the regulation of SOD production (Costa 2010; Hassett 1999). Although the biological role of the membrane antigenic protein has not been studied in *G. diazotrophicus* PAL5, Lery et al. (2011) found this protein, similar to DnaK, overexpressed when the strain was co-cultured with sugarcane plantlets and suggested that is involved in the early recognition process between the microbe and the host. 6-phosphogluconate dehydrogenase is part of the pentose phosphate pathway of *G. diazotrophicus* PAL5, which metabolize gluconic acid obtained from the oxidation of glucose. Comparable to DnaK and the membrane antigenic protein, this dehydrogenase enzyme is overexpressed when *G. diazotrophicus* PAL5 is co-cultured with sugarcane plantlets (Lery 2011). It has been proposed that, similar to the alcohol dehydrogenase of *E. coli*, 6-phosphogluconate dehydrogenase could be part of a biological barrier against the oxidative damage (Lery 2011).

The results presented in this report show that the QS system of *G. diazotrophicus* PAL5 is active and that the environmental factors can modulate its activity. This regulatory mechanism could play a role in the defense against certain stress conditions. In addition, data presented here suggest that the QS of *G. diazotrophicus* PAL5 is involved in the host-microbe interactions. Other properties of this regulatory system cannot be ruled out in this plant–associated microorganism. The host plant could also modify its

physiology in response to the AHL signal molecules produced by *G. diazotrophicus* PAL5, as has already been shown for *Medicago truncatula* and *Arabidopsis thaliana* (Hartmann 2012; Mathesius 2003; Von Rad 2008). Further studies are required to test these hypotheses

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