

Identification of *N*-acyl homoserine lactones produced by *Gluconacetobacter diazotrophicus* PAL5 cultured in complex and synthetic media

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Abstract The endophytic diazotrophic *Gluconacetobacter diazotrophicus* PAL5 was originally isolated from sugarcane (*Saccharum officinarum*). The biological nitrogen fixation, phytohormones secretion, solubilization of mineral nutrients and phytopathogen antagonism allow its classification as a plant growth-promoting bacterium. The recent genomic sequence of PAL5 unveiled the presence of a quorum sensing (QS) system. QS are regulatory mechanisms that, through the production of signal molecules or autoinducers, permit a microbial population the regulation of the physiology in a coordinated manner. The most studied autoinducers in gram-negative bacteria are the *N*-acyl homoserine lactones (AHLs). The usage of biosensor strains evidenced the presence of AHL-like molecules in cultures of *G. diazotrophicus* PAL5 grown in complex and synthetic media. Analysis of AHLs performed by LC-APCI-MS permitted the identification of eight different signal molecules, including C6-, C8-, C10-, C12- and C14-HSL. Mass spectra confirmed that this diazotrophic strain also synthesizes autoinducers with carbonyl substitutions in the acyl chain. No differences in the profile of AHLs could be determined under both culture conditions. However,

although the level of short-chain AHLs was not affected, a decrease of 30% in the production of long-chain AHLs could be measured in synthetic medium.

Keywords *Gluconacetobacter diazotrophicus* · Quorum sensing · *N*-Acyl homoserine lactone · Autoinducer · Diazotrophic

Introduction

Gluconacetobacter diazotrophicus is an endophytic and diazotrophic gram-negative bacterium originally isolated from sugarcane. It has also been found in association with other sucrose-rich crops, such as sweet potato, sweet sorghum, pineapple, banana and coffee (Reis et al. 1994; Saravanan et al. 2008; Martínez et al. 2003; Jimenez-Salgado et al. 1997). Several important biotechnological features related to plant growth promotion have been described for this Alphaproteobacterium. In addition to its capacity to fix atmospheric nitrogen (Stephan et al. 1991), it secretes phytohormones such as auxins and gibberellins (Bastián et al. 1998); it inhibits the growth of the phytopathogens *Xanthomonas albilineans*, *Fusarium* sp. and *Helminthosporium carbonum* (Saravanan et al. 2008), and can increase the availability of insoluble phosphate and zinc salts (Intorne et al. 2009). These characteristics allow the classification of this microorganism as a plant growth-promoting bacterium. The recent genomic sequencing of *G. diazotrophicus* PAL5 permitted the identification of several genes related to these features of agricultural importance, and others involved in microbe– and plant–microbe interactions. A complete quorum sensing system has also been detected in this diazotrophic strain (Bertalan et al. 2009).

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Quorum sensing (QS) systems, firstly described in the marine bacterium *Vibrio fischeri*, are regulatory mechanisms of the microbial physiology, which depend on the biosynthesis of small signaling molecules (Fuqua et al. 1994). The measurement of the extracellular concentration of these compounds allows the cells to coordinate their behaviors in a coordinate manner. When the microbial population density reaches a threshold level, an intracellular complex formed between the signal molecule, also called autoinducer, and a protein receptor binds regulatory sequences in the DNA modifying the expression of genes usually related to microbe–microbe, and host–microbe interactions (Ng and Bassler 2009). Autoinducers belonging to the *N*-acyl homoserine lactone (AHLs) family produced by gram-negative bacteria are the most studied signaling molecules. The biosynthesis of AHLs is catalyzed by LuxI-type enzymes encoded by *luxI*-homolog genes. *luxR*-homolog genes encode the LuxR-type receptors of AHLs that bind the *lux*-box regulatory sequences through a HTH domain localized in the carboxy terminus of the protein. QS systems described in both beneficial and pathogenic bacteria have been related, among others, to the production of exopolysaccharide, siderophores, antibiotics, biofilm formation, regulation of motility and conjugation (Aguilar et al. 2003; McGowan et al. 2005; Fuqua and Greenberg 2002).

As stated above, *G. diazotrophicus* PAL5 possesses at least one complete QS system. In spite of its biotechnological importance, the description of this system has not yet been accomplished in this strain. This work aims at detecting and describing the QS signaling molecules produced by this diazotrophic endophytic bacterium.

Materials and methods

Bacteria and culture conditions

Gluconacetobacter diazotrophicus PAL5 (ATCC 49037) was cultured aerobically at 30°C in LGIP synthetic medium and DYGS complex medium (Reis et al. 1994; Stephan et al. 1991), both supplemented with glucose at a final concentration of 0.2%. *Pseudomonas putida* F117 (pKR-C12) and *P. putida* F117 (pAS-C8) were cultured aerobically at 30°C in LB broth; *Agrobacterium tumefaciens* NTL4 (pCF218 pCF372) was cultured aerobically at 30°C in AT broth (Riedel et al. 2001; Zhu et al. 1998). When required, the culture media were supplemented with the following antibiotics: kanamycin 50 µg ml⁻¹, spectinomycin 50 µg ml⁻¹, tetracycline 5 µg ml⁻¹ and gentamicin 20 µg ml⁻¹. Growth was measured turbidimetrically at 600 nm (OD₆₀₀).

Bioassays

Concentrated extracts of whole cultures (cell and supernatant) of PAL5 strain were prepared essentially as described elsewhere (Marketon et al. 2002). Briefly, a volume of culture was extracted twice with one volume of ethyl acetate acidified with 0.1% acetic acid. Residual water was removed with anhydrous magnesium sulfate, and organic extracts were concentrated 500-fold. For both conditions, samples equivalent to 2 ml of supernatants were analyzed by C₁₈ RP-TLC chromatography (Merck) and developed with an overnight culture of *A. tumefaciens* NTL4 (pCF218 pCF372), as described elsewhere (Shaw et al. 1997); 0.5 nmol of hexanoyl homoserine lactone and 0.25 nmol of octanoyl homoserine lactone were used as positive controls. Five microlitres of a liquid culture of *G. diazotrophicus* PAL5 was also spotted on a DYGS agar plate, close to 5 µl of an overnight culture of *P. putida* F117 (pKR-C12). After 14–18 h of incubation at 30°C, plates were observed using an Olympus BX51 microscope equipped with an Olympus Q-Color5 digital camera in order to detect induction of the production of green fluorescence protein (GFP).

LC-APCI-MS analysis of AHLs

Identification of AHLs present in concentrated extracts of cultures of *G. diazotrophicus* PAL5 was performed by high-resolution LC–MS. Analyses were carried out with a Rheos 2200 HPLC system (Flux Instruments, Basel, Switzerland) coupled to an LTQ-Orbitrap (Thermo Fisher Scientific, Waltham, MA) equipped with an atmospheric pressure chemical ionization probe, and a Gemini C18 analytical column (150 × 2.0 mm, particle size 3 mm; Phenomenex, Torrance, CA, USA) at a flow rate of 200 µl min⁻¹. Injection volume was 10 µl. MS analysis was done in the positive FTMS mode at a resolution of 60,000. The analytes were identified by the exact masses of the corresponding [M+H]⁺ ions.

Relative quantification of AHLs

G. diazotrophicus PAL5 was cultured in LGIP and DYGS liquid media as described above until early stationary growth phase. Quantification of the relative concentration of AHLs produced was performed essentially as described elsewhere with slight modifications (Riedel et al. 2001). Cultures were centrifuged at room temperature, and cell-free supernatants were stored at –20°C until further use in the quantification assays. Overnight cultures of the biosensor *P. putida* F117 (pKR-C12) and *P. putida* F117 (pAS-C8) were diluted, supplemented with cell-free supernatants of PAL5 strain and grew 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 OD₆₀₀. Background fluorescence of the biosensor was measured by incubation of the strains in LB medium supplemented with sterile DYGS or LGIP media. Specific fluorescence units were calculated as the RFU minus the background fluorescence per unit of OD₆₀₀ of the corresponding PAL5 culture. Quantifications were performed at least three times.

Results

Detection of AHLs produced by *G. diazotrophicus* PAL5

As stated above, the genome sequence of *G. diazotrophicus* PAL5 has recently been published (Bertalan et al. 2009). The presence of one *luxI* homolog encoded in GDI2836 and two *luxR* homologs encoded in GDI2837 and GDI2838 suggests that this diazotrophic bacterium possesses at least one complete quorum sensing system based on acyl homoserine lactone (AHL) production. PAL5 strain was cultured aerobically in both DYGS organic complex to detect and to study the profile of autoinducers released and LGIP mineral synthetic media until late exponential growth phase. In concentrated extracts analyzed by C₁₈ RP-TLC chromatography plates and developed with *A. tumefaciens* NTL4 (pCF218 pCF372), two spots could clearly be observed in both samples, which suggested the presence of at least two different AHLs (Fig. 1a). After comparison with the migration of synthetic standards, the spots were tentatively assigned to hexanoyl- and octanoyl-HSL (C6-HSL and C8-HSL). Interestingly, a third spot between C6-HSL and C8-HSL was visualized in the extract prepared from DYGS medium. No spot could be observed when concentrated extracts of uninoculated culture media were analyzed with both biosensor strains. In addition, the diameter and intensity of all the spots were superior under that condition, suggesting that the concentrations of signal molecules could be higher in complex medium (see below). However, in DYGS medium, the OD₆₀₀ value near 2.70 measured at the onset of stationary phase was higher to that observed in LGIP medium, which was about 0.90.

Both long- and short-chain AHLs have been identified in culture supernatants of phylogenetically distant bacteria. However, biosynthesis of autoinducer molecules with long acyl chain seems to be a characteristic of Alphaproteobacteria. A colony of the biosensor *P. putida* F117 (pRK-C12) was cultured next to a colony of PAL5 strain in DYGS agar plates to analyze whether *G. diazotrophicus*

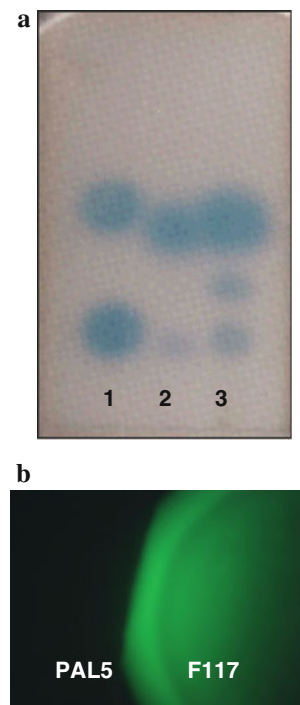


Fig. 1 Detection of AHLs produced by *G. diazotrophicus* PAL5. Cultures in LGIP (lane 2) and DYGS (lane 3) media were extracted and analyzed by RP-TLC developed with *A. tumefaciens* NTL4 (pCF218 pCF372) (a). For comparison purposes, C6- and C8-HSL were chromatographed next to analyzed samples (lane 1). PAL5 strain was also cultured in DYGS medium next to *P. putida* F117 (pRK-C12). As controls, both strains were inoculated separately. After 16–18 h of incubation, the observation by fluorescence microscopy showed induction of GFP production in the biosensor (b)

PAL5 could synthesise AHLs with long acyl chains. As controls, in the same plate, both strains were cultured separately. After 16–18 h of incubation at 30°C, the biosensor showed an intense fluorescence under the assayed conditions. When the colony of *G. diazotrophicus* was at a relative distance, the biosensor showed no fluorescence indicating that the PAL5 strain was producing molecules that could induce the production of green fluorescence protein (GFP), as depicted in Fig. 1b. Taking into account that short-chain AHLs including hexanoyl-HSL and octanoyl-HSL cannot activate *P. putida* F117 (pRK-C12) (Riedel et al. 2001), this result suggested that in addition to C6- and C8-HSLs, *G. diazotrophicus* PAL5 could also synthesize quorum sensing molecules with long acyl chains.

Chemical identification of AHLs by LC-APCI-MS

In order to determine the chemical structure of these compounds, concentrated extracts were prepared as described above and analyzed by high-resolution LC-APCI-MS. For identification, theoretical *m/z* values were compared with the measured *m/z* values (Table 1). Mass

Table 1 Comparison of theoretical and measured m/z values of AHLs identified in organic extracts of *G. diazotrophicus* PAL5 cultures

Identified compound	Theoretical m/z^a	Measured m/z^a
C6-HSL	200.12812	200.1279
C8-HSL	228.15942	228.1593
C10-HSL	256.19072	256.1902
C12-HSL	284.22202	284.2215
C14-HSL	312.25332	312.2531
3-oxo-C10-HSL	270.17000	270.1696
3-oxo-C12-HSL	298.20130	298.2010
3-oxo-C14-HSL	326.23259	326.2323

^a m/z values corresponding to $[M+H]^+$

spectra showed the presence of positive adduct ions of $[M+H]^+$ with a m/z 200.1279 and m/z 228.1593, which allowed the confirmation of the presence of C6-HSL and C8-HSL in the concentrated extracts, as expected from the TLC bioassays (Fig. 2a, b). Long-chain AHLs detected with the *P. putida* F117 biosensor could also be identified by LC-APCI-MS. Adduct ions of $[M+H]^+$ with m/z 256.1902, m/z 284.2215 and m/z 312.2531 confirmed the presence of C10-HSL, C12-HSL and C14-HSL in the analyzed samples (Fig. 2c–e). In addition, the nature of the substitution at the third position of these long-chain autoinducers varied from fully reduced to partially oxidized: the measurement of the m/z 270.1696, 298.2010 and 326.2323 permitted the identification of 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL that possess molecular weights of 269, 297 and 325, respectively (Fig. 2f–h). Both groups of quorum sensing molecules with long acyl chains could not be detected by RP-TLC. These results agree with previous reports that evidence the lower sensitivity of *A. tumefaciens* NTL4 (pCF218 pCF372) for detecting long-chain AHLs (see above) and highlight the importance of using more than one biosensor for the detection of these signaling molecules. By LC-APCI-MS, no difference in the AHL profile could be detected when the PAL5 strain grew in DYGS complex medium and LGIP synthetic medium. A spot observed by RP-TLC in samples from DYGS medium and absent in LGIP extracts could not be attributed to any AHL with this sensitive technique (see above). Under the assayed condition, it was not possible to estimate the relative concentration of the signal molecules present in the concentrated extracts, due to the ion suppression effect observed (Annesley 2003).

Relative quantification of AHL activity in supernatants of *G. diazotrophicus* PAL5

The relative level of inducing activity was measured in DYGS and LGIP spent media to analyze whether the

concentration of AHLs was influenced by the culture conditions. Cell-free samples were used to supplement cultures of the biosensors *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12). These biosensors synthesize GFP in response to short- and long-AHLs, respectively. The measurements of RFU after 6 h of induction were normalized to the OD₆₀₀ of the corresponding *G. diazotrophicus* culture. As shown in Fig. 3, the relative level of GFP induction determined with supernatants withdrawn from DYGS medium was between 3.5 and 4 times higher than that observed for samples from LGIP. Similar results were found with both *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12) biosensors. When cell densities attained in each culture medium by *G. diazotrophicus* PAL5 were considered, no significant differences could be determined in samples from DYGS and LGIP media analyzed with *P. putida* F117 (pAS-C8). However, a decrease of about 30% in the specific fluorescence measured with *P. putida* F117 (pKR-C12) was observed in supernatants obtained from LGIP synthetic medium, revealing that the extracellular concentration of long-chain AHLs could be affected under this culture condition. The influence of alkaline conditions as an abiotic factor on the deactivation of quorum sensing molecules has largely been studied (Yates et al. 2002; Byers et al. 2002). The relative diminution in the concentration of autoinducers in mineral medium could not be attributed to lactonolysis produced by alkalization, since the pH at the onset of stationary phase was below 4.00 (data not shown).

Discussion

The obligate endophyte *G. diazotrophicus* provides significant quantities of nitrogen to sugarcane (*Saccharum officinarum*) through biological nitrogen fixation. In addition, other biochemical features, such as the production of phytohormones, and the solubilization of nutrients highlight the strong interactions of this bacterium with the host plant. Although QS has been largely studied in the last years as a key mechanism in the regulation of the microbial interactions with the environment, its description has not been attained in *G. diazotrophicus*. As mentioned above, the genome sequencing of *G. diazotrophicus* PAL5 revealed a complete quorum sensing system in this strain, composed of one LuxI- and one LuxR-homolog encoded in GDI2836 and GDI2838, respectively. GDI2837 encodes a putative LuxR-related protein that lacks an AHL-binding domain.

The low G+C content of GDI2836 (49.2%), GDI2837 (50.4%) and GDI2838 (49.7%), compared to the average content of the *G. diazotrophicus* PAL5 genomic sequence (66.19%), suggests that this regulatory mechanism was

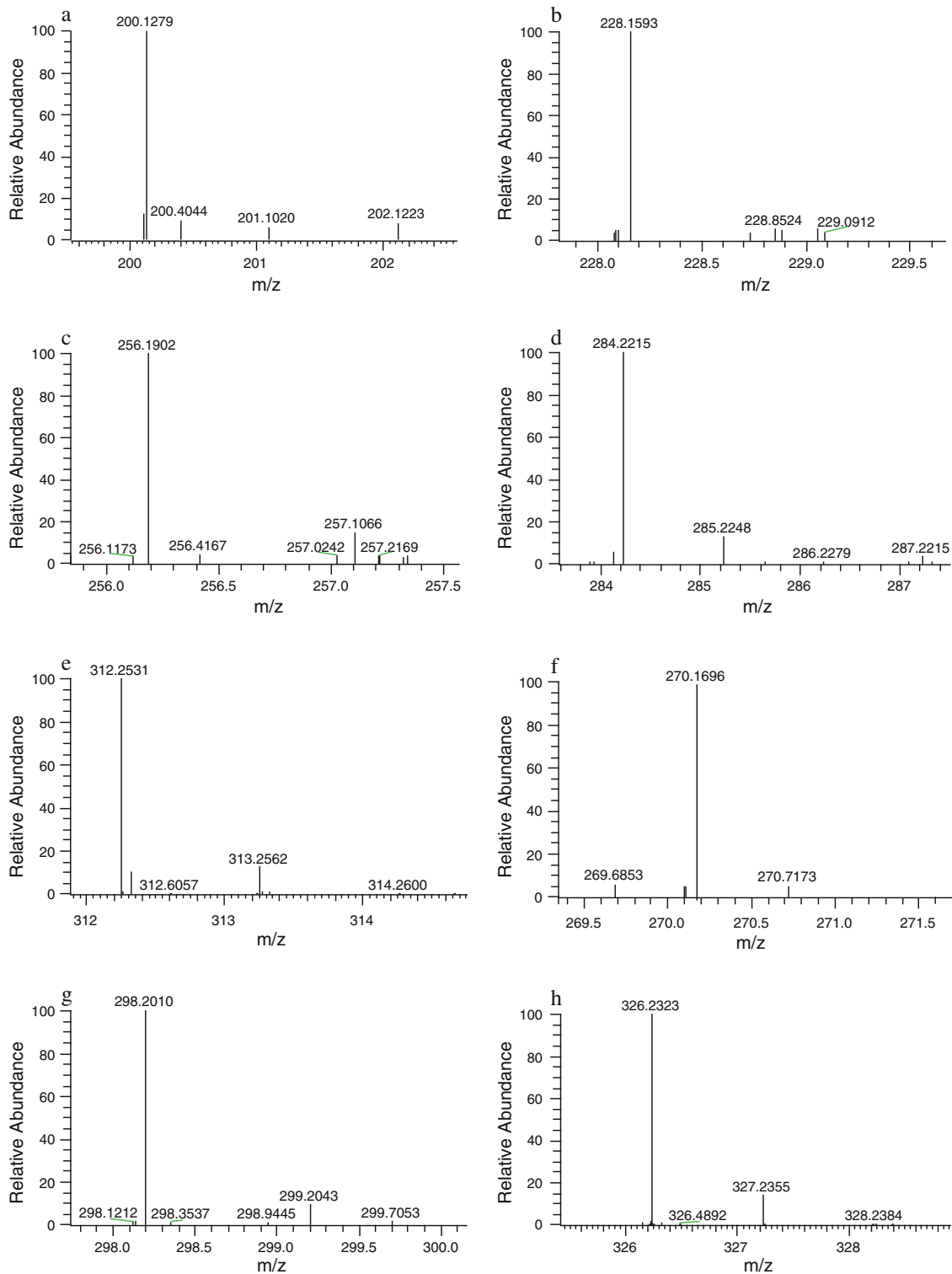


Fig. 2 Mass spectra of AHLs identified in DYGS cultures. *G. diazotrophicus* PAL5 was incubated in DYGS medium, and organic concentrated extracts were analyzed by LC-APCI-MS. Eight AHLs

were identified. **a** C6-HSL. **b** C8-HSL. **c** C10-HSL. **d** C12-HSL. **e** C14-HSL. **f** 3-oxo-C10-HSL. **g** 3-oxo-C12-HSL. **h** 3-oxo-C14-HSL. Similar results were obtained in LGIP cultures (data not shown)

acquired through horizontal gene transfer. The report presented by Bertalan et al. suggests that the QS system of *G. diazotrophicus* PAL5 would be part of a genome island.

The genetic organization around the putative *luxI* homolog also supports this hypothesis. The stop codon of *luxI* gene is 2,516 bp downstream the stop codon of GDI2833, which

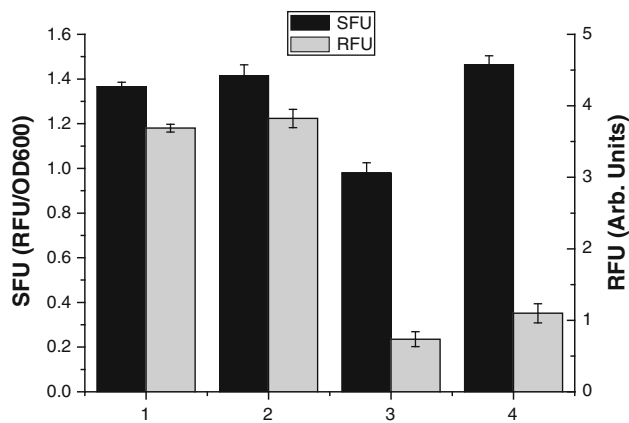


Fig. 3 Relative quantification of AHLs. Spent supernatants from DYGS and LGIP media were utilized to supplement cultures of *P. putida* F117 (pAS-C8) and *P. putida* F117 (pKR-C12). After 6 h, relative fluorescence units (RFU) were measured as indicated in “Materials and methods”. SFU refers to RFU normalized to the corresponding OD₆₀₀ of *G. diazotrophicus* PAL5. 1 DYGS supernatant analyzed with *P. putida* F117 (pKC-C12). 2 DYGS supernatant analyzed with *P. putida* F117 (pAS-C8). 3 LGIP supernatant analyzed with *P. putida* F117 (pKC-C12). 4 LGIP supernatant analyzed with *P. putida* F117 (pAS-C8)

codes for a putative nonribosomal peptide synthase. As stated elsewhere (Bertalan et al. 2009), GDI2833 seems to be part of a genomic island that is absent in almost all the *G. diazotrophicus* strains analyzed. According to the report presented by Bertalan et al., in addition to PAL5, only *G. diazotrophicus* PBD4 isolated from sweet potato from Brazil, harbors the GDI2833. Considering that the *luxI* homolog and the two *luxR* homologs are also part of this genomic element, these findings suggest that quorum sensing systems based on AHL production are not widely distributed in *G. diazotrophicus* strains, independently of the origin of the isolates. It is interesting to contrast these findings with *Azospirillum*, a genus that possesses several features in common with *G. diazotrophicus*. Both are plant growth-promoting bacteria that fix nitrogen under non-symbiotic conditions. In addition, these Alphaproteobacteria can live in the inner tissues of several plants. Recently, Vial and collaborators presented evidence that these signaling systems are only present in *A. lipoferum* TVV3 and *A. lipoferum* B518, and absent in several strains of *A. brasilense*, *A. amazonense* and *A. irakense*, among others (Vial et al. 2006). Noteworthy, the quorum sensing system of *A. lipoferum* TVV3, which also seems to be acquired by horizontal gene transfer, only has a minor effect on the physiology of this bacterium (Boyer et al. 2008). To this day, AHL production has not been analyzed in any other *G. diazotrophicus* strain. However, it cannot be ruled out that quorum sensing systems based on the production of different molecules are present in other strains of *G. diazotrophicus*. Recently, Iida et al.

characterized the quorum sensing system of *G. intermedium* NCI1051 and showed that GinI, a LuxI homolog in this strain, is responsible for the biosynthesis of C10-HSL, C12-HSL and C12:1-HSL (Iida et al. 2008). Although *G. diazotrophicus* PAL5 and *G. intermedium* NCI1051 synthesize long-chain AHLs, GinI and GDI2836 only share 38% of similarity at the amino acid level.

The two different biosensor strains utilized [i.e., *A. tumefaciens* NTL4 (pCF218 pCF372) and *P. putida* F117 (pKR-C12)], in combination with high-resolution LC-APCI-MS allowed the identification of eight different AHLs synthesized by *G. diazotrophicus* PAL5. C6-HSL and C8-HSL have also been isolated from cultures of diverse microorganisms including plant-associated bacteria such as *P. aureofaciens* and *Pantoea ananatis* (Wood and Pierson 1996; Morohoshi et al. 2007). Long-chain AHLs are usually synthesized by Alphaproteobacteria. For instance, *Rhodobacter capsulatus* was found to produce C16-HSL (Puskas et al. 1997); marine Alphaproteobacteria biosynthesize the longer AHLs discovered to this day, including unsaturated C18-HSLs (Wagner-Döbler et al. 2005). Among the long-chain autoinducers synthesized by *G. diazotrophicus* PAL5, 3-oxo-C10-HSL and 3-oxo-C12-HSL are also produced by the biocontrol *P. putida* IsoF (Steidle et al. 2002). It is interesting to note that the legume *Medicago truncatula* responds to long-chain autoinducers identical to some of those produced by *G. diazotrophicus* PAL5 (Mathesius et al. 2003). It would be interesting to address whether sugarcane or other hosts also respond to AHLs synthesized by *G. diazotrophicus*.

Several reports presented evidence that one LuxI-type enzyme can be responsible for the biosynthesis of several AHL molecules. The genome sequence of *G. diazotrophicus* PAL5 presents only one gene, GDI2836, which codes for a LuxI-like enzyme (Bertalan et al. 2009); there is no evidence for the presence of genes with homology to *ainS* or *luxM*, which encode autoinducer synthesis proteins that do not belong to the LuxI-type family of AHL synthases (Laue et al. 2000). Therefore, it is plausible to assume that the enzyme encoded in GDI2836 is responsible for the biosynthesis of the eight AHLs produced by this strain. Interestingly, the utilization of highly sensitive analytical techniques permitted Ortori et al. (2007) the identification of more than twenty different AHLs produced by *Yersinia pseudotuberculosis*. At present, the reason for the production of several signaling molecules by the same strains remains unclear. It is improbable that each LuxR homolog (GDI2837 and GDI2838) binds a different set of signal molecules, since GDI2837 lacks the AHL binding domain characteristic of these regulatory proteins (Zhang et al. 2002). Decho et al. (2010) have recently suggested that the variability in the physicochemical properties (i.e., solubility, diffusivity, resistance to hydrolysis) among short- and long-chain AHLs could restrict the

latter for the communication over a short range of distance between two cells; the former would be better suited for longer distances.

Culture conditions have strong influences on AHLs produced by diverse bacteria. Supplementation of the culture media with different carbon sources or salts modifies the extracellular concentration of C6-, C8- and C10-HSL when *Burkholderia* sp. O33 is cultured in nutrient broth (Keum et al. 2009). Biosynthesis of 3-oxo-C6-HSL by *Erwinia carotovora* ssp. *carotovora* is maximal when the strain is grown in sucrose supplemented medium and is minimal in glycerol supplemented medium (McGowan et al. 2005). In contrast, the specific level of long-chain signal molecules produced by *G. diazotrophicus* PAL5 was not affected by the two culture conditions assayed. Although the production of long-chain AHLs was significantly increased in complex medium, the more elevated intensity of GFP induction in *P. putida* F117 (pKR-C12) bioassay could only be attributed to the higher biomass yields attained under that conditions by *G. diazotrophicus* PAL5. In contrast, short-chain AHLs were 30% reduced in spent mineral medium. The cultivation of *Rhizobium leguminosarum* in mineral medium also produced a decrease in the production of AHLs with short acyl chain (Lithgow et al. 2001). Bazire et al. (2005) evidenced that decrease in phosphate concentration affects negatively the biosynthesis of 3-oxo-C12-HSL and C4-HSL by *P. aeruginosa*. It is worthy of note the differences in phosphate concentration between DYGS and LGIP media, which are 27 and 5 mM, respectively. It is plausible that the former group of cell–cell signaling molecules in this diazotrophic bacterium is less dependent on the concentration of this macronutrient than the latter group. The influence of alkaline conditions as an abiotic factor on quorum sensing molecules has largely been studied (Byers et al. 2002). Under this condition, AHLs are reversibly deactivated being the short molecules more susceptible. Nevertheless, pH levels under both culture conditions at the onset of stationary phase were below 4.00, as mentioned above. Substrate availability or enzyme affinity could be modified when changes in environmental compositions are detected by this strain. Further experiments are required to validate this hypothesis, considering that only one LuxI homolog should be responsible for the biosynthesis of all AHLs produced by *G. diazotrophicus* PAL5. To the best of our knowledge, this is the first report describing the signaling system of the plant growth-promoting *G. diazotrophicus* PAL5. Molecular and physiological characterization of this regulatory mechanism will reveal its importance for the microbe–microbe and microbe–plant interactions.

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