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Laherradurin, a natural stressor, stimulates QS mechanism involved in biofilm formation of a PAHs degrading bacterium

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ABSTRACT

Laherradurin is an annonaceous acetogenin (ACG) extracted from the seeds of *Annona cherimolia* (Annonaceae). It has been shown to increase biofilm production of *Pseudomonas plecoglossicida* J26, a polycyclic aromatic hydrocarbon degrading bacterium. It is not an autoinducer agonist, and is indirectly involved in quorum sensing mechanism by inducing a stress related increase in autoinducer production, which in turn results in more biofilm formation without any significant detriment on bacterial growth rates.

The results of the analysis of cells and supernatants obtained from J26 cell cultures by chromatographic techniques (TLC revealed with the Kedde reagent and RP-HPLC) and mass spectrometry (EI-MS) allowed us to determine that the ACG laherradurin and squamocin are located in the bacterial pellet, as was expected given their theoretical log *p* values. Thus, results suggest that these ACG act as bacterial membrane natural stressors.

1. Introduction

Annonaceous acetogenins (ACG) are secondary metabolites with a widely reported biological activity (Londershausen et al., 1991). These molecules are exclusively isolated from plants of the Annonaceae family (Makabe et al., 2008). They present a long alkyl chain whose terminal end usually exhibits a γ-methyl-α,β-unsaturated-γ-lactone. Attached in position 2 of the lactone there is a hydrocarbon chain that frequently has one or more tetrahydrofuranic rings (Bermejo et al., 2005). These compounds represent a class of bioactive compounds whose cytotoxicity seems to be related to the conformation adopted in the membrane revealed by the intermolecular interactions between the headgroups of membrane (specifically phosphate groups) and the hydroxylated-THF of ACG (Shimada et al., 1998; Di Toto Blessing et al., 2010). In addition, the hydrocarbons tend to reside within the hydrophobic region between membrane monolayers composed by the acyl chains of phospholipids (Van Hamme et al., 2003). Bacteria in the environment, are often exposed to several stressors, that may trigger adaptive responses. So, many bacteria, in response to this situation, form biofilm, and would express a phenotype of general response to stress.

Previous investigations conducted in our laboratory showed that *Pseudomonas plecoglossicida* J26 (Parellada et al., 2012) increased its biofilm formation when the β-hydroxylated lactone, laherradurin was added to the culture medium. This result was consistent with the hypothesis that laherradurin might act as an autoinducer or quorum-sensing signaling molecule affecting the expression of genes involved in biofilm formation. To test this hypothesis we analyzed the effects of laherradurin on the expression of genes in reporters strains.

Biofilms are surface associated communities whose cells are embedded in an extracellular matrix that they themselves produce and that holds them together. They exhibit an altered phenotype with respect to their planktonic form (Rodney et al., 2002), which provides them with a better chance of adaptation and survival especially during periods of stress. The fact that bacteria are better able to survive environmental insults when growing in biofilm highlights the advantage of co-operation and a multicellular lifestyle (Stanley and Lazazzera, 2004). This is the reason for which biofilm-mediated bioremediation is considered to be safer and more efficient than that produced by planktonic organisms (Decho, 2000). From this we can deduce that stimulating biofilm formation may represent an important goal (Parellada et al., 2012).

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Since polycyclic aromatic hydrocarbons (PAHs) can come from many sources, they are ubiquitous in the environment (Finlayson-Fitts and Pitts, 1997; Norse, 2005; Johnsen et al., 2005). As a result, many organizations have considered them priority pollutants for bioremediation (Liu et al., 2001) and their microbial degradation has become crucial (Meckenstock et al., 2000; Johnsen et al., 2005). Naphthalene serves as a model for understanding the properties of a wide range of environmentally relevant PAHs (Johnsen et al., 2005).

A variety of bacteria employs quorum sensing (QS) to coordinate communal behavior. QS consists in the regulation and coordinated expression of genes in response to cell density (González and Keshavan, 2006). The process of biofilm formation in diverse bacteria has been shown to specifically involve the recognition of and response to self-generated secreted small molecules called type 1 autoinducers (AI-1). In Gram negative bacteria they are *N*-acyl-homoserine lactones (AHLs) (Bassler and Losick, 2006). AHLs play an important role in the regulation of biofilm formation in some bacteria (Waters and Bassler, 2005). Like many other ACG, laherradurin (Fig. 1a) and AHLs (Fig. 1b) structurally share the γ-lactone moiety.

Strain J26 was employed for the present study. It was isolated from intertidal sediments of the southern coasts of Argentina by selective enrichment with naphthalene as its sole carbon and energy source. Its naphthalene degrading capacity was demonstrated by other authors and was identified as *P. plecoglossicida* (Riva Mercadal et al., 2010). Previously described by Nishimori, it has a respiratory but not a fermentative metabolism (Nishimori et al., 2000) and exhibits biofilm formation capacity (Li et al., 2009). *P. plecoglossicida* J26 regulates its biofilm production by an AHL-mediated QS mechanism (Parellada et al., 2011).

The aim of this work was determine the mode of action of the annonaceous acetogenin laherradurin as a stimulant the biofilm formation in comparison with results previously reported for squamocin. *P. plecoglossicida* J26 cell growth was evaluated by kinetic studies in presence of laherradurin or squamocin.

In addition, we investigated if laherradurin, a γ-lactone, could be recognized by the reporter strain like an AHLs analog.

2. Materials and methods

2.1. Isolation and purification of laherradurin and squamocin

2.1.1. Plant material

Annona cherimolia seeds from "cherimoya" fruits were collected from Tucumán, Argentina. A voucher specimen is deposited at the Miguel Lillo Foundation herbarium, Tucumán, Argentina, under the reference LIL 515092.

2.1.2. Extraction

The dried and powdered seeds of *A. cherimolia* (1000 g) were macerated with methanol. The methanolic extract was evaporated and the residue partitioned in a mixture of chloroform–H_2O (50:50). Chloroformic and aqueous subextracts were obtained by vacuum evaporation. The chloroformic subextract was partitioned with a mixture of hexane–methanol (50:50). The methanolic subextract contained squamocin, laherradurin and many other ACG.

2.1.3. Purification and characterization

The methanolic subextract was subjected to column chromatography. Chromatographic conditions used were the same as presented earlier in Parellada et al. (2012). Column was eluted with a gradient of chloroform–ethyl acetate–methanol and fractions were collected and pooled according to their similar TLC patterns. Plaques were revealed with Kedde reagent and fuchsia stained spots were observed in positive cases. Laherradurin was eluted with AcOEt:MeOH (80:20) and squamocin was eluted with AcOEt:MeOH (97:3). Both were exhaustively purified by reverse phase high performance liquid chromatography (RP-HPLC) with RI detector. Characterization of squamocin and laherradurin was assessed by spectroscopic techniques (IR, ^{1}H NMR, ^{13}C NMR), and MS as well as α_D determination, in comparison with previously reported data. Laherradurin was isolated as a white powder EI-MS (70 eV): m/z = 625 $[MH]^+$, 607 $[MH - H_2O]^+$, 571 $[MH - 3H_2O]^+$, 553 $[MH - 4H_2O]^+$, 435, 417, 399, 365, 347, 313, 295, 267, 241, 223; HPLC fractions yielded squamocin in the form of a viscous oil, EI-MS (70 eV): m/z = 623 $[MH]^+$, 605 $[MH - H_2O]^+$, 587 $[MH - 2H_2O]^+$, 569 $[MH - 3H_2O]^+$, 519, 399, 365, 347, 329, 317, 295, 267. All spectral information confirms the identity and purity of the isolated ACG and shows excellent correlation with data previously obtained by Kawasu et al. (1989) for squamocin and Warmerdam et al. (1998) for laherradurin.

2.1.4. ACG solutions

Several ACG solutions in aqueous dimethyl sulfoxide 2.75%, with concentrations ranging between 2.5 and 100 μg ml^{-1} were prepared to be used in reporter strain assays. Also, standard solutions of laherradurin and squamocin were prepared for the determination of retention times by HPLC analysis (MeOH:H_2O, 90:10 and 80:20, respectively).

2.2. Bacterial strains

2.2.1. P. plecoglossicida J26

P. plecoglossicida J26 was selected from 15-day enrichment cultures of marine sediments of Patagonia, Argentina in a minimal

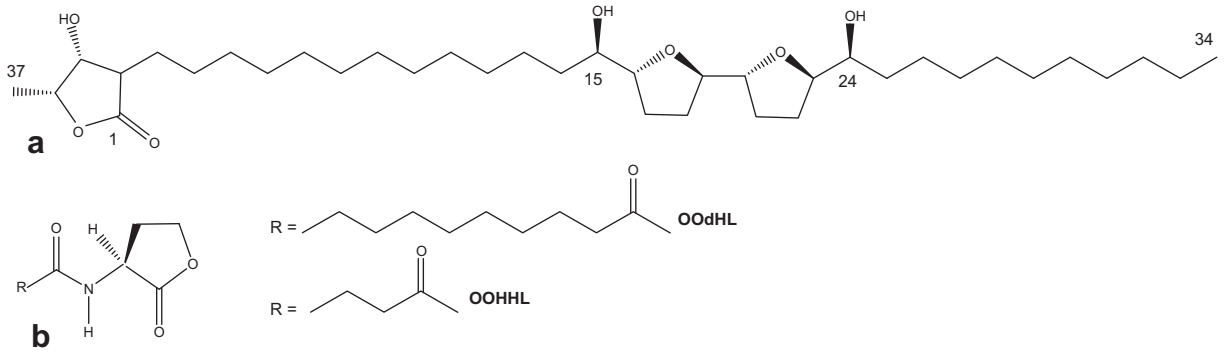


Fig. 1. a-Structure of laherradurin (β-hydroxy-γ-lactone acetogenin); b-Structure of AHL or autoinducers type 1.

seawater medium with naphthalene crystals as the sole carbon and energy source. Riva Mercadal et al. (2010) demonstrated that planktonic cultures of *P. plecoglossicida* J26 were able to degrade naphthalene (in methanol) supplemented in the culture medium. They identified J26 strain as *Pseudomonas* sp., closely related to *P. plecoglossicida*. The partial 16S rRNA gene sequence (99.7% identity with *P. plecoglossicida*) was deposited in a GenBank database under the accession number FR668235. The strain was called *P. plecoglossicida* J26 (Riva Mercadal et al., 2010). Recently, Parellada et al. (2011) have shown that biofilm formation of *P. plecoglossicida* J26 is regulated by an AHL mediated quorum sensing process. The biochemical characterization of the species was reported by Nishimori et al. (2000).

2.2.1.1. Preparation of P. plecoglossicida J26 inocula. *P. plecoglossicida* J26 was maintained at 30 °C in Luria–Bertani (LB) agar medium (Gibco, Rockville, MD, USA) in presence of naphthalene crystals. The strain was kept in contact with naphthalene vapors to promote a bacterial resistance phenotype. Selected colonies were used to inoculate LB broth and incubated overnight.

2.2.2. Reporter strains

In this study we used three quorum sensing reporter strains. Their use allowed us to determine the presence of AHL in the supernatants and also to quantify their activity.

These strains have been constructed specifically to detect and respond to AI-1.

Pseudomonas aeruginosa qsc mutant (qsc129b) strain was obtained by Whiteley et al. (1999), and generously donated by E.P. Greenberg and K. Lee, University of Iowa, USA. This strain has a transcriptional fusion of lacZ gene in the chromosome that produces a double mutant *las*I-*rhl*I called qsc129b. Hence, it does not produce AHLs but responds to them through the production of the β-galactosidase enzyme. As the strain was grown in restrictive conditions, the LB medium was supplemented with gentamicin (100 μg ml^{-1}, Sigma). A culture without antibiotic was used for the Miller reaction (O'Toole and Kolter, 1998; Whiteley et al., 1999).

CV026 is a mini-Tn5 mutant of *Chromobacterium violaceum* ATCC 31532 (Throup et al., 1995; Winson et al., 1995; Latifi et al., 1996) with no AHLs production. The strain recognizes short-chain AHLs (4, 6 and 8 carbons) with or without substituent in C3 (McLean et al., 1997). As it was grown in restrictive conditions, the LB medium was supplemented with kanamycin (50 μg ml^{-1}, Sigma). CV026 responds to AHLs by producing the purple pigment violacein.

VIR07 is a mutant derived from *C. violaceum* ATCC 12472 with no AHLs production by means of a deletion in the *cvi*I gene for the insertion of a kanamycin resistance cassette (*cvi*I::*Km*r). The strain recognizes long-chain AHLs (10, 12, 14 and 16 carbons) with or without substituent in C3 (Morohoshi et al., 2008). As it was grown in restrictive conditions, the LB medium was supplemented with kanamycin (50 μg ml^{-1}, Sigma). VIR07 responds to AHLs by producing violacein.

2.3. Bioassays

Different cultures in presence of laherradurin were prepared and the supernatants of these cultures were used to stimulate biofilm formation and to quantify AI-1 production. The AI-1 in the supernatant of J26 cells were quantified using *P. aeruginosa* qsc129b reporter strain.

2.3.1. Growth under stress conditions

Since it has been reported that laherradurin and squamocin at a concentration of 2.5 μg ml^{-1} are stimulants of naphthalene consumption of J26 strain (Parellada et al., 2012), a kinetic study was conducted to determine the manner in which these ACG affect bacterial growth during a 24 h (28 °C) incubation period. For this study 175 μl of LB medium were inoculated with 20 μl (10%) of an overnight culture of *P. plecoglossicida* J26 (see 2.2.1. Preparation of *P. plecoglossicida* J26 inocula) and supplemented with 5 μl of a 100 μg ml^{-1}ACG ethanolic solution (final concentration 2.5 μg ml^{-1}) in a 96 wells microtiter plate. Then OD 600 nm was measured at regular intervals in a spectrophotometer (Biotek- Power Wave XS2 with GEN5 data analysis software). Assays were conducted by octuplicate with EtOH controls.

2.3.2. Supernatants and bacterial pellets

In order to obtain supernatants of J26 cell cultures grown in presence of laherradurin, a set of 10 ml of LB medium tubes were inoculated with *P. plecoglossicida* J26 (see 2.2.1. Preparation of *P. plecoglossicida* J26 inocula) and supplemented with 2.5, 25, 50 and 100 μg ml^{-1} of laherradurin (lh, ACG stress). Tubes were incubated for 22 h at 30 °C. A culture of J26 cells grown in the absence of any stress was prepared to be used as the control supernatant. The experiments were performed in triplicate. The supernatants from these cultures were obtained by centrifugation at 10,000 rpm for 15 min at 25 °C and then sterilized with 0.22 μm Millipore filters. Additional supernatants and bacterial pellets were prepared for TLC, RP-HPLC and EI-MS analysis with ACG at 2.5 μg ml^{-1}.

2.3.3. AI-1 production

The biological activity of AI-1 present in the supernatant was quantified using the reporter strain *P. aeruginosa* qsc129b.

To address this question, we mixed equal parts (0.5 ml) of the supernatant and *P. aeruginosa* qsc129b overnight culture in several test tubes. Induction time was 1 h at 37 °C. During this period, the reporter strain recognized AHL from the samples and responded with β-galactosidase formation. Its activity was measured by Miller reaction (O'Toole and Kolter, 1998; Whiteley et al., 1999).

2.3.4. C. violaceum bioassays

Knowing the structural similarity between laherradurin and AHLs, we determined whether they had similar biological behaviors with reporter strains *C. violaceum* VIR07 and CV026. Aforementioned mutant strains were inoculated in LB broth and incubated 6 h at 30 °C with stirring. Next, 10 ml of the referred cultures were mixed with an equal volume of molten LB agar (1.5% agar) at 40 °C and allowed to solidify. Subsequently, sterile Whatman filter paper discs (diameter: 5 mm) were disposed on the surface of the medium. Then, the discs were embedded with 10 μl of a laherradurin solution.

The negative control was an aqueous solution of 2.75% dimethyl sulfoxide and the positive control were extracts from supernatants of *P. aeruginosa* containing C4-AHL and 3-oxo-C12-AHL. Petri dishes were incubated at 30 °C for 18 h in a moist chamber. Purple halos were observed in positive samples.

2.3.5. Biofilm assay

P. plecoglossicida J26 biofilm production was quantified in the presence of a supernatant of the same strain (See 2.3.2. Supernatants and bacterial pellets). The biofilm quantification assay is based on the ability of bacteria to form biofilms on polystyrene. The technique requires the addition of violet crystal which stains the cells but not the polystyrene (O'Toole and Kolter, 1998).

The objective was to establish a relationship between AI-1 production in the supernatants of the culture strain in presence of laherradurin and the stimulation of biofilm production. A supernatant of J26 cells grown in the absence of any stressor was

required as control. A volume of 170 μl of LB broth and 10 μl of each stressed culture supernatant or control supernatant were placed in each well of polystyrene microtiter plate. Then, each well was inoculated with 20 μl of an overnight (OD_{600nm} = 1.0) *P. plecoglossicida* culture in LB (see 2.2.1. Preparation of *P. plecoglossicida* J26 inocula). The microplates were incubated at 30 °C for 6 h in a moist chamber. After this time period, 25 μl of violet crystal (1%) were added to the wells, incubated 15 min at room temperature and then rinsed thoroughly and repeatedly with water. Biomass-attached dye was solubilized with ethanol 95% (v:v); absorbance was then measured at 560 nm in a microplate spectrophotometer (Biotek-Power Wave XS2 with GEN5 data analysis software).

2.4. Thin layer chromatography, liquid chromatography and mass spectrometry

Both cells and supernatants from cultures grown in presence of laherradurin and squamocin were analyzed by chromatographic techniques to investigate the presence of the ACG. Purified compounds obtained from cells (bacterial pellets) and supernatants extracts were identified by EI-MS.

2.4.1. TLC

The absence of ACG in the supernatants of J26 cell cultures grown in their presence would suggest that these lipophilic molecules have been retained within bacterial membranes or other lipophilic cell materials.

To explore this possibility, TLC were performed with a naphthalene solution as negative control, and squamocin and laherradurin standards as positive controls. The CH_2Cl_2 extract from a culture supernatant grown in absence (control) and presence of laherradurin or squamocin were obtained by partitioning 200 ml of each culture supernatant in 100 ml of CH_2Cl_2 successively, followed by drying with anhydrous Na_2SO_4 and vacuum evaporation of the organic phases. Concentrated portions were used for TLC analysis.

In addition, bacterial pellets were obtained by centrifugation (See 2.3.2. Supernatants and bacterial pellets) and then washed with small volumes of distilled water and finally transferred to a mortar. The pellets were repeatedly frozen with liquid nitrogen and crushed by mechanical action. This process was repeated until obtaining a powder that was transferred to a vial and subjected to disruption of bacterium by exposure to high-frequency sound waves (30 min) using CH_2Cl_2 as a partition solvent. The aim of this process was to transfer the ACG into the dichloromethanic phase for further analysis by TLC. Analytical TLC was performed on Merck precoated silica gel 60 F_{254} plates using hexane:ethyl acetate (50:50). The plates were revealed with Kedde reagent (dinitrobenzoic acid 10% in MeOH, and KOH 2 N in MeOH) selective for ACG (pink–magenta color).

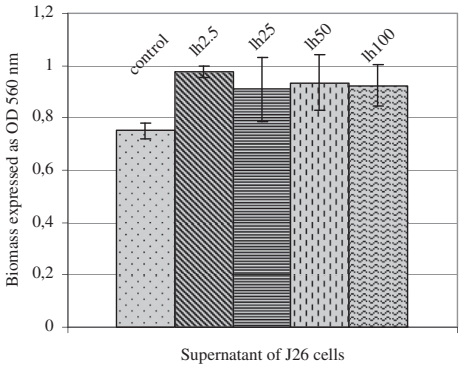


Fig. 3. Biofilm formation expressed as $OD_{560\ nm}$ in presence of a supernatant of J26 cells grown in presence of laherradurin (lh). Numbers are referred to concentrations expressed in μg ml^{-1}.

2.4.2. RP-HPLC

Extracts from supernatants and bacterial pellets were obtained by vacuum evaporation, redissolved in 2 ml of MeOH:H_2O (90:10) and filtered through 0.22 μm filters before HPLC analysis. Semi-preparatives HPLC of ACG standard (lh and sq) were carried out on a Phenomenex RP-C18 column (25 × 1 cm i.d., 5 μm particle size), flow rate 2.0 using MeOH/H_2O (90:10) for laherradurin and 1.8 ml min^{-1} using MeOH/H_2O (80:20) for squamocin. Retention time values and positive reactions with Kedde reagent of collected peaks were applied as preliminary identification criteria.

2.4.3. EI-MS

HPLC peaks were concentrated and re-dissolved in CH_2Cl_2 to determine their fragmentation patterns at 70 eV by direct introduction into a low resolution mass spectrometer (EIMS Polaris Q (Thermo Electron); gas carrier He; ion trap detector; ion source temperature 200 °C; full scan from 50 to 700 uma). Identification of ACG, laherradurin and squamocin was achieved by comparison with the fragmentation patterns of their standards.

2.5. Statistical analysis

Differences in the mean values were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pair wise multiple comparisons of groups. In all analyses, values of $p < 0.05$ were considered statistically different (Statistix 7.1, 2002).

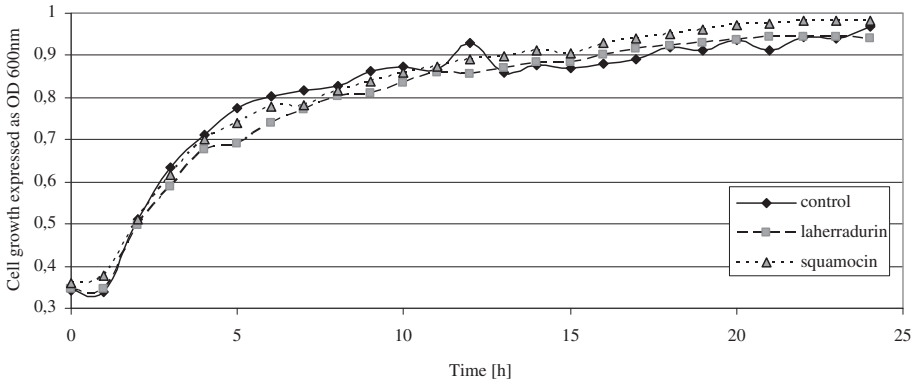


Fig. 2. *Pseudomonas plecoglossicida* J26 growth expressed as OD 600 nm over time [h]. ACG concentrations were of 2.5 μg ml^{-1} and were supplemented to the culture medium in an ethanol solution.

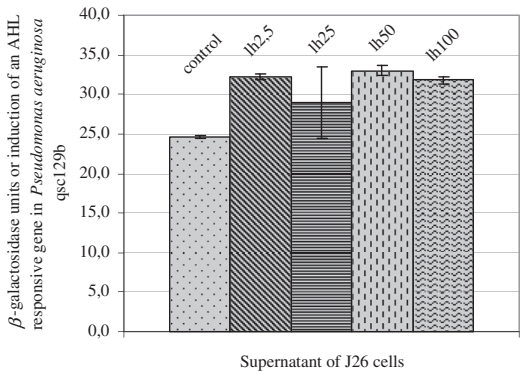


Fig. 4. β-Galactosidase units ± SD or induction of an AI-1 responsive gene in *Pseudomonas aeruginosa* qsc129 in presence of a supernatant of J26 cells grown in presence of laherradurin (lh). Numbers represent concentrations expressed in μg ml^{-1}.

3. Results and discussion

3.1. Effect of laherradurin on P. plecoglossicida J26 growth and biofilm formation

Parellada et al. (2012) demonstrated that squamocin and laherradurin (2.5 μg ml^{-1}) stimulated *P. plecoglossicida* J26 biofilm formation. That fact, led to increased consumption of naphthalene. Fig. 2, shows the growth kinetics of *P. plecoglossicida* J26 in presence of laherradurin and squamocin (2.5 μg ml^{-1}). After 24 h of incubation bacterial growth was not affected.

Supernatants of cultures grown in presence of laherradurin at different concentrations significantly stimulated biofilm formation ($p < 0.05$). The highest stimulation (130%) was achieved at 6 h of incubation for the lowest concentration ($OD_{560} = 0.976 \pm 0.022$) (Fig. 3). The effect of these supernatants was compared with that of another supernatant from J26 cells grown in the absence of any stress (100%, $OD_{560} = 0.751 \pm 0.031$).

The biofilm formation of *P. plecoglossicida* J26 was stimulated in presence of laherradurin in agreement with the results obtained by Parellada et al. (2011, 2012), for squamocin at 2.5 μg ml^{-1}.

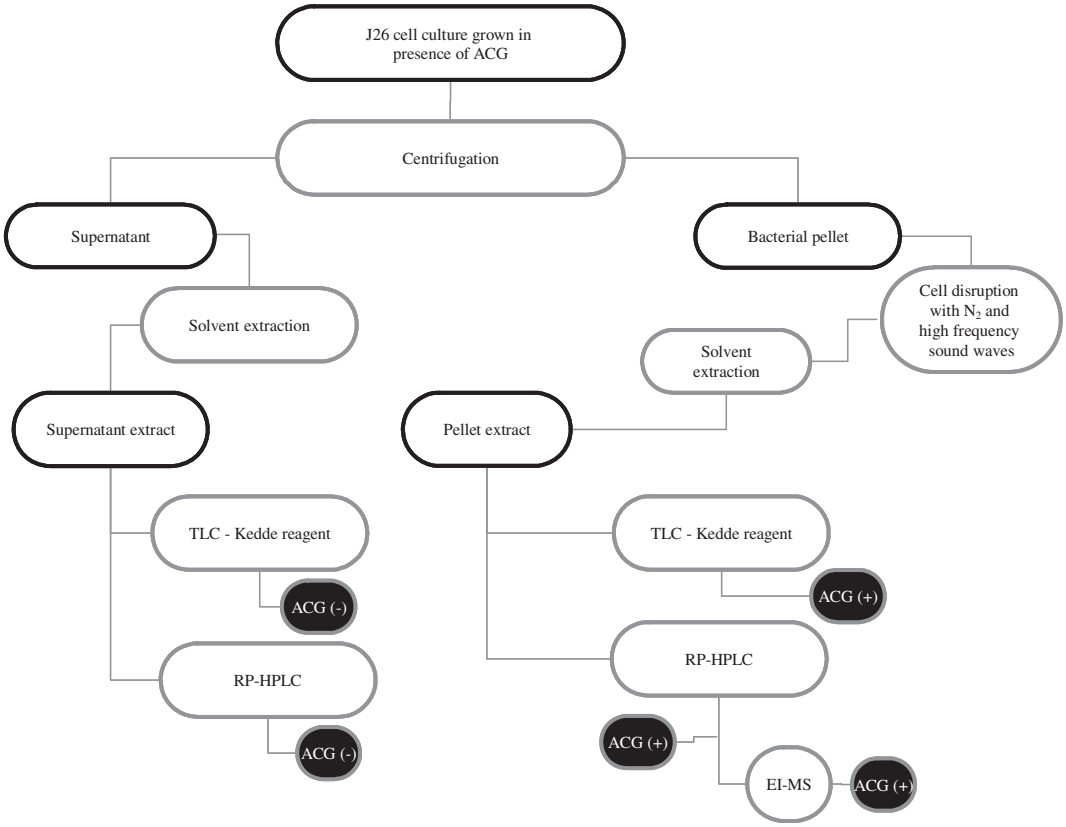
3.2. Effect of laherradurin on AI-1 production

Fig. 4 shows the activity of AI-1 (AHL) present in the supernatants, represented as β-galactosidase units. β-galactosidase activity in all supernatants was higher than in control (supernatant of J26 cells grown in the absence of any stress) ($p < 0.05$). Similar results were obtained in a previous report for squamocin (Parellada et al., 2011).

It is important to note that increments on laherradurin concentration did not result in a greater induction of the β-galactosidase activity. Laherradurin (2.5 μg ml^{-1}) increased autoinducers production of the strain by 30% (β-galactosidase units = 32.3 ± 0.3), compared with a supernatant of J26 cells grown in the absence of any stressor (β-galactosidase units = 24.7 ± 0.2). Previous reports indicated that squamocin 2.5 μg ml^{-1} stimulated production of AI-1 by 56%, in the same conditions (Parellada et al., 2011). These results show that the ACG laherradurin and squamocin, differentially, stimulate the production of AI.

3.3. Reporter strains bioassays with laherradurin

Given the structural similarity between ACG and AHLs, that structurally share the γ-lactone moiety (Fig. 1a, b), it was first



Scheme 1. Chromatographic and spectroscopic methods employed. Results summary. (+) Involves the formation of a pink–magenta compound by reaction with Kedde reagent, coincidence or similarity in retention times during a chromatographic run or excellent correlation between fragmentation patterns.

Table 1
HPLC studies results. The first two columns are referred to the composition of the mobile phase used; st is referred to a standard; sup: extract from a cell culture supernatant grown in presence of lh: laherradurin, sq: squamocin or c: in the absence of any stressor.

Experimental conditions			Retention time [min.]							
MeOH	H_2O	Flow rate [ml min^{-1}]	$pellet_c$	$pellet_{lh}$	lh_{st}	$pellet_{sq}$	sq_{st}	sup_c	sup_{lh}	sup_{sq}
90	10	2.0	–	50	46	–	–	–	–	–
80	20	1.8	–	–	–	24	22	–	–	–

thought that they might act as AI-1 agonists. This approach to the problem was explored by assays with three different reporter strains: *C. violaceum* VIR 07 and CV026 and *P. aeruginosa* qsc129b. Laherradurin did not stimulate the production of β-galactosidase or violacein by any of the reporter strains. Despite structural similarities, laherradurin is not an autoinducer agonist. It indirectly stimulates autoinducer production and increases *P. plecoglossicida* J26 cell growth. The same results were obtained previously with squamocin (Parellada et al., 2011).

3.4. Chromatographic and spectrometric assays

3.4.1. Analysis of supernatants

TLC with Kedde reagent did not reveal the presence of any of the ACG in extracts of supernatants (Scheme 1). The RP-HPLC analysis showed no peak with retention time similar to any of the ACG standards (Table 1).

The absence of ACG in the supernatants of *P. plecoglossicida* J26 cell cultures grown in their presence might indicate that these lipophilic molecules have remained associated with bacterial membranes or other cell materials.

3.4.2. Analysis of bacterial pellets

Positive reactions with Kedde reagent revealed the presence of the ACG in the pellet extracts (Scheme 1). The RP-HPLC analysis showed a peak with retention time (50 min) similar to laherradurin standard (46 min) as shown in Table 1. The presence of squamocin in the pellet extract was determinated by the same methodology. The retention time (24 min) was similar to squamocin standard (22 min) as shown in Table 1. The small differences observed in retention times in both cases, could be attributed to the difference in complexity of injected samples.

These peaks were collected, evaporated under vacuum and subjected to EI-MS analysis. The presence of laherradurin and squamocin in the pellet extracts was confirmed by comparison of the molecular ions and EI-MS fragmentation patterns with the corresponding standards at the same conditions (Scheme 1). Also, the presence of ACG (hydrophobic compounds, MW >500 Da) in the bacterial pellet extracts, is predictable by theoretical calculations of their octanol/water partition coefficients (log $P_{lh} = 8.539$; log $P_{sq} = 9.818$). These log *p* values suggest that ACG might reside in the lipid membranes of cells. Furthermore, ACG cytotoxicity would be strongly related to the conformation adopted in the membrane (Shimada et al., 1998). In this regard, Bombasaro et al. (2011) and Di Toto Blessing et al. (2012), indicated that specific sites of interaction of the ACG in the lipid bilayers are the lipid headgroups (glycerol and phosphate) of POPC through hydrogen bonds with THF/flanking hydroxyls of ACG.

4. Conclusions

Laherradurin significantly stimulated AI-1 production. *C. violaceum* bioassays showed that laherradurin is not an autoinducer agonist. *P. plecoglossicida* J26 does not recognize these natural products as autoinducers, but senses a change produced by them that results in an increased biofilm formation. The exacerbation of biofilm formation achieved with a supernatant of J26 cells grown in presence of laherradurin is a response to the stress caused by this ACG.

Laherradurin and squamocin, are indirectly involved in quorum sensing mechanism by inducing a stress related increase in AI production which in turn results in more biofilm formation. These ACG could not be found in any of the supernatants, but instead were successfully isolated from bacterial pellet extracts (obtained from cell cultures grown in their presence). The localization of the ACG, without structural modifications, in the bacterial pellet extracts, corroborates that they act only as stressors. The log *p* values suggest that this stress is exerted on the bacterial membrane.

Acknowledgments

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