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Squamocin mode of action to stimulate biofilm formation of *Pseudomonas plecoglossicida* J26, a PAHs degrading bacterium

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ABSTRACT

Squamocin, an annonaceous acetogenin (ACG) extracted from *Annona cherimolia* (Annonaceae), has been shown to increase biofilm production of *Pseudomonas plecoglossicida* J26 (closely related to *P. plecoglossicida*), a polycyclic aromatic hydrocarbon degrading bacterium. PAHs have become priority pollutants for bioremediation due to their carcinogenicity and toxicity. The effect of various stressful stimuli (naphthalene, octanol, HCl, and NaCl) on cell growth, biofilm formation and autoinducer production of *P. plecoglossicida* were evaluated and compared with the effect of squamocin to establish its mode of action on biofilm formation. All stressors that inhibited growth stimulated autoinducer production while squamocin was growth stimulant at concentrations above 2.5 μg ml^{-1}. Despite structural similarities, squamocin is not an autoinducer agonist. It indirectly stimulates autoinducer production and increases *P. plecoglossicida* J26 cell growth. This is the first report on an ACG mode of action in the formation of biofilm in a naphthalene-degrading strain.

1. Introduction

Annonaceous acetogenins (ACG) are secondary metabolites from acetyl CoA biosynthesis in the polyacetates pathway (Gleye et al., 1998). They usually present 35 to 37 carbon atoms and consist of 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 molecules are isolated from plants of the Annonaceae family (Makabe et al., 2008) and display a wide range of biological activities like a potent cytotoxicity by specific inhibition of complex I of the mitochondrial respiratory chain (Londershausen et al., 1991). Such cytotoxicity is evident in their insecticide, acaricide, deworming, herbicide (Zafra-Polo et al., 1996; Zeng et al., 1996) and tumor cell growth inhibition activities (Oberlies et al., 1995, 1997) and implies a membrane level interaction. Furthermore, acetogenin cytotoxicity would be strongly related to the conformation adopted in the membrane (Shimada et al., 1998).

Previous investigations conducted in our laboratory showed that *P. plecoglossicida* J26 (Parellada et al., 2010) and *Pseudomonas aeruginosa* PA100 (Cartagena et al., 2007) increased their biofilm formation when squamocin was added to the culture medium. Biofilms are sessile microbial communities whose cells are anchored to a substrate or to an interface of other bacteria that produce and exhibit an altered phenotype with respect to their growth rate and gene transcription. These bacteria are embedded in a matrix of extracellular polymeric substances that they themselves produce (Rodney et al., 2002). Biofilm associated microorganisms play a crucial role in terrestrial and aquatic nutrient cycling and environmental contaminant biodegradation (Li et al., 2009). Biofilm-mediated bioremediation is safer and more efficient than that produced by planktonic organisms. This is because biofilm cells have a better chance for adaptation and survival (especially during periods of stress) because they are protected within the matrix (Decho, 2000).

Since polycyclic aromatic hydrocarbons (PAHs) come from many sources they are easily found in (Finlayson-Fitts and Pitts, 1997; Norse, 2005) air, water and soil (Johnsen et al., 2005). Many

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organizations have listed different PAHs as priority pollutants for bioremediation (Liu et al., 2001) because of their carcinogenic and toxic properties (Bispo et al., 1999) and their microbial degradation has become crucial (Johnsen et al., 2005; Meckenstock et al., 2000). 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 (Gonzalez and Keshavan, 2006). This phenomenon is caused by the production, release and detection of small signal 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).

Squamocin (Fig. 1a) and AHLs (Fig. 1b), structurally share the γ-lactone moiety. Besides, they have a long, lipophilic carbon chain, and are therefore expected to be quantitatively associated with membranes. Previous reports determined the positions of both THF and lactone rings, within liposomal membranes by proton (^{1}H) nuclear magnetic resonance spectroscopy. They proposed that THF rings with their flanking hidroxil groups, act as a hydrophilic anchor in the lipid membrane while the terminal carbon chain of lipophilic nature interacts with the acyl side chains of phospholipids (Shimada et al., 1998). Hydrocarbons tend to reside within the hydrophobic region between membrane monolayers composed by the acyl chains of phospholipids. Many bacteria, in response to this situation, form biofilms, and would express a phenotype of general response to stress (Van Hamme et al., 2003). In addition, the specific interaction sites of ACG with 1-palmitoyl-2-oleoyl-*syn*-glycero-3-phosphatidylcholine (POPC) lipidic bilayers (FTIR spectrometry) have been reported by our laboratory (Di Toto Blessing et al., 2010).

Strain J26 was employed for the present study. It was isolated from intertidal sediments of Patagonia, 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).

The aim of this work was to determine if *P. plecoglossicida* J26 regulates its biofilm production by a QS mechanism and to establish squamocin mode of action on *P. plecoglossicida* J26 biofilm formation. Cell growth in presence of squamocin and different stressors was evaluated by preparing different cultures. Supernatants of these cultures were used to stimulate biofilm formation and to quantify AI-1 production. The AI-1 in the different supernatants of J26 cells was quantified using *P. aeruginosa* qsc129b reporter strain. In addition, we investigated if squamocin, a γ-lactone, could be recognized by the reporter strain like an AHLs analogue.

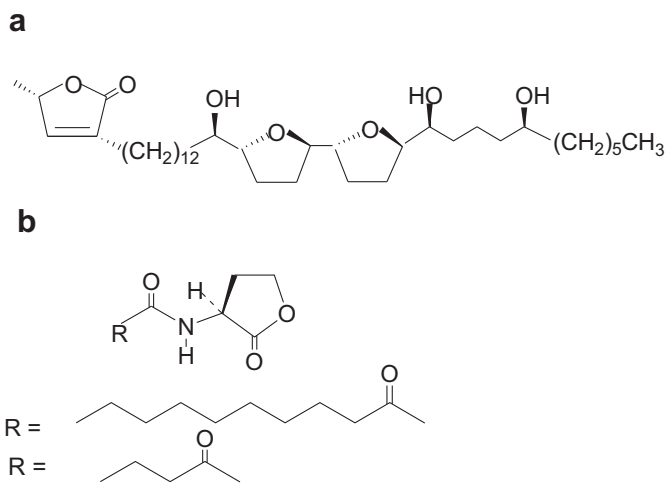


Fig. 1. Squamocin (a), and AHLs (b), structurally share the γ-lactone moiety.

2. Materials and methods

2.1. Isolation and purification of squamocin

2.1.1. Plant material

Annona cherimolia seeds from "cherimoya" fruits were collected in January 2008, in El Corte, Yerba Buena, 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 (1:1). The subextracts in chloroform and H_2O were obtained by vacuum evaporation. The chloroformic subextract was partitioned with a mixture of hexane–methanol (1:1). The methanolic subextract contained squamocin.

2.1.3. Purification and characterization

The methanolic subextract was subjected to column chromatography over silica gel 60 (Merck 70–230 Mesh) and eluted with a gradient of chloroform–ethyl acetate–methanol. Fractions were collected and pooled according to their similar TLC patterns. Squamocin was eluted with ethyl acetate–methanol (97:3), and exhaustively purified by reverse phase high performance liquid chromatography (RP-HPLC), using a Phenomenex C18 column (25 × 1 cm i.d., 10 μm particle size) and eluted with MeOH–H_2O (94:6). Characterization of squamocin was assessed by spectroscopic techniques (IR, ^{1}H NMR, ^{13}C NMR, and MS) as well as α_D determination. Squamocin was isolated as a viscous oil; $[\alpha]^{20}{}_D > = +15.0$ (*c* 1.7, MeOH); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 0,83 (t, 3H, *J*> = 7 Hz), 1.20–1.27 (m), 1.3 (m), 1.36 (3H, d, *J* = 6.8 Hz), 1.35–1.40 (m), 1.50 (m), 1.6 (m), 1.76 (m), 1.87 (m), 1.90 (m), 2.21 (tt, 2H, *J* = 7.7, 1.4 Hz), 3.33 (dt, 1H, *J* = 11, 7.5 Hz), 3.52 (m), 3.76 (m), 3.86 (m), 4.95 (qq, 1H, *J* = 6.8 Hz, 1.4), 6.96 (q, 1H, *J* = 1.4 Hz); ^{13}C NMR (200 MHz, $CDCl_3$) δ: 173.9, 148.9, 134.3, 83.3, 82.8, 82.5, 82.1, 77.3, 74.0, 71.6, 71.5, 37.5, 37.2, 33.1, 32.5, 31.9; IR ($CHCl_3$) cm^{-1}: 3680, 3585, 3460, 3015, 2940, 2855, 1755; EI-MS (70 eV): *m/z* = 221, 239, 267, 295, 317, 329, 347, 365, 399, 417, 435, 519, 568, 586, 604. Chemical shifts, coupling constants and the peaks corresponding to fragmentation of the molecule at 70 eV (EI-MS) showed excellent correlation with those obtained previously (Kawasu et al., 1989).

2.1.4. Squamocin solution

Several squamocin 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.

2.2. Bacterial strains

2.2.1. Pseudomonas sp. J26

Pseudomonas sp. J26 was selected from 15-day enrichment cultures of marine sediments of Patagonia, Argentina in a minimal seawater medium and naphthalene crystals as the sole carbon and energy sources. Aliquots of these enrichment cultures (100 μl) were

spread on the minimal seawater–agar medium and incubated for 48 h with naphthalene crystals on the lids of Petri dishes. In planktonic cultures, *Pseudomonas* sp. J26 was able to degrade 1 mM of naphthalene after 10 h growth, when naphthalene (in methanol) was supplemented in the culture medium (data not shown). This strain showed the highest biofilm formation capability when a screening program was conducted for biofilm-forming capacity in presence of naphthalene vapors and squamocin. J26 strain was identified as *Pseudomonas* sp., closely related to *P. Plecoglossicida* (Riva Mercadal et al., 2010).

2.2.2. J26 identification

DNA extraction from a culture of J26 was performed by the CTAB method (Ellis et al., 1999). Almost full-length 16S ribosomal ribonucleic acid (rRNA) gene sequences were amplified from DNA extracted with oligonucleotide primers 27f (*E. coli* 16S rDNA positions 8–27) and 1492r (*E. coli* 16S rDNA positions 1492–1512), according to Lane (Lane, 1991). After amplification, aliquots (10 μl) of the 16S DNA amplicons were visualized by electrophoresis on a 0.8% agarose gel after staining with ethidium bromide and then purified with Wizard PCR Preps (Promega, Madison, WI). Sequencing was performed directly on amplification products (Macrogen, Korea) and aligned with the reference 16S rRNA gene sequence using the basic local alignment search tool analysis according to the method of Altschul (Altschul et al., 1997; Anzai et al., 2000). 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).

2.2.3. Preparation of P. plecoglossicida J26 inoculums

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.

2.3. Reporter strains

2.3.1. P. aeruginosa qsc mutant (qsc129b)

P. aeruginosa qsc mutant (qsc129b) strain was obtained by Whiteley et al 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).

2.3.2. Chromobacterium violaceum (CV026)

CV026 is a mini-Tn5 mutant of *Chromobacterium violaceum* ATCC 31532 (Latifi et al., 1996; Throup et al., 1995; Winson et al., 1995) 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.

2.3.3. C. violaceum (VIR07)

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.

2.4. Bioassays

2.4.1. Growth under stress conditions

In order to evaluate the effect of different stressors and ACG on the growth of *P. plecoglossicida* J26, a set of 10 ml of LB medium tubes were inoculated with *P. plecoglossicida* J26 (see 2.2.3. Preparation of *P. plecoglossicida* J26 inoculums) and supplemented with the following stressors: 2.5, 25, 50 and 100 μg ml^{-1} squamocin (sq, ACG stress), naphthalene (naph, PAHs stress) and octanol (oc, solvent stress). Other assays were conducted with HCl (5N, until pH 5, acid stress) and NaCl (5%, osmotic stress). All tubes were incubated for 22 h at 30 °C and the OD_{600nm} was measured using a Shimadzu 160 A UV spectrophotometer. A culture of J26 cells grown in the absence of any stress was used as control. The experiments were performed in triplicate.

2.4.2. Supernatants

The supernatants from the cultures carried out in 2.4.2 (See 2.4.1 Growth under stress conditions) were obtained by centrifugation at 8000 rpm for 15 min at 25 °C and then sterilized with 0.22 μm Millipore filters.

2.4.3. AI-1 production (Miller reaction)

The biological activity of AI-1 present in the different supernatants was quantified using the reporter strain *P. aeruginosa* qsc129b. This bioassay was conducted to assess the effect of different stressors on the *P. plecoglossicida* J26 QS system. Also, we tested whether squamocin could be recognized by *P. aeruginosa* qsc129b like an AHL analogue.

For this purpose, equal parts (0.5 ml) of different supernatants or squamocin solution (See 2.1.4 Squamocin solution) and *P. aeruginosa* qsc129b overnight culture were mixed 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. β-galactosidase activity was measured by Miller reaction (O'Toole and Kolter, 1998; Whiteley et al., 1999).

2.4.4. C. violaceum bioassays

Given the structural similarity between squamocin 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 an squamocin solution (See 2.1.4 Squamocin solution).

The negative control was an aqueous solution of 2.75% dimethyl sulfoxide and the positive control were extracts from supernatants of *P. aeruginosa* (they contained 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.

2.4.5. Biofilms assay

P. plecoglossicida J26 biofilm production was quantified in the presence of supernatants of the same strain obtained in 2.4.3

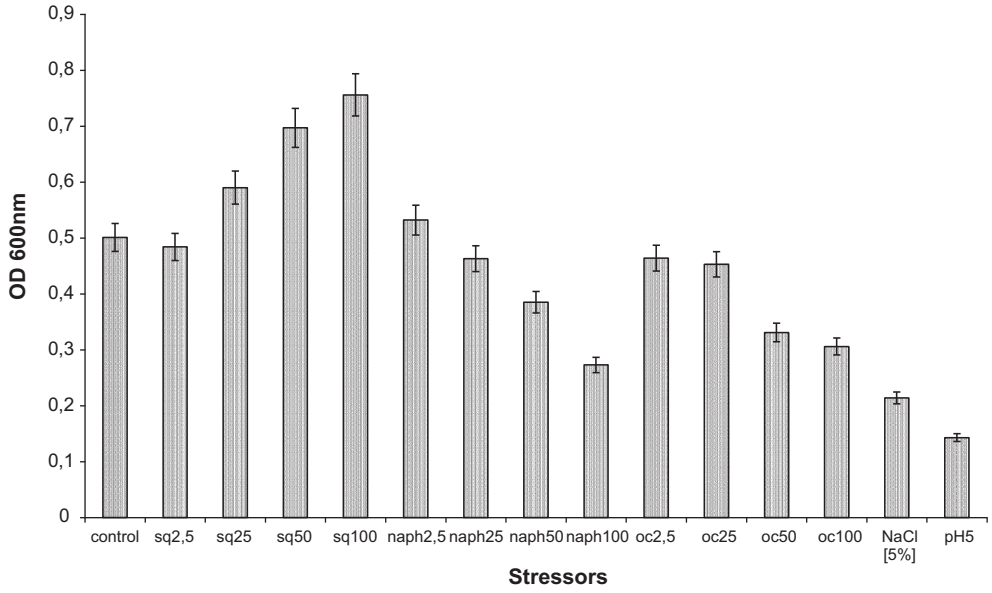


Fig. 2. J26 cell growth expressed as OD_{600nm} in presence of squamocin (sq); naphthalene (naph); octanol (oc); NaCl 5%; HCl (pH5). Numbers are referred to concentrations expressed in μg ml^{-1}.

(See 2.4.2. Supernatants) using the technique of O'Toole and colleagues with minor modifications (O'Toole and Kolter, 1998). 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.

Since the objective was to establish a relationship between AI-1 production in the supernatants grown in presence of stressors and stimulation of biofilm production, different controls were required: LB and a supernatant of J26 cells grown in the absence of any stressor. A volume of 170 μl of LB broth and 10 μl of either supernatants or controls were placed in each well of a 96-well polystyrene microtiter plate. Then, each well was inoculated with 20 μl of an overnight (OD_{600nm} = 1.0) *P. plecoglossicida* culture in LB (See 2.2.3. Preparation of *P. plecoglossicida* J26 inoculums). 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).

2.5. 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).

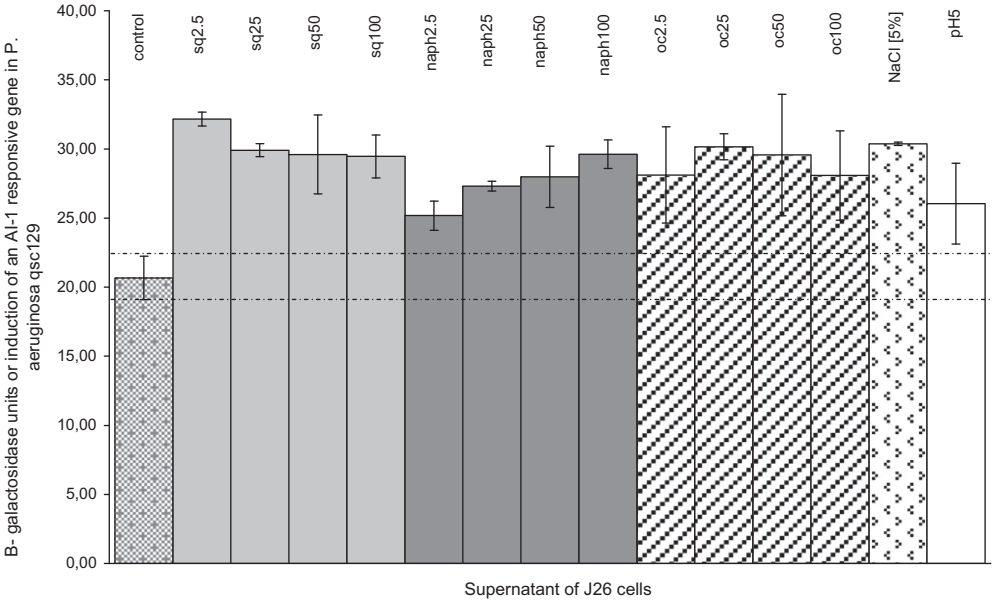


Fig. 3. β-galactosidase units or induction of an AI-1 responsive gene in *P. aeruginosa* qsc129 in presence of a supernatant of J26 cells grown in presence of squamocin (sq); naphthalene (naph); octanol (oc); NaCl 5%; HCl (pH5). Numbers are referred to concentrations expressed in μg ml^{-1}.

Table 1
Reporter strains bioassays.

Reporter strain	Positive control[a]	Negative control[b]	Squamocin solutions
C. violaceum vir07	+	–	–
C. violaceum cv026	+	–	–
P. aeruginosa 129b	+	–	–

[a] Extracts from supernatants of *P. aeruginosa* (contains C4-AHL and 3-oxo-C12-AHL).
[b] Aqueous solution of 2.75% dimethyl sulfoxide.

3. Results and discussion

3.1. Effect of different stressors on P. plecoglossicida J26 growth

Fig. 2 shows the effect of different stressors on *P. plecoglossicida* J26 growth expressed as OD_{600nm}. High concentrations of squamocin and naphthalene at 2.5 μg ml^{-1} significantly stimulated cell growth ($p < 0.05$). Naphthalene at 50 and 100 μg ml^{-1}, octanol at all concentrations, NaCl (5%) and HCl (pH 5) significantly inhibited cell growth ($p < 0.05$).

3.2. AI-1 production

Fig. 3 shows the activity of AI-1 (AHL) present in the supernatants, represented as β-galactosidase units. AHLs were recognized by the *P. aeruginosa* qsc129b reporter strain.

β-galactosidase activity in all supernatants was higher than in control (supernatant of J26 cells grown in the absence of any stress) ($p < 0.05$). Induction of β-galactosidase by the supernatants obtained in presence of octanol and squamocin was not concentration dependent while the supernatants obtained in presence of naphthalene induced β-galactosidase production in a concentration dependent manner.

3.3. Bioassays with squamocin

Given the structural similarity between squamocin and AHLs and in order to rule out a possible AI-1 agonistic activity of squamocin we conducted three bioassays with different reporter strains: *C. violaceum* VIR07 and CV026 and *P. aeruginosa* qsc129b. Table 1 summarizes the results obtained. Squamocin did not stimulate the production of β-galactosidase or violacein by any the reporter strains (Fig. 5).

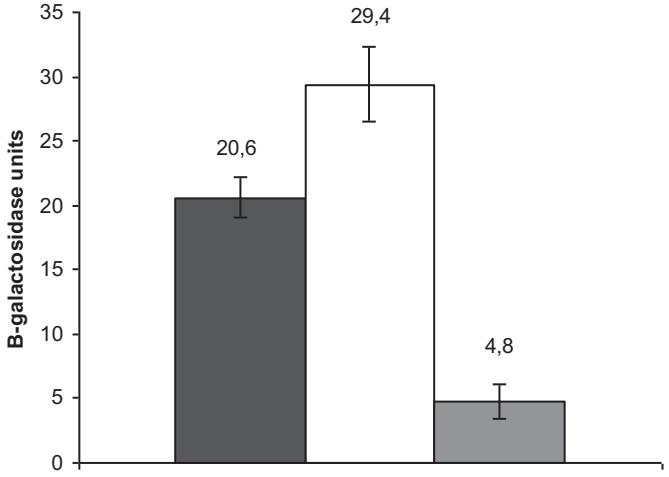


Fig. 5. β-galactosidase units or induction of an AI-1 responsive gene in *P. aeruginosa* qsc129: ■- in presence of a supernatant of J26 cells grown in absence of any stressor; □-in presence of a supernatant of J26 cells grown in presence of squamocin 100 μg ml^{-1}; ■- in presence of squamocin 100 μg ml^{-1} solution.

3.4. Biofilm formation

All supernatants stimulated biofilm formation ($p < 0.05$). It is noteworthy that the addition of a stress free *P. plecoglossicida* J26 supernatant to another culture of the same bacterium significantly stimulated its biofilm production ($OD_{560nm} = 0.751 \pm 0.031$; 169% compared with control, which in this case consisted of the biofilm formation in an LB culture without the addition of a supernatant $OD_{560nm} = 0.445 \pm 0.061$, 100%).

The supernatant of J26 cells grown in presence of squamocin at all concentrations, significantly stimulated biofilm formation ($p < 0.05$). The highest stimulation was achieved at 2.5 μg ml^{-1}

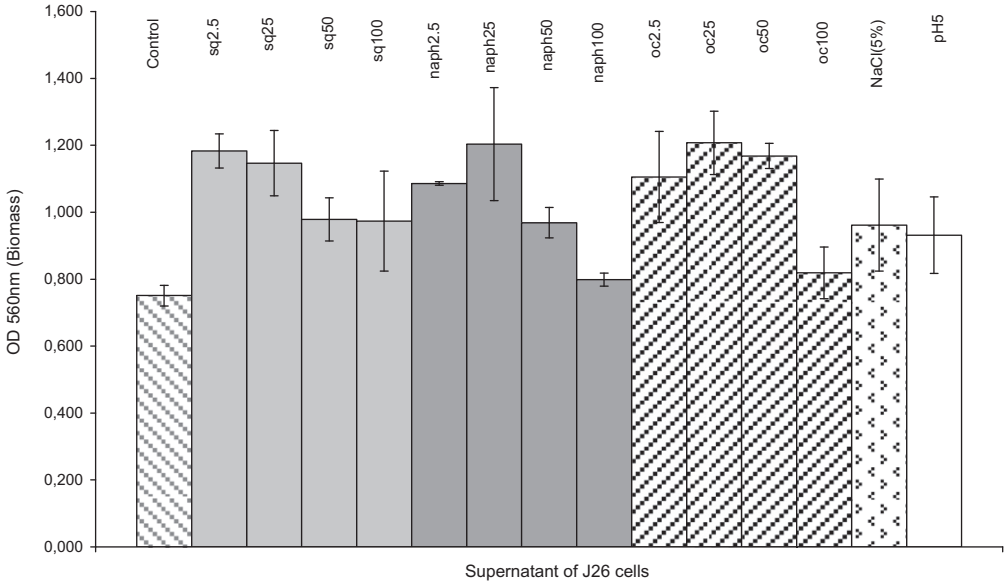
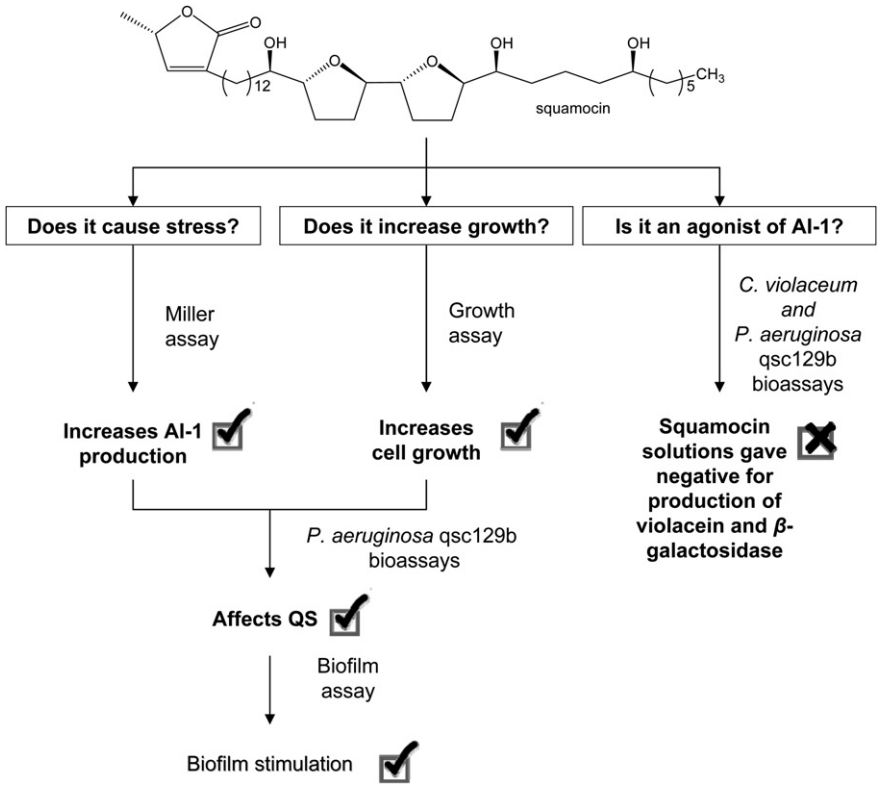


Fig. 4. Biofilm formation expressed as OD_{560nm} in presence of a supernatant of J26 cells grown in presence of squamocin (sq); naphthalene (naph); octanol (oc); NaCl 5%; HCl (pH5). Numbers are referred to concentrations expressed in μg ml^{-1}.



Scheme 1.

(Fig. 4). Observed stimulations decreased with the concentration of the ACG. The effect of these supernatants was compared with that of another supernatant from J26 cells grown in the absence of any stress.

The naphthalene stressed culture supernatants produced a significant stimulation ($p < 0.05$). The most relevant one was observed at 25 μg ml^{-1} and began to decrease with increasing concentrations of naphthalene in the cell cultures.

Supernatants from octanol stressed cultures produced a stimulation similar to that of naphthalene. Both naphthalene and octanol assays showed a similar behavior with increasing concentrations of the stressor.

Supernatants of cell cultures with osmotic stress (NaCl 5% in the culture medium), significantly increased the formation of biofilm. So did the culture supernatants stressed at pH 5 (HCl 5N).

4. Discussion

Scheme 1 summarizes the main results and methodologies used. Autoinducers produced by *P. plecoglossicida* J26 under different stress situations were evaluated. Although different in mean value, the stimulations achieved at high concentrations of naphthalene and octanol (concentrations above 25 μg ml^{-1}) did not present major differences. Yet, it is important to note that cell growth was significantly affected by the presence of higher concentrations of these stressors. Octanol showed an important concentration dependent growth inhibition. Acid and osmotic stress showed a significant growth inhibition. In some cases, for example octanol, increments on the stressor concentration did not result in a greater induction of the β-galactosidase activity. Showing an opposite trend, squamocin simultaneously stimulated production of AI-1 and significantly increased cell density (See Figs. 2 and 3).

Supernatants of J26 cells grown in the presence of squamocin, naphthalene and octanol, generated in *P. aeruginosa* qsc129b reporter strain a production of β-galactosidase units significantly higher than their control. When compared with a supernatant of J26 cells grown in the absence of any stressor (β-galactosidase units = 20.6 ± 1.5, 100%), squamocin at 2.5 μg ml^{-1} produced 156% stimulation (β-galactosidase units = 32.2 ± 0.5).

The addition of a stress free *P. plecoglossicida* J26 supernatant to another culture of the same bacterium significantly stimulated its biofilm production. Even more, squamocin alone does not behave like a bacterial AI agonist. No significant differences with the basal β-galactosidase production of the reporter strain (3 β-galactosidase units) were detected in this case (Fig. 5). These results were confirmed by *C. violaceum* bioassays (Table 1).

5. Conclusions

Squamocin significantly stimulated both growth and AI-1 production. Even those stressors that caused cell growth inhibition produced supernatants that significantly stimulated biofilm formation. *C. violaceum* bioassays showed that squamocin is not an autoinducer agonist. The exacerbation of biofilm formation achieved with a supernatant of J26 cells grown in the presence the squamocin would be the stress response of the strain caused by squamocin.

P. plecoglossicida J26 regulates its biofilm production by *N*-acyl-homoserine lactones in a population dependent manner. Two facts support this conclusion: the presence of AHLs in supernatants was confirmed by *P. aeruginosa* qsc129b reporter strain recognition and the addition of a stress free *P. plecoglossicida* J26 supernatant to another culture of the same bacterium significantly stimulates its biofilm production. Finally, we propose that squamocin is indirectly involved in quorum sensing mechanism by inducing a stress

related increase in AI production for a given incubation time. Therefore, the exacerbation of biofilm formation is due to increased production of AI-1.

Acknowledgments

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