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Effect of the annonaceous acetogenins, squamocin and laherradurin, on the degradation of naphthalene mediated by *Pseudomonas plecoglossicida* J26

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ABSTRACT

The effects of squamocin and laherradurin, two annonaceous acetogenins (ACG) from *Annona cherimolia* seeds on naphthalene degradation and biofilm formation of *Pseudomonas plecoglossicida* J26 were evaluated. These PAHs degrading bacteria have been shown to increase their biofilm formation when small concentrations of squamocin or laherradurin were added to the culture medium. An increase of initial naphthalene degradation rate was observed in ACG treated cultures that permitted us to establish that there is a relationship between naphthalene consumption and biofilm formation. The stimulation of biofilm formation led to increased consumption of naphthalene. Naphthalene consumption rate has been tripled in presence of ACG. This fact could be employed to increase biofilm formation and efficiency of PAHs bioremediation.

1. Introduction

Bacterial biofilms are clusters of cells that growth attached to a surface or interface and are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) (Singh et al., 2006). Biofilm formation is often regulated by a cell density-dependent signaling system called quorum sensing. In quorum sensing, bacteria monitor their own population density through the production, release and detection of molecules called type 1 autoinducers (AI-1) which in Gram negative bacteria are *N*-acid homoserine lactones (AHLs) (Bassler and Losick, 2006).

Biofilm cells have a better chance of adaptation and survival, especially during periods of stress, since they are protected within the biofilm matrix. Because of this, the bioremediation process mediated by bacteria in biofilm presents a proficient and safer alternative to bioremediation compared to planktonic bacteria (Davey and O'Toole, 2000).

Polycyclic aromatic hydrocarbons (PAHs) are a diverse class of hydrophobic organic compounds consisting of two or more fused aromatic rings. Due to their carcinogenic, mutagenic and toxic properties, some PAHs are considered priority pollutants for remediation by the Environmental Protection Agency United States (EPA) and the European Environment Agency (Keith and Telliard, 1979; Smith et al., 1989). Among PAHs, naphthalene has been commonly taken as a model compound for biodegradation studies (Ferrero et al., 2002).

The strain employed for the present study (called J26 and subsequently identified as *Pseudomonas plecoglossicida*), was isolated from intertidal sediments and has naphthalene degrading capability (Riva Mercadal et al., 2010). *P. plecoglossicida* has a respiratory metabolism (Nishimori et al., 2000) and exhibits biofilm formation capacity (Li et al., 2009).

Annonaceous acetogenins (ACG) are secondary metabolites derived from polyketides. They structure consist in a long alkyl chain that usually presents a terminal, α-β-unsaturated, γ-methyl-γ lactone. Such hydrocarbon chain generally shows one, two, or more rarely three tetrahydrofuranic rings (THF). In previous work, two ACG, squamocin and laherradurin (Fig. 1) were extracted from the seeds of *Annona cherimolia*, a tropical tree of the Annonaceae family and were evaluated for their ability to stimulate *P. plecoglossicida* J26 biofilm production. We demonstrated that both ACG, were able to stimulate biofilm formation at 2.5 μg ml^{-1} (Parellada et al., 2010). Squamocin has shown to stimulate *P. plecoglossicida* J26 AHLs production, which in turn causes an

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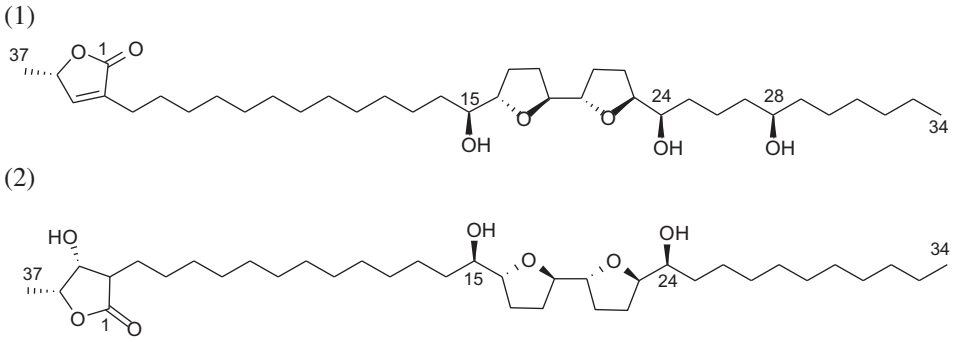

Fig. 1. ACG used in the assays: (1) squamocin, (2) laherradurin.

increase on biofilm formation. Despite that it possesses a γ-lactone moiety in its structure, which is similar to the lactone moiety present in AHLs, it was found not to act as an AI-1 agonist (Parellada et al., 2011).

The primary objective of the research presented in this article was to investigate the effect of squamocin and laherradurin on the degradation of naphthalene mediated by *P. plecoglossicida* J26.

This study seeks to establish a relationship between naphthalene degradation and biofilm formation. For this, PAHs and biofilm were quantified simultaneously. In order to study the degradation capabilities of *P. plecoglossicida* J26 biofilm, naphthalene degradation in the absence of planktonic cells was also assessed.

2. Material and methods

2.1. Isolation and purification of acetogenins

2.1.1. Plant material

The seeds of *A. cherimolia* were obtained from "cherimoya" fruits from El Corte, Tucumán. A voucher specimen is deposited in the herbarium of the Miguel Lillo Foundation, Tucumán, Argentina, under the reference LIL 515092.

2.1.2. Extraction

A. cherimolia seeds (1000 g), were dried, powdered and then macerated with methanol. The methanolic phase was evaporated and the extract partitioned in a mixture of chloroform and H_2O (1:1). The sub-extracts in chloroform and H_2O were obtained by vacuum evaporation. The chloroformic sub-extract was partitioned with a mixture of hexane and methanol (1:1). The methanolic sub-extract contained squamocin and laherradurin. All procedures were monitored using TLC and Keddes reagent.

2.1.3. Purification and characterization

The methanolic sub-extract was subjected to column chromatography. Silica gel 60 (Merck 70–230 Mesh) was used. Column was eluted with a gradient of chloroform-ethyl acetate-methanol and fractions were collected and pooled according to their similar TLC patterns. Squamocin was eluted with ethyl acetate-methanol (97:3) and laherradurin was eluted with ethyl acetate-methanol (80:20). Both were exhaustively purified by reverse phase high performance liquid chromatography (RP-HPLC with RI detector), using a Phenomenex C18 column (25 × 1 cm i.d., 10 μm particle size). A mixture methanol-water 94:6 was employed for eluting squamocin and methanol-water 90:10 for eluting laherradurin. Characterization of squamocin and laherradurin were 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. HPLC fractions yielded squamocin in the form of a viscous oil; $[\alpha]_D^{20} = +15.0$ (*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 (*d*, 3H, *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, 1.4 Hz), 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. Laherradurin was isolated as a white powder; $[\alpha]_D^{20} = +21°$ (*c* = 0.09, MeOH); ^{1}H-NMR (200 MHz, $CDCl_3$) *δ*: 0.9 (*t*, 3H, H-34), 1.4 (*d*, 3H, H-37), 2.6–2.3 (*m*, 5H, H-2, −3, −35), 3.8 (*m*, 5H, H-4, −16, −19, −20, −23), 4.2 (*m*, 4H, H-15, −24), 4.5 (dd, 1H, H-36); ^{13}C-NMR (200 MHz, $CDCl_3$) *δ*: 14.1 (C-34), 19.0 (C-37), 43.7 (C-2), 71.3 (C-24), 74.1 (C-15), 82.7 (C-16), 82.2 (C-23), 82.6 (C-19, -20), 73.5 (C-35), 174.7 (C-1), 82.5 (C-36) IR (film, $CHCl_3$) cm^{-1}: 3590, 2930, 2860, 1765, 1450; EI-MS (70 eV): *m/z* = 453, 435, 417, 399, 383, 365, 347, 313, 311, 295, 385, 267, 241, 223, 203, 141; CI-MS: 624 $(M)^+$, 453, 435, 417, 399, 383, 365, 347, 331, 295, 285, 241, 223, 203. 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 for squamocin (Kawasu et al., 1989) and laherradurin (Warmerdam et al., 1998).

2.2. ACG solutions for the stimulation of biofilm formation

Solutions of squamocin and laherradurin 500 μg ml^{-1} in ethanol (96%) were prepared. These solutions were used in all assays performed in this work.

2.3. Pseudomonas plecoglossicida J26

The strain J26 was selected from 15-day enrichment cultures of intertidal sediments of the coasts of Patagonia, Argentina. Aliquots of these enrichment cultures (100 μl) were spread on the minimal seawater-agar medium and incubated for 48 h with naphthalene crystals (as the sole carbon and energy source) on the lids of Petri dishes. This strain showed the highest biofilm formation capability when a screening program was conducted in presence of naphthalene vapors and ACG. J26 strain was identified as *Pseudomonas* sp. and its 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). *P. plecoglossicida* J26 regulates its biofilm production by a quorum sensing mechanism (Parellada et al., 2011).

2.3.1. Preparation of Pseudomonas plecoglossicida J26 inoculums

P. plecoglossicida J26 was maintained at 30 °C in Luria-Bertani (LB) agar medium (Gibco, Rockville, MD, USA) in contact with naphthalene vapors to promote a bacterial resistance phenotype.

Selected colonies were used to inoculate LB broth and incubated overnight (Parellada et al., 2011).

2.4. Quantification of biofilm formation on polystyrene microplates: kinetic assay

P. plecoglossicida J26 biofilm formation in the presence of squamocin and laherradurin was quantified by the technique described by O'Toole and Kolter (1998) modified. Briefly, 180 μl of LB medium, 1 μl of an ethanolic solution (500 μg ml^{-1}) of squamocin or laherradurin (final concentration 2.5 μg ml^{-1}) and 2 μl of an ethanolic solution (100 mM) of naphthalene (final concentration 1 mM) were placed in 96-well microtitre dishes. After this, 20 μl from an overnight LB culture (10^8 CFU ml^{-1}) were inoculated. These cells have reached quorum and therefore have a phenotype according to the biofilm mode of growth. After inoculation, microplates were incubated at 28 °C in a moist chamber. Experiments were conducted in quintuplicate using ethanol and naphthalene (without squamocin or laherradurin) as controls. Measurements were performed at 1, 4, 6, 8, 10 and 12 h. After incubation, 10 μl of crystal violet solution (1%) were added to each well and incubated at room temperature for 15 min. Then, wells were thoroughly rinsed thrice with water to remove planktonic cells and unattached dye. The crystal violet staining the biofilm formed in each well was re-dissolved with the addition of 200 μl of 95% ethanol. Absorbance of the resulting solution was measured at 560 nm in a microplate spectrophotometer (Biotek-Power Wave XS2 with GEN5 data analysis software).

2.5. Quantification of biofilm formation on glass beads

Glass beads provide an excellent substrate for adhesion and growth of bacterial biofilm (Bielefeldt et al., 2002). Based on this, *P. plecoglossicida* J26 biofilm formation on glass beads in presence and absence of ACG was assessed.

Approximately 30 g of glass beads (diameter: 4 mm) were placed respectively into two series of Erlenmeyer flasks with LB medium (50 ml). In the first series 250 μl of squamocin or laherradurin (See Section 2.2. ACG solutions for the stimulation of biofilm formation) were added (final concentration 2.5 μg ml^{-1}). The second series was used as control (ethanol without ACG). Both series were inoculated (10% $^{v}/_{v}$) with an overnight culture of J26 cells ($DO_{600} = 1.0$) and flasks were incubated for 12 h at 28 °C. Then, the culture medium was discarded and the glass beads were gently washed with PBS to remove planktonic cells and all traces of culture medium. The biomass on the beads were stained for 15 min with crystal violet (1%) at room temperature and then washed repeatedly with distilled water to remove unattached dye. The crystal violet staining the biofilm formed on the beads was re-dissolved with 50 ml of ethanol 96% and the OD_{560} of the resulting solution was measured in a Shimadzu 160 A UV spectrophotometer. Assays were performed in triplicate. By considering controls as 100% of biofilm production, stimulations of biofilm formation were expressed as increased percentage.

2.6. Naphthalene degradation assay in batch planktonic cultures with developing biofilm

In this assay, the concentration of naphthalene was determined in a batch planktonic culture by HPLC measurements according to Manohar et al. (2001). Observed degradation corresponds to the joint action of planktonic and biofilm bacteria.

Two set of dark glass flasks with screw cap (to prevent loss by volatilization of naphthalene) containing 5 ml of LB were prepared. The first set of flasks (set with ACG), was supplemented with 25 μl of a squamocin or laherradurin solution (500 μg ml^{-1} in methanol to obtain a 2.5 μg ml^{-1} final concentration) and 50 μl of a naphthalene solution (100 mM in methanol to obtain a 1 mM final concentration). The second set (control) was supplemented only with naphthalene (set without ACG). Methanol was permitted to evaporate for 2 h at 25 °C before inoculation. After that, the flasks were inoculated with an overnight culture of *P. plecoglossicida* J26 (10% $^{v}/_{v}$) and incubated in darkness at 28 °C and 150 rpm of stirring. All assays were performed in triplicate.

At 0, 2, 3, 4 and 6 h, three flasks of each set were used to quantify residual naphthalene. In order to stop growth and dissolve all residual naphthalene, 45 ml of methanol were added in all flasks after incubation. Also, 250 μl of a stock solution of acenaphthene (10 mM in methanol, MW = 154.2 g mol^{-1}, Sigma Aldrich) were added to the flasks to be used as internal standard (Manohar et al., 2001). Finally, the samples were filtered with a 0.22 μm nylon filter (Microclar, Argentina) to separate the cells and stored at −20 °C until analysis. Quantification of naphthalene was carried out by reverse phase high performance liquid chromatography (RP-HPLC: Perkin Elmer series 200 chromatograph; UV/visible spectrophotometric detector fixed at 276 nm wavelength; series 600 analogical interphase). A C8 column (25 × 1 cm i.d., 10 μm particle size) was employed and a solution of methanol-water (9:1) was used as mobile phase (flow rate 1.5 ml min^{-1}) (Manohar et al., 2001). Peaks areas were calculated with a peak analyzer (Total Chrom analyzer) and naphthalene concentrations were estimated by comparison with the internal standard area. Both analyte and standard have slight differences between their respective detector responses. For this reason, we calculated a relative response factor (RRF) which was introduced in the calculations and therefore the ratio of signals obtained can be considered independent of fluctuations inherent in the method and instruments.

A mixture of naphthalene and acenaphthene was injected in triplicate (known in concentration, both 1 mM approximately). The areas, which are obtained from the chromatograms, are used to calculate the relative response factor of the detector (RRF).

Samples were filtered and then injected in triplicate (20 μl, loop exceeded). We determined the peak areas corresponding to naphthalene and acenaphthene in each chromatogram. Naphthalene concentrations were calculated from those areas and expressed as micromoles per liter.

2.7. Naphthalene degradation assay with biofilm formed on glass beads

In this assay, the naphthalene degradation produced by *P. plecoglossicida* J26 biofilm formed on glass beads was assessed.

Approximately 30 g of glass beads (diameter: 4 mm) were placed respectively into two series of Erlenmeyer flasks with LB medium (50 ml). In the first series 250 μl of squamocin or laherradurin (See Section 2.2. ACG solutions for the stimulation of biofilm formation) were aggregated (final concentration 2.5 μg ml^{-1}). The second series was used as control (ethanol without ACG). Both series were inoculated (10% $^{v}/_{v}$) with an overnight culture of J26 cells ($DO_{600} = 1.0$) and flasks were incubated for 12 h at 28 °C. After this, LB medium was discarded and the beads were washed with PBS to remove planktonic cells and culture medium. Then, 50 ml of a solution of naphthalene (1 mM in PBS) were aggregated and incubated at 28 °C. Under these conditions, only biofilm bacteria on the glass beads produce the consumption of naphthalene.

Aliquots (1 ml) were taken every 2 h and immediately diluted with methanol (1:10) to cease naphthalene consumption. Then, cells were separated with a 0.22 μm nitrocellulose filters and the liquid phase stored at −20 °C until injection into the HPLC column. Quantification of residual naphthalene was carried out by RP-HPLC

following the same chromatographic conditions as it was shown in Section 2.6. Peaks areas were calculated with an analyzer (Total Chrom analyzer) and concentrations were estimated with the use of a calibration curve obtained by the least squares method.

2.8. Statistical analysis

The differences in the mean values (Abs ± SD) were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pair wise multiple comparisons of groups. In all statistical analysis, values of $p > 0.05$ were considered not significant (Statistix 7.1, 2000).

3. Results and discussion

3.1. Quantification of biofilm formation on polystyrene microplates: kinetic assay

Fig. 2 shows the biofilm formation expressed as biomass (OD_{560}) in batch planktonic cultures under the presence or absence of squamocin and laherradurin. Both compounds stimulated biofilm formation. Squamocin is indirectly involved in quorum sensing mechanisms by inducing an increment of AI production and therefore increasing biofilm formation (Parellada et al., 2011).

The inoculum of *P. plecoglossicida* J26 had an $OD_{600} = 1.0$ (10^8 cells ml^{-1}). Under these conditions, J26 cells have reached quorum and therefore have a phenotype according to a biofilm mode of growth. This means that biofilm formation began at the moment of inoculation. However, the concentration of AHL decreased with dilution of inoculum resulting in a lower rate of biofilm formation until the quorum was reached again between 4 and 6 h of incubation. In presence of ACG, steeper slopes of biofilm formation were observed (Fig. 2). Experimental data (DO_{560}) were fitted to lineal curves using the least squares method. The rate of biofilm formation in presence of squamocin or laherradurin was respectively 1.7 (slope = 0.4056) and 1.5 (slope = 0.3566) times higher than control (slope = 0.2414) (Fig. 2).

Squamocin (2.5 μg ml^{-1}) significantly stimulated biofilm formation of *P. plecoglossicida* J26 ($p < 0.05$). At 6 h of incubation the stimulation was 167% ($OD_{560} = 2.381 \pm 0.109$) compared to control 100% ($OD_{560} = 1.426 \pm 0.138$). The biomass percentage was similar until 12 h of incubation.

Laherradurin (2.5 μg ml^{-1}) also significantly stimulated biofilm formation ($p < 0.05$). At 6 h of incubation the stimulation was 148% ($OD_{560} = 2.109 \pm 0.11$) compared to control 100% ($OD_{560} = 1.426 \pm 0.138$).

Results demonstrate the ability of these ACG to stimulate biofilm formation confirming preliminary results (Parellada et al., 2010).

3.2. Naphthalene degradation in batch planktonic cultures with developing biofilm

Planktonic cultures of *P. plecoglossicida* J26 were conducted to study the effect of squamocin and laherradurin on naphthalene degradation. Along with planktonic growth, an increasing amount of biofilm is being formed in all interphases within the batch cultures.

Fig. 3 shows the concentration of residual naphthalene in batch planktonic cultures with developing biofilm in presence or absence of squamocin and laherradurin, respectively. The internal standard method was used. The calculated RRF was 1.07.

Squamocin and laherradurin were able to increase the initial naphthalene degradation rate (0–6 h). As shown in Fig. 3, in presence of squamocin, 42.69% of initial naphthalene was degraded during the first 6 h (naphthalene μM concentration dropped from 767.10 ± 8.82 to 439.60 ± 6.11). In presence of laherradurin, 42.75% of initial naphthalene was degraded during the first 6 h (naphthalene μM concentration dropped from 771.70 ± 2.00 to 441.84 ± 3.15). Only 15% of naphthalene was degraded in the control flasks without ACG (naphthalene μM concentration dropped from 771.70 ± 1.43 to 653.23 ± 3.15).

The largest differences in naphthalene concentration between control and treated assays were registered at 6 h of incubation (Fig. 3). Experimental data (naphthalene concentration vs time) were fitted to lineal curves using the least squares method. Naphthalene consumption rate has been tripled in presence of ACG (Fig. 3). The rates of naphthalene degradation (slopes of lineal curves) in presence of squamocin or laherradurin were 52.860 μmol l^{-1} h^{-1} and 55.512 μmol l^{-1} h^{-1}, respectively. The slope of the control was 19.716 μmol l^{-1} h^{-1}. This could be because ACG stimulate growth and biofilm formation (Parellada et al., 2011) and therefore there is, from the beginning of the process, a higher number of naphthalene degrading-cells.

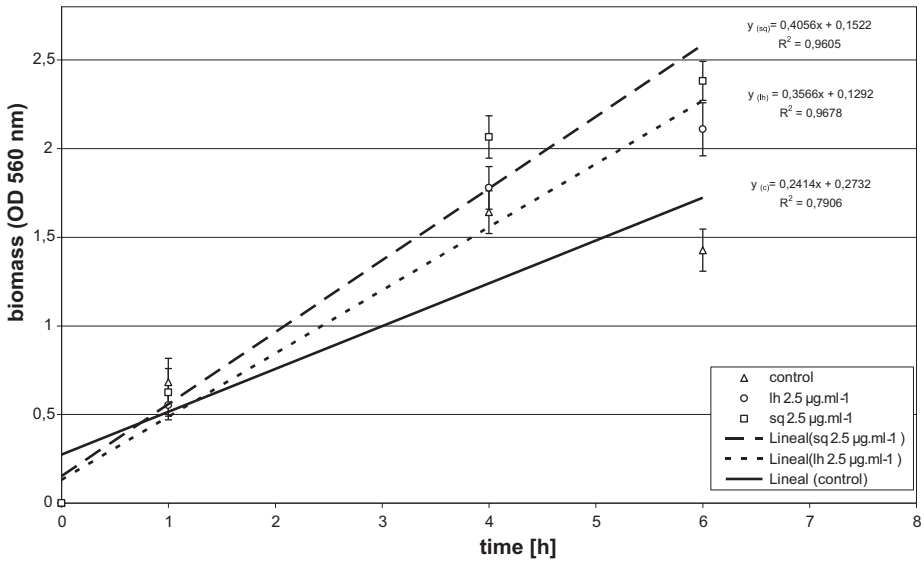

Fig. 2. Kinetics of *Pseudomonas plecoglossicida* J26 biofilm formation on 96 wells microtitre plates in presence or absence of ACG. Biomass was measured by crystal violet technique and expressed as OD_{560}. Linear regressions of experimental data and their respective equations are shown in the upper right corner of the figure.

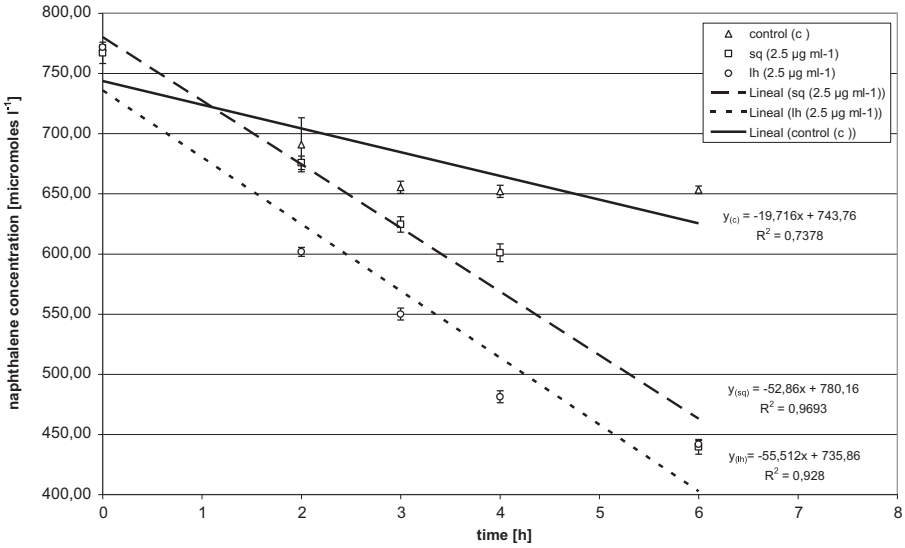

Fig. 3. Degradation of naphthalene produced by planktonic and biofilm cells of *Pseudomonas plecoglossicida* J26 in batch cultures in presence or absence of ACG. Linear regressions of experimental data and their respective equations are shown in the right side of the figure. Concentrations were expressed in μmol l^{-1}.

Table 1
Pseudomonas plecoglossicida J26 biomass formed on glass beads over 12 h of incubation in presence or absence of ACG. Biomass is expressed as OD560 ± SD. Stimulations are given in increased % taking control as 100% stimulation.

	Control	Squamocin	Laherradurin
Biomass (OD_{560}) ± SD	1.328 ± 0.049	1.928 ± 0.058	1.590 ± 0.022
Stimulation (%)	100	145	120

3.3. Biofilm quantification and naphthalene degradation on glass beads

The biofilm quantification and naphthalene degradation assays on glass beads were carried out in presence of squamocin and laherradurin.

The amount of biofilm formed on the beads (biomass) after 12 h of incubation was measured by crystal violet (Table 1). Assay described in Section 2.5 provides indirectly measures of the amount of biofilm or biomass used in this assay. We found that both laherradurin and squamocin, significantly stimulated biofilm formation of *P. plecoglossicida* J26 ($p < 0.05$).

The least squares method was applied over experimental data (naphthalene μM concentration vs. time [*h*]) as it is shown in Fig. 4. This figure represents the degradation of naphthalene produced by the biomass obtained on the beads in the presence or absence of ACG.

From the beginning of incubation naphthalene consumption is evident in all cases (control, squamocin and laherradurin assays), indicating that *P. plecoglossicida* J26 is capable of degrading naphthalene even in the absence of ACG as described earlier by Riva Mercadal et al. (2010). However, in presence of ACG, the slopes of degradation curves were more pronounced indicating greater degradation rates (Fig. 4).

Twelve hours of incubation yielded the consumption of 47% of initial naphthalene in the control (naphthalene μM concentration

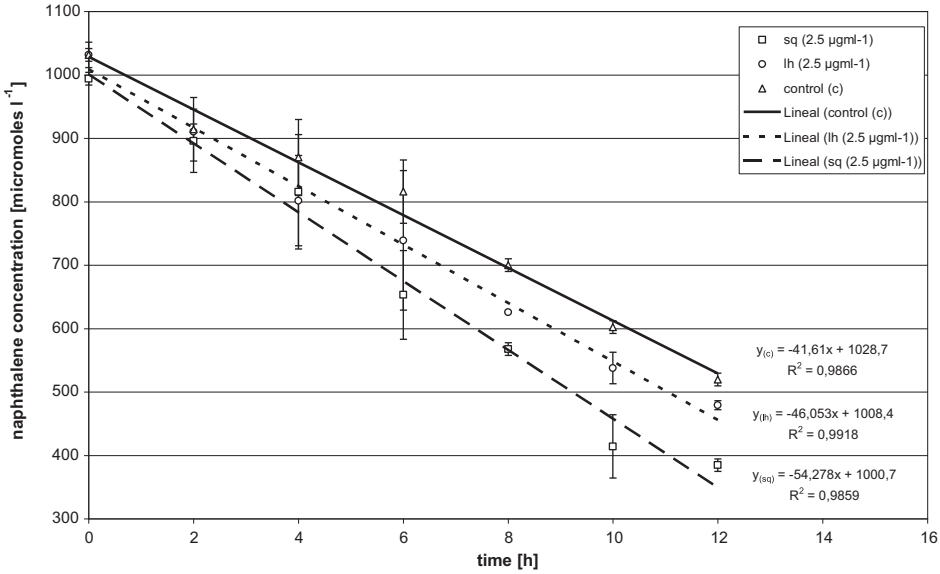

Fig. 4. Degradation of naphthalene produced by the biomass obtained on the beads in presence or absence of ACG. Linear regressions of experimental data and their respective equations are shown in the bottom right of the figure. Concentrations were expressed in μmol l^{-1}.

dropped from 1031.44 ± 0.01 to 519.44 ± 0.01) while the biofilm produced in presence of squamocin has consumed 61% (naphthalene μM concentration dropped from 993.91 ± 0.01 to 384.56 ± 0.01) and the biofilm produced in presence of laherradurin has consumed 54% (naphthalene μM concentration dropped from 1031.44 ± 0.01 to 479.05 ± 0.01).

In presence of ACG, higher quantities of biofilm were formed on the beads. Thus, the amount of biofilm at the beginning of the degradation was higher in these cases, leading to an increased rate of naphthalene consumption. Squamocin stimulated biofilm production to a greater extent than laherradurin. Because of this, a steeper slope (or naphthalene degradation rate) was observed in its presence (slope control = 41.6 μmol l^{-1} h^{-1}, slope laherradurin = 46.1 μmol l^{-1} h^{-1}, slope squamocin = 54.3 μmol l^{-1} h^{-1}).

4. Conclusions

Our results show that squamocin and laherradurin stimulate *Pseudomonas plecoglossicida* J26 biofilm formation. This fact, led to increased consumption of naphthalene yet in the absence of planktonic cells, that has been tripled in presence of ACG.

Therefore, this work provides experimental evidence that supports the use of laherradurin and squamocin as biofilm formation promoters that could make more efficient, safer and durable naphthalene bioremediation processes.

Acknowledgments

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