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ORIGINAL ARTICLE

Biofilm formation in marine bacteria and biocidal sensitivity: interplay between a potent antibiofilm compound (AS162) and quorum-sensing autoinducers

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Abstract
The capacity of two homoserine lactones to stimulate the marine bacteria *Pseudoalteromonas ulvae* (TC14 strain) for its capacity to form a biofilm when exposed to a potent antibiofilm compound AS162 is reported. Effective concentrations (EC_{50}) of AS162 at 24 h, 48 h, and 72 h were, respectively, of 4.3, 4.4, and 6.0 µM. When tested in combination with HSLs, results showed that quorum-sensing signal molecules 3-oxo-C6 and 3-oxo-C8 homoserine lactones do not act directly on the biofilm formation, but are able to interfere positively with AS162 to promote biofilm growth with EC_{50} ranging from 30 to 50 µM. The same results were obtained with two other marine bacterial strains: *Pseudoalteromonas lipolytica* TC8 and *Paracoccus* sp. 4M6. These findings suggest that HSLs can significantly affect the biocidal sensitivity of marine bacteria to antifouling agents.

Keywords Quorum sensing · Biofilm · Antimicrobial · Biocontrol

Introduction

In the marine environment, biofouling is defined as a rapid colonization of microorganisms (bacteria, microalgae, etc.) on artificial or natural surfaces causing large and significant economic damages (Yebra et al. 2004; Fitridge et al. 2012; Qian et al. 2007). To prevent such an undesirable process, antifouling coatings have been routinely used to protect the hulls of ships, as well as of smaller vessels. Such coatings are based on the use of broad-spectrum biocides. As their use is now strictly regulated by International Marine organization (IMO), there is a great need of friendly environmentally antifouling solutions which should act specifically on biofouling without killing targeted and non-targeted microbial species (Ciriminna et al. 2015). For this purpose, non-toxic molecules that could have the ability to inhibit the formation of biofilms in a non-permanent way are of major interest in a goal of respect for ecosystems, and a biomimetic approach should offer a practical solution. In this way, the observation of marine organisms such as corals, sponges, or macroalgae which conserve their surfaces free of fouling (Hamann et al. 1993; Steinberg et al. 1997; Müller et al. 2003; Krug 2006) led to the discovery of secondary metabolites possessing antifouling properties (Tsukamoto et al. 1996a, b, c; De Nys and Steinberg 2002; Bhadury and Wright 2004; de Nys and Fusetani 2004; Hellio et al. 2005; Sipkema et al. 2005; Maréchal and Hellio 2009; Fusetani 2011; Gerwick and Moore 2012; Stowe et al. 2011; Sun et al. 2018). However, although effectives, such molecules inhibit irreversibly the formation of biofilms and generally remain still toxic for marine organisms. In this context, there is a great urge for the conception of smart biocides which could exhibit temporary antibiofilm properties allowing their use in an environmentally friendly way without affecting aquatic life. As a part of our studies aimed to the discovery of original compounds able to modulate biofilm formation, we have initiated a program aimed to a biomimetic approach. This program involved the synthesis of analogues of natural products, with the advantage to be non or poorly toxic (Linares et al. 2011; Andjouh and Blache 2015, 2016), and led

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to the discovery of an original analogue of psammaplin A alkaloid AS162 (Andjouh 2017, 2019) (Fig. 1). To investigate, furthermore, the eco-friendly aspect of such molecule, we report here the potential role of quorum-sensing signal molecules in the biofilm growth of marine bacteria when exposed to this antibiofilm molecule. Quorum sensing (QS) is a signaling pathway present in almost Gram-negative bacteria (Passos da Silva et al. 2017) and is termed as the ability of bacteria to perceive rapidly changes in concentrations of small secreted signalling molecules known as autoinducers (AI-1 and AI-2) (Waters and Bassler 2005). In the marine environment, this pathway of intra- and inter-communication in the bacterial community is of high prevalence and a recent study showed that a large number of marine bacteria can produce autoinducers of type I (homoserine lactones: HSLs) (Muras et al. 2018). In response to these changes, several modifications in the metabolic engine appear, such as biosurfactant production, exopolysaccharide (EPS) production, and biofilm formation. To our knowledge, although QS inhibitors have been largely described to interfere with biofilm formation, no studies showed that QS signal molecules (HSLs) were able to counteract an antibiofilm effect for the restoration of biofilms in bacteria when treated with antibiofilm compounds. For this purpose, we have identified a Mediterranean Sea-isolated strain, *Pseudoalteromonas ulvae*, TC14 to produce violacein and which possesses a functional QS receptor capable of sensing extrinsic homoserine lactones (HSLs) (Ayé et al. 2015). Among all the HSLs tested, two of them (3-oxo-C6-HSL and 3-oxo-C8-HSL) retained our attention for their capacity to interact with the production of violacein. We now report the capacity of these two HSLs to modulate biofilm production and biocidal susceptibility when the bacteria are submitted to the highly efficient antibiofilm compound AS162 (Fig. 1).

Results and discussion

Effect of 3-oxo-C6 and 3-oxo-C8-HSLs on biofilm growth of TC 14

Since the exposition to exogenous HSLs was previously reported to affect positively the bacterial growth and biofilm formation of isolated marine bacteria (*Pseudomonas* genus) (Mangwani et al. 2016), the first point of our study was to check the intrinsic effect of HSLs (3-oxo-C6-HSL and 3-oxo-C8-HSL) on biofilm formation and on bacterial growth of *Pseudoalteromonas ulvae* TC14 over a period of 72 h. It was reported that none of these two HSLs modified TC14 growth compared to the control sample (without added AHLs) over a short time of 7 h (Ayé et al. 2015), suggesting that they do not present any toxicity on this strain in these conditions. Taking into account these observations and preliminary results, biofilm formation as well as viability of the bacteria were monitored over 72 h for a set of concentrations ranging from 10 to 200 µM. Results at 72 h are reported in Fig. 2 (results at 24 h and 48 h for biofilm growth, bacterial growth over 72 h are reported in Supplementary data, Figs. A1 and A2). In spite of the fact that the regulation of biofilm by quorum sensing has often been described in Gram-negative bacteria, our results suggest that these two HSLs did not affect the biofilm growth after 72 h at low-to-medium concentrations. Furthermore, the viability of the bacterial strains was also found to be not affected when exposed to increasing concentrations of HSLs over the 72 h period. According these results, the concentration of 50 µM was retained for studying the combination.

Interplay between HSLs and AS162

Although the mode of action of this molecule is not yet elucidated, AS162 is a potent antibiofilm and non-biocidal compound against various marine bacteria strains at low concentrations (EC_{50} in the range of 5 µM), while at high concentrations (> 100 µM), a biocidal effect was observed decreasing the viability (more than 50% after 7 h) (Andjouh 2017, 2019). Taking into account these results, long-term effect of AS162 and the potential role of HSLs to limit biocidal effect over 72 h were investigated by monitoring the viability of the bacterial population. In this way, the effect of AS162 (respectively, at 10, 25, 50, 100, 150, and 200 µM) over 72 h and the effect of its combination with 3-oxo-C6-HSL and 3-oxo-C8-HSL are reported in Fig. 3. Results showed that AS162 has a long-term effect on viability and that these effects are dose dependent with an LC_{50} (72 h) of 100 µM (Table 1). The same experiment was conducted with AS162 administrated in combination with 50 µM of the two AHLs. No modification of the toxicity profile was

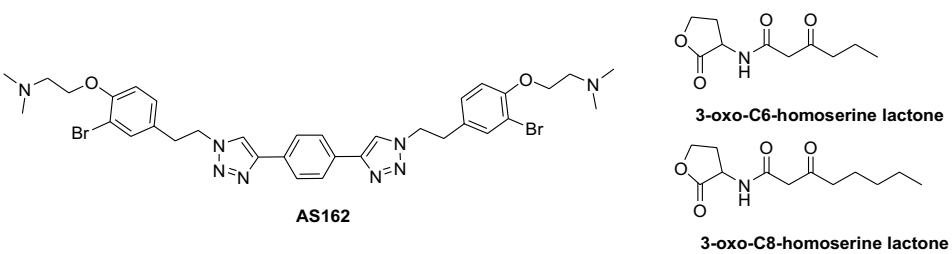

Fig. 1 Structure of the antibiofilm compound AS162, and of the two homoserine lactones studied

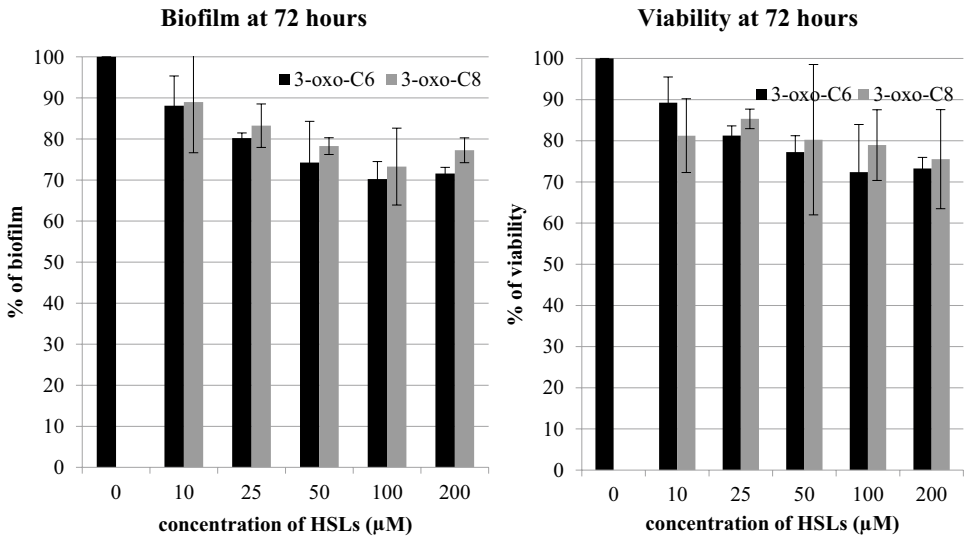

Fig. 2 Left, effect of 6-oxo-HSL (■ 3-oxo-C6) and 3-oxo-C8-HSL (■ 3-oxo-C6) at concentrations of 10–200 µM on biofilm growth of *Pseudoalteromonas ulvae* TC14 at 72 h. Data are expressed as % of biofilm when compared to an untreated sample (100%). Right, effect on viability of *Pseudoalteromonas ulvae* TC14 at 72 h. Data are expressed as % of viable bacteria when compared to an untreated sample with 100% of viability

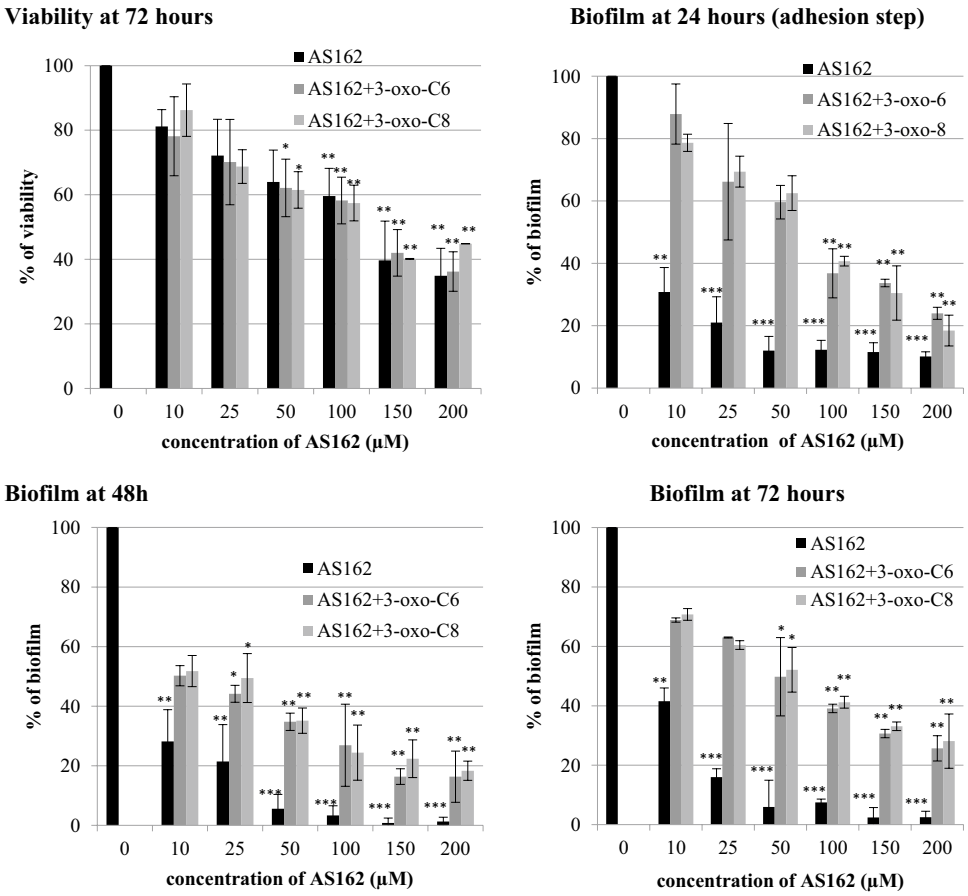

Fig. 3 Effect of combinations on viability of *Pseudoalteromonas ulvae* TC14, data are expressed as percentage of viable bacteria when compared to untreated sample (100%) (up, left). Effect of combinations on the biofilm growth of *Pseudoalteromonas ulvae* TC14 at 24 h (up, right), 48 h (down, left), and 72 h (down, right). Data are expressed as percentage of biofilm when compared to untreated sample (100%) (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$)

noted, indicating that AHLs do not interact directly with the toxicity pathway of AS162. These results finally are in good agreement with our first report (over 7 h) and confirm the potential long-term antimicrobial effect of AS162 at high concentrations. With these elements in hand, the effect of AS162 in combination with AHLs on biofilm growth was studied over 72 h. Results were unambiguous and showed that the two AHLs were able to limit the antibiofilm effect of AS162 (Fig. 3 and Table 1). More precisely, the initial stage of biofilm at 24 h was greatly impacted at a low concentration of 10 µM of AS162, since the biofilm is recovered to more than 80% when compared to untreated

Table 1 Biological screening of compound AS162 and its combination with 3-oxo-C6-HSL and 3-oxo-C8-HSL

Compounds	Antibiofilm activity: EC_{50} (µM)			Toxicity: LC_{50} at 72 h
	24 h	48 h	72 h	
Pseudoalteromonas ulvae TC14				
AS162	4.3 ± 1.0	4.4 ± 1.2	6.0 ± 1.4	100.1 ± 25.9
AS162 + 3-oxo-C6 (50 µM)	64.2 ± 5.7	20.0 ± 3.4	50.9 ± 4.7	97.9 ± 18.7
AS162 + 3oxo-C8 (50 µM)	62.9 ± 8.7	23.0 ± 4.7	55.0 ± 3.4	105.0 ± 1.96
Pseudoalteromonas lipolytica TC8				
AS162	23.2 ± 2.4	29.3 ± 4.0	36.5 ± 2.8	96.5 ± 12.3
AS162 + 3-oxo-C6 (50 µM)	50.8 ± 2.8	37.1 ± 2.7	38.0 ± 7.0	105.7 ± 28.5
AS162 + 3oxo-C8 (50 µM)	52.9 ± 4.7	46.7 ± 1.7	45.2 ± 1.7	105.6 ± 11.5
Paracoccus sp. 4M6				
AS162	8.0 ± 0.8	18.8 ± 2.1	25.9 ± 2.4	78.4 ± 1 2.4
AS162 + 3-oxo-C6 (50 µM)	41.1 ± 1.8	47.8 ± 2.4	44.6 ± 2.6	92.4 ± 4.1
AS162 + 3oxo-C8 (50 µM)	35.2 ± 2.4	53.3 ± 1.4	47.0 ± 3.8	95.5 ± 3.2

Left: antibiofilm activity against bacterial biofilms of *Pseudoalteromonas ulvae* (TC14). *Pseudoalteromonas lipolytica* (TC8), *Paracoccus* sp. (4M6). Results are expressed as effective concentration to inhibit 50% of biofilm formation (EC_{50}) in micromoles/L (µM). Right: toxicity of compound AS162 and its combination with 3-oxo-C6-HSL and 3-oxo-C8-HSL against TC14, TC8, 4M6. Results are expressed as the concentration which causes the death of 50% of the bacterial population (lethal concentration, LC_{50}) in micromoles/L (µM). Data represent means ± standard deviations values from three independent experiments

sample. The same observation was made at 48 and 72 h. Taking in account that at this concentration, only 80% of cells are viable, we can consider that biofilm is recovered at 100% of viable bacteria. When the sample was treated with higher concentrations of AS162 (> 50 µM), we can remark that restoration is lower, but this result is not surprising, since the capacity to form a biofilm is directly connected with the number of viable bacteria [for example, LC_{50} (72 h) = 100 µM and % of biofilm (72 h) at this concentration are only of 50% when compared to untreated sample and these finally correspond to 100% of viable cells).

Taken together, our results indicated that the QS regulation pathway is not directly implicated in biofilm formation of TC14, but indirectly, the two AHLs tested are able to interfere with biofilm growth when the bacteria are treated with an effective antibiofilm compound. This highlights a crucial role of QS in the remediation pathway of this marine strain to bypass the effect of antibiofilm compounds. In addition, these experiments were also extended to two additional strains of marine bacteria responsible of micro-fouling (*Pseudoalteromonas Lipolytica* TC8 and *Paracoccus* sp. 4M6 strains). All the results are reported in Table 1 and Supplementary data (Figs. A3–A7). In a similar manner, it was interesting to note that AHLs did not impact biofilm formation no more than bacterial growth and viability of these two strains, and the resulting EC_{50} of the combinations highlight analogous results, especially at 24 h.

In summary, we have shown that marine bacterial biofilms are able of being positively responsive to exogenous HSLs when submitted to environmental changes such as exposure to antibiofilm molecules. HSLs were observed to promote the biofilm growth of the three strains when exposed to efficient concentrations of the antibiofilm compound AS162. This suggests that in bacterial communities, the interplay with quorum-sensing autoinducers appears of primary importance to maintain the capacity to form biofilms by modulating the efficiency of non-biocidal antibiofilm compounds. In addition, such results should to be taken in account as specific bioassays for assessing a reversible or permanent efficiency of antibiofilm compounds in the course for designing eco-friendly antibiofilm solutions.

Materials and methods

Bacterial strains

Three marine Gram-negative bacterial strains *Pseudoalteromonas ulvae* (TC14), *Pseudoalteromonas lipolytica* (TC8), and *Paracoccus* sp. (4M6) were used in the study (Brian-Jaisson et al. 2014). TC14 was isolated in June 2010 in Little Bay of Toulon (1 m depth, Mediterranean Sea, France) (Brian-Jaisson et al. 2014). The strain 4M6 was provided by the LBCM (Université de Bretagne Sud). It was isolated on glass slides immersed during 6 h at 1 m depth in March 2000 in the Morbihan Gulf (Bailleron Island, 47_3403700 N-2_4405400 W, Atlantic Ocean) (Grasland et al. 2003). TC8 was isolated in February 2008 in Little Bay of Toulon y (1 m depth, Mediterranean Sea, France) (Camps et al. 2011).

Antibiofilm bioassay at 24 h (adhesion bioassays) (Camps et al. 2011; Othmani et al. 2014)

Bacterial strains were grown on Väätänen nine-salts solution (VNSS). When the stationary phase was reached, bacterial suspension was centrifuged. Cells were then diluted in sterile artificial sea water (ASW) and introduced into microtiter plates (sterile black PS; Nunc, Fisher Scientific, France) with tested molecules at eight concentrations (2, 5, 10, 20, 50, 100, 150, and 200 µM) in three replicates in the presence of controls: (i) non-specific staining control and (ii) adhesion control. The maximum percentage of solvent (final concentration = DMSO 2%) used for the dilution of biocides was also tested in triplicate as additional control to insure the non-effect of solvent. After incubation during 24 h, the non-adhered bacteria were eliminated and the adhered cells were quantified after SYTO® 61 (Molecular Probes®Invitrogen, France) (1 µM) staining. A percent of inhibition was calculated per well:

$$\frac{\text{Mean } FIi - nsCi}{\text{Mean } FIc - \text{Mean } B} \times 100$$

with *FIi* as the fluorescence intensity in a treated well (tested compound + bacteria + SYTO® 61), *FIc* as the fluorescence intensity in a control well (bacteria + SYTO® 61), *nsCi* as the non-specific control (tested compound without bacteria + SYTO® 61), and *B* as the blank, i.e., stain control (only SYTO® 61). A sigmoid dose–response curve was obtained when plotted the percentage of inhibition with the log of compound concentrations, after mean ($n = 3$) and standard deviation (SD) calculation per triplicate for each concentration. EC_{50} values were then calculated for each compound using GraphPad Prism® (GraphPad Software, USA). This software allowed also to perform statistical tests dedicated to the analysis of two variables simultaneously, such as the difference between strains and between biocides (two-way ANOVA). Significant differences were accepted when $p < 0.05$ ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

Antibiofilm bioassays at 48 h and 72 h

The evolution of biofilm formation was analyzed using the Crystal Violet (CV) assay following the protocol of Liu et al. (2012) with some modifications. Post-exponential phase *Pseudoalteromonas ulvae* (TC4), *Pseudoalteromonas lipolytica* (TC8), and *Paracoccus* sp. (4M6) subcultures in ASW were centrifuged and resuspended in ASW to reach an OD_{600} of 0.4. The bacterial suspension was then inoculated in 96-well microplates with or without the addition of AS162 and/or AHLs at the corresponding concentrations. After 48 h of incubation at 20 °C under humid conditions, the planktonic cells and media were removed, and the bacteria were washed twice with 100 µL of a solution of sodium chloride (36 g/L) to remove the remaining non-adherent bacteria. The cells were then fixed at a temperature of 50 °C for 30 min. Biofilm was marked with 200 µL of 0.01% (w/v) CV in distilled water for 15 min. Microplates wells were then washed three times with 200 µL of distilled water, followed by a 15 min drying time at room temperature. To release the CV adsorbed by the biofilm cells, a solubilization step was performed by the addition of 100 µL of absolute ethanol per well followed with an agitation of 5 min at 120 rpm. Then, the solution containing ethanol and solubilized CV was transferred from the microplate into another sterile 96-well microplate and the OD of each well was measured at 570 nm (OD570) using a Tecan microplate reader. The data are reported as the CV absorbance at 570 nm of bacterial samples subtracted from CV absorbance of the sterile media containing AS162 and/or AHLs and divided by CV absorbance of the sterile media which were used as controls into a microplate and treated in the same manner as wells containing bacteria.

Toxicity tests: (Camps et al. 2011; Othmani et al. 2014)

After growth on VNSS, bacterial strains were picked up during the exponential phase. The microtiter plates (sterile transparent PS) were filled as described in the protocol of the antiadhesion assay but using VNSS instead of ASW to allow bacterial growth. The bacterial growth was followed by measuring the turbidity ($OD_{600\ nm}$) during 72 h. Then, resazurin (50 µM) was added in all the wells, and fluorescence was measured after 2 h to quantify the percent of bacterial viability. The same methodology used with SYTO® 61 was applied to calculate a percent of viability after resazurin staining. Only compounds with EC_{50} lower than 200 µM were tested and experiments were performed at a concentration of 100 µM of each compound (AS 162 and combinations) with an untreated sample as a reference.

Statistical tests and calculations of EC_{50} and LC_{50}

EC_{50} and LC_{50} values were calculated for each compound using GraphPad Prism® (GraphPad Software, USA). This software allowed also to perform statistical tests dedicated to the analysis of two variables simultaneously, such as the difference between strains and between compounds (two-way ANOVA). Significant differences were accepted when $p \leq 0.05$ ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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