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Biofilm formation by *Pseudomonas aeruginosa*: role of the C_4-HSL cell-to-cell signal and inhibition by azithromycin

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***Objectives*: In *Pseudomonas aeruginosa*, biofilm formation is controlled by a cell-to-cell signalling circuit relying on the secretion of 3-oxo-C_{12}-HSL and C_4-HSL. Previous studies suggested that C_4-HSL plays no significant role in biofilm formation. However the wild-type PAO1 strain PAO-BI, used as a control in these studies is itself impaired in the production of C_4-HSL. We wondered therefore whether the role of C_4-HSL in biofilm formation might have been underestimated, and whether azithromycin inhibits biofilm formation by interfering with cell-to-cell signalling.**

***Methods*: We used isogenic mutants of wild-type PAO1 strains PAO-BI and PT5 in a static biofilm model. Biofilm formation was quantified using Crystal Violet staining and exopolysaccharide measurements.**

***Results*: Wild-type strain PAO-BI, as a result of its reduced C_4-HSL secretion, produced 40% less biofilm compared with the wild-type PAO1 strain PT5. Using isogenic mutants of strain PT5 we have shown that whereas a *lasI* mutant (deficient in 3-oxo-C_{12}-HSL) produced similar amounts of biofilm to the wild-type, a *rhlI* mutant (deficient in C_4-HSL) produced 70% less biofilm. In the latter strain, biofilm formation could be restored by addition of exogenous C_4-HSL. Azithromycin, known to reduce the production of both 3-oxo-C_{12}-HSL and C_4-HSL, inhibited biofilm formation of wild-type PT5 by 45%. This inhibition could be reversed by the addition of both cell-to-cell signals.**

***Conclusions*: Our results indicate that C_4-HSL also plays a significant role in biofilm formation. Furthermore, we demonstrate the potential of using cell-to-cell signalling blocking agents such as azithromycin to interfere with biofilm formation.**

Keywords: quorum sensing, autoinducers, homoserine-lactones, 3-oxo-C_{12}-HSL

Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is responsible for severe ventilator-associated pneumonia (VAP) in intubated patients. In these patients, colonization of the upper respiratory tract by *P. aeruginosa,* which almost always precedes pneumonia, is related to the presence and duration of an endotracheal intubation. The colonization of inert surfaces by *P. aeruginosa* requires a sessile bacterial growth pattern, called a biofilm.[1] The acyl homoserine lactone based cell-to-cell signals (autoinducers, AHLs) 3-oxo-C_{12}-HSL and C_4-HSL participate in a cell-to-cell signalling (quorum-sensing) regulatory network that controls the production of several extracellular virulence factors and are important for biofilm formation.[2] Synthesis of these molecules depends on two autoinducer-synthase genes, *lasI* and *rhlI*, respectively.[2] Both AHLs are produced *in vitro* by *P. aeruginosa* isolates from chronically infected cystic fibrosis (CF) patients,[3] *ex vivo* in sputum from CF patients infected by *P. aeruginosa*,[4] as well as in lungs of mice chronically infected by *P. aeruginosa*.[5] AHLs have been detected *in situ* on urethral catheters and *in vitro* using a *P. aeruginosa* biofilm model,[6] as well as in lung secretions of CF patients.[7,8] Based on studies using the wild-type PAO1 strain PAO-BI and its derived *lasI* mutant PAO-JP1, it has been shown that 3-oxo-C_{12}-HSL is required for biofilm differentiation.[9] Using the same strain PAO-BI, it was observed that the autoinducer synthase gene *lasI*, required for 3-oxo-C_{12}-HSL production, is expressed in a large number of cells during the initial phase of biofilm development and that this number decreases progressively over time.[10] In contrast, *rhlI*, the autoinducer synthase gene required for C_4-HSL production, was expressed in fewer cells but remained constant over time. Based on these observations, it has

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Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Source or reference
E. coli		
MG4λ$I_1$4	λ lysogen carrying a chromosomal *lasI::lacZ* fusion	40
P. aeruginosa		
PAO-BI	PAO1 wild-type (*nfxC*)	14
PAO-JP1	PAO-BI (*nfxC*, Δ*lasI*)	41
PT918	PAO-BI (*nfxC*, *mexE*::ΩHg)	this study
PDO100	PAO1 (*rhlI*::Tn*501*)	42
PAO-JP2	PDO100(Δ*lasI)*	41
PT5	PAO1 wild-type	14
PT466	PT5(Δ*lasI*)	18
PT454	PT5(Δ*rhlI*)	18
PT502	PT5(Δ*lasI,* Δ*rhlI*)	31
Plasmids		
pECP61.5	*rhlA′-lacZ* transcriptional fusion	41

been suggested that C_4-HSL might be of little importance in biofilm formation.[10] This is surprising as it has recently been shown that *ex vivo, P. aeruginosa* isolates from CF patients grown in biofilm produce higher amounts of C_4-HSL compared with 3-oxo-C_{12}-HSL.[4] It has also been suggested that this AHL production pattern could be used as a marker for *P. aeruginosa* growing in biofilm in CF patients.[4] We have detected AHLs *in situ* directly in the lung tissue of CF patients infected by *P. aeruginosa* and found a similar elevated C_4-HSL/3-oxo-C_{12}-HSL ratio.[11] Moreover, the *rhl* cell-to-cell signalling system (dependent on the production of C_4-HSL) has been suggested to be important for survival of bacteria during the anaerobic biofilm mode of growth encountered in CF lung disease,[12] and rhamnolipids whose production is controlled by C_4-HSL[2] has been shown to be required for the maintenance of biofilm architecture.[13] Together, these observations suggest that C_4-HSL might be more important for biofilm formation than previously thought.

It has been recently understood that wild-type PAO1 strains from various laboratories might differ in cell-to-cell signal production.[14] In particular, strain PAO-BI produces less C_4-HSL than other PAO1 strains.[14] We have recently described that the macrolide antibiotic azithromycin inhibits virulence factor production of *P. aeruginosa* by interfering with the cell-to-cell signalling circuit.[15] We wondered whether the reduced production of C_4-HSL by strain PAO-BI might have affected previous biofilm studies using this strain as a control strain, and whether azithromycin affects biofilm formation by interfering with AHL production.

Materials and methods

Media, antibiotics and strains

Unless otherwise stated, bacteria were freshly streaked from frozen stocks onto LB-agar medium supplemented with antibiotics when appropriate. Liquid LB cultures, containing antibiotics when appropriate, were grown overnight at 37°C with agitation (250 rpm). Minimal AP medium supplemented with 0.3 M NaCl was used for biofilm growth experiments.[16] Antimicrobials were supplied at the following concentrations: gentamicin (15 mg/L), tetracycline (50 mg/L), mercury chloride (12.5 mg/L). All chemicals and antimicrobials were purchased from Sigma, except azithromycin which was kindly provided by Pfizer. The bacterial strains and their genetic linkage are presented in Table 1. To construct strain PT918 (PAO-BI *mexE*::ΩHg), the *mexE*::ΩHg mutation from strain PT121[17] was transferred by transduction using phage E79*tv*2[18] into strain PAO-BI.

Autoinducer measurements

For extraction of autoinducers from liquid cultures, bacterial strains were grown in LB medium until they reached stationary phase (6 h). Aliquots of filtered culture supernatants were subjected to two extractions by addition of one volume of acidified ethyl acetate (0.01% acetic acid). For extraction of autoinducers from biofilms, polyvinyl chloride (PVC) fragments on which biofilms had been formed were rinsed three times and immersed into 1 mL of 0.9% NaCl. One volume of acidified ethyl acetate was added and the mixture incubated in closed tubes at 4°C overnight. A second extraction was carried out for 1 h, and the two extracts were pooled. The extracted autoinducers were quantified using specific bioassays using *E. coli* MG4λ$I_1$4 (pPCS1) for 3-oxo-C_{12}-HSL and *P. aeruginosa* PAO-JP2 (pECP61.5) for C_4-HSL as reporter strains.[19] β-Galactosidase (β-gal) activity was determined as previously described.[20]

Static biofilm formation assay

Sterile PVC strips of approximately 1 cm^2 were cut from original endotracheal devices (Mallinckrodt Inc., St Louis, MO, USA) and biofilm was allowed to develop under static conditions for 3 days as previously described.[21] All PVC strips were weighed and experiments were standardized by weight. Bacterial strains were grown in LB medium for 6 h with agitation at 37°C. PVC strips were immersed in the bacterial culture medium for 1 h without agitation to allow bacteria to adhere. PVC strips were then transferred into AP medium and incubated for 3 days without agitation at 37°C. Azithromycin (2 mg/L) and/or autoinducers (10 µM each, once a day) were added as indicated. After the 3 day incubation period, the PVC strips were individually washed twice in 0.9% NaCl and subjected to biofilm measurements (see below). We had previously observed that initial growth of wild-type PT5 was slightly retarded in the presence of 2 mg/L azithromycin when grown under agitation in liquid media.[15] This effect was restricted to early and mid-exponential growth phases, when quorum sensing is not yet active. In order to determine

whether this small delay could affect final growth in our 3 day static biofilm assay, we measured colony forming units (cfu) of strain PT5 grown without agitation after 3 days in the biofilm medium in the presence and absence of 2 mg/L azithromycin. Azithromycin did not affect the number of viable counts of wild-type strain PT5.

Crystal Violet staining and exopolysaccharide determinations

PVC strips, on which biofilm had developed, were incubated for 15 min in a Crystal Violet staining solution (0.1% in distilled water),[22] and washed three times in distilled water. The stain was then dissolved in ethanol and absorbance was measured at 570 nm as previously described.[22]

Exopolysaccharide determinations were carried out by total carbohydrate assays.[23] PVC strips were washed in 0.9% NaCl, and incubated in an equal volume of 5% phenol to which 5 volumes of H_2SO_4 containing hydrazine sulphate was added. The mixture was incubated for 1 h in the dark and absorbance was measured at 490 nm.

Results and discussion

Role of C_4-HSL and 3-oxo-C_{12}-HSL in total biofilm formation

Most *in vitro* studies have investigated the potential role of cell-to-cell signals in the formation of *P. aeruginosa* biofilm using a continuous flow system.[9,10,24,25] In such bioreactors a constant medium flux brings new nutrients and eliminates metabolic products. These experimental conditions are therefore very different from *in vivo* situations such as biofilm formation on endotracheal intubation devices. Indeed the bacteria growing inside the secretions that accumulate above the cuff of intubation devices are in static growth conditions without regular change of their growth medium. We therefore decided to adopt a static *in vitro* biofilm model[21] using sterile PVC strips derived from original intubation devices. Previous experiments, using continuous flow systems, have shown that the total amount of biofilm formed by the *lasI* mutant PAO-JP1 is not reduced compared with its parent strain.[9,25] As experimental conditions greatly influence biofilm formation,[25] we first compared biofilm formation by mutant PAO-JP1 and its parent strain the PAO1 wild-type strain PAO-BI in our static biofilm assay. Biofilm measurements using Crystal Violet staining gave similar results for both strains (Figure 1a), confirming that the *lasI* mutation does not affect total biofilm for-mation in our biofilm model.

In previous studies, strain PAO-BI and its derived *lasI* mutant PAO-JP1, as well as the non-*nfxC rhlI* mutant PDO100, have been used to determine the respective roles of autoinducers in biofilm formation.[9,10,24,25] Such studies have suggested that C_4-HSL does not play a significant role in biofilm formation.[9,10] However, we have recently shown that strain PAO-BI bears the *nfxC* mutation.[14] This mutation leads to overexpression of the MexEF-OprN multidrug efflux system, and to resistance towards quinolones, chloramphenicol, trimethoprim and tetracycline. More importantly, this mutation also leads to a reduction in *rhlI* expression, responsible for lower C_4-HSL production, compared with the non-*nfxC* wild-type PAO1 strain PT5.[14] Therefore, we wondered whether the reduced expression of *rhlI* by strains PAO-BI and PAO-JP1 might have led to an underestimation of the role of C_4-HSL in biofilm formation. In accordance with a recent report,[24] we have shown that the *rhlI* mutant PDO100 formed slightly less biofilm compared with strain PAO-BI (Figure 1a). More importantly, strain PAO-BI only produced 60% of the total biofilm compared with wild-type PT5 (Figure 1a), suggesting that overexpression of the MexEF-OprN multidrug efflux system reduces the biofilm production of strain PAO-BI. In order to confirm this result we deleted the *mexE* gene that encodes the membrane fusion protein of the MexEF-OprN efflux pump[17] in strain PAO-BI to obtain mutant PT918. Such a deletion leads to complete inactivation of the MexEF-OprN efflux system, therefore suppressing the phenotype associated with pump overexpression.[14] When tested in our static biofilm model, mutant PT918 was completely restored in biofilm formation to wild-type PT5 levels (Figure 1a). We also measured C_4-HSL production in culture supernatants of strains PT5, PAO-BI and PT918 by a specific bioassay. C_4-HSL production, reduced by 50% in strain PAO-BI, was restored to strain PT5 levels by the inactivation of the MexEF-OprN efflux system in strain PAO-BI (Table 2). These results confirm that the *nfxC* mutation is responsible for a reduction in biofilm formation by strain PAO-BI resulting from decreased C_4-HSL production.

In order to determine the role of C_4-HSL during biofilm formation, we decided to compare the total biofilm formed by wild-type PT5 and its derived *lasI* (PT466), *rhlI* (PT454), and *lasI/rhlI* (PT502) mutants. First, the production of C_4-HSL was measured in the culture supernatants of these mutants. Some reduction in C_4-HSL production can be expected in a *lasI* mutant as the *las* quorum-sensing system controls the transcription of *rhlR*, itself required for optimal C_4-HSL production.[26] Indeed the C_4-HSL production of the *lasI* mutant PT466 was reduced by 55% compared with its parent strain, probably as a result of the *las/rhl* hierarchy. The *lasI* mutant PAO-JP1 was even more severely affected and produced only 2.5% compared with its parent strain and 1.5% compared with strain PT5 (Table 2). As expected, culture supernatants of mutants PT454, PT502 and PDO100 contained no detectable C_4-HSL (Table 2).

When biofilm production was assessed, the *lasI* mutant PT466 produced slightly more biofilm than strain PT5 (Figure 1b). In contrast, the biofilm formed by the *rhlI* mutant PT454 was reduced by 70% compared with its parent strain (Figure 1b), correlating with the reduction in biofilm formed by the *rhlI* mutant PDO100 (Figure 1a). Furthermore, the double *lasI/rhlI* mutant PT502 formed a similar amount of biofilm as the *rhlI* mutant PT454 (Figure 1b). These results indicate that C_4-HSL is required for full biofilm formation. To confirm this hypothesis, we complemented mutant PT454 by adding exogenous C_4-HSL during the biofilm formation assay. As shown in Figure 1(b), addition of exogenous C_4-HSL partially restored the biofilm formation of *rhlI* mutant PT454 from 30% to 65% of the wild-type PT5 level. Similarly, in the presence of both exogenous AHLs, the biofilm formed by mutant PT502 increased from 30% to 75% of the PT5 level (Figure 1b). The lack of complete complementation of both mutants through addition of a constant concentration of exogenous autoinducers to the biofilm assay is likely to result from suboptimal AHL concentrations as these vary both in time and space during biofilm formation.[10] We do not believe that secondary mutations in *vfr* and *algC* in our cell-to-cell signalling mutants could be responsible for the lack of complete complementation. Mutations in these genes affect twitching motility, which is cell-to-cell signalling independent.[27,28] As twitching motility did not differ between parent strain PT5 and its derived cell-to-cell signalling mutants PT466, PT454 and PT502 (data not shown), such secondary mutations are unlikely to have occurred in our strains.

These results clearly demonstrate that, at least under our experimental conditions, C_4-HSL is required for optimal biofilm formation by *P. aeruginosa*. Indeed it appears that a previously undiscovered reduced expression of *rhlI*, leading to lower C_4-HSL production, in the wild-type strain PAO-BI, is responsible for an underestimation of the role of this autoinducer in biofilm formation. The importance of C_4-HSL is indeed not visible when PAO-BI is used as the control

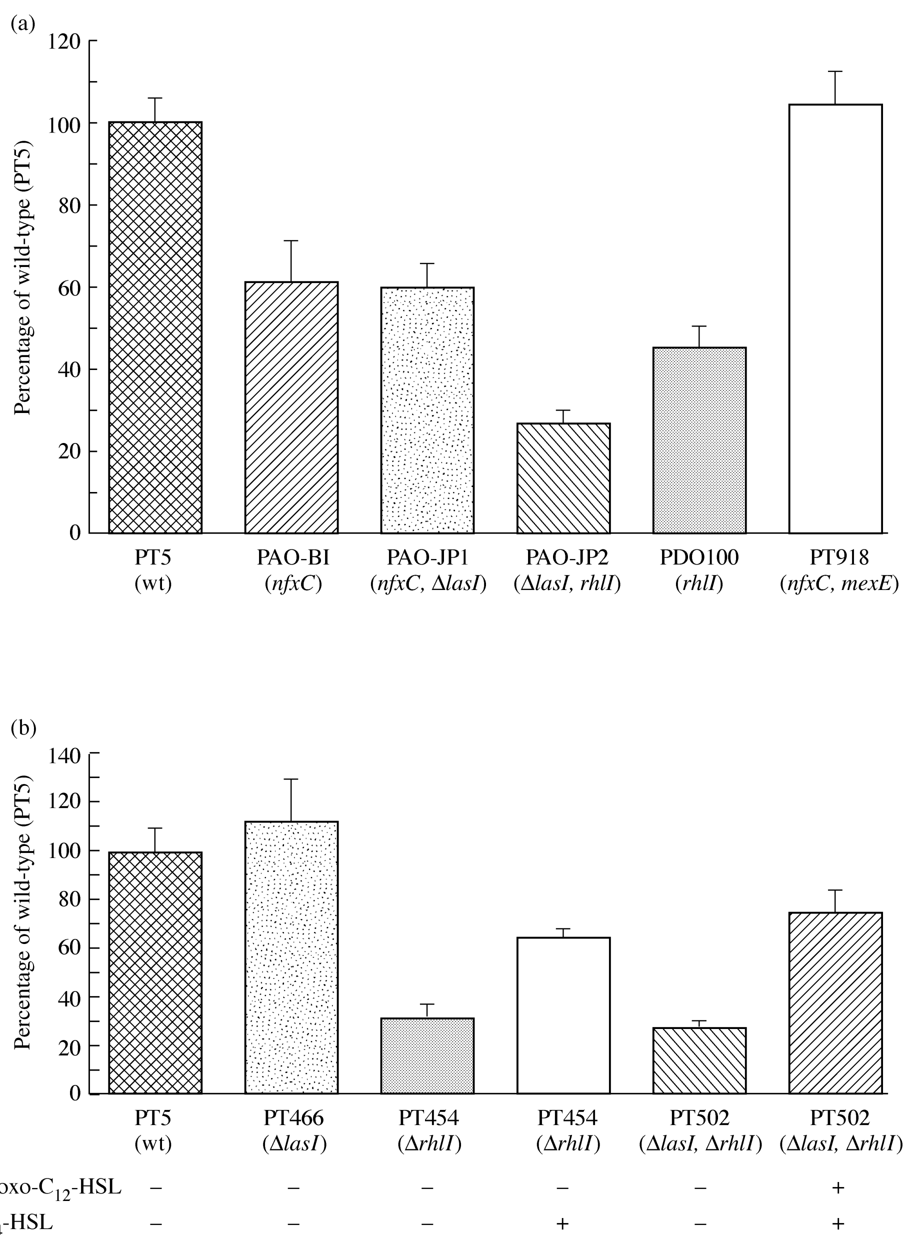

Figure 1. (a) Biofilm formation in wild-type *Pseudomonas aeruginosa* and AHL-deficient mutants. (b) Effects of AHLs on biofilm formation.

strain,[9] or when carrying an *rhlI* reporter fusion.[10] In contrast, the importance of this autoinducer becomes apparent when both the parent and the *rhlI* mutant do not overexpress the MexEF-OprN efflux system. These results correlate well with the recent observation that the *rhl* system is activated during the maturation stage of biofilm development.[29] It appears that while the *las* system (LasR/3-oxo-C_{12}-HSL) is essential for biofilm differentiation,[9] and plays a role during the irreversible attachment stage,[29] the *rhl* system (RhlR/C_4-HSL) also plays an important role.

The finding that *P. aeruginosa* strains overexpressing the MexEF-OprN multidrug efflux system are impaired in biofilm formation is interesting. Such strains can be selected during quinolone treatment in an animal model,[30] and are less virulent in a rat model of acute pneumonia.[31] Whether such multidrug-resistant isolates could be selected during antimicrobial treatment of CF patients, and affect the evolution of the disease deserves further investigation.

Azithromycin inhibits biofilm formation by reducing cell-to-cell signalling

The potential clinical value of antibacterials that would control and/or prevent *P. aeruginosa* infections by interfering with cell-to-cell signalling has recently been underlined.[2,32] The macrolide antibiotic azithromycin is neither bactericidal nor bacteriostatic against *P. aeruginosa* (MIC 64–128 mg/L) at achievable tissue concentrations (1–4 mg/L).[33,34] However, even at low concentrations (1–2 mg/L) azithromycin reduced the adherence of *P. aeruginosa* to polystyrene,[35] as well as the production of exopolysaccharides by *P. aeruginosa*, and has therefore been suggested to inhibit biofilm

Table 2. Concentration of C_4-HSL in culture supernatants of *P. aeruginosa* cell-to-cell signalling mutants after 6 h of growth in LB medium

P. aeruginosa strains	C_4-HSL (μM)
PT5	10.24 ± 0.51
PT466 (*nfxC*Δ*lasI*)	4.41 ± 1.6
PT454 (Δ*rhlI*)	$<10^{-3}$
PT502 (Δ*lasI*, Δ*rhlI*)	$<10^{-3}$
PAO-BI (*nfxC*)	5.57 ± 0.27
PAO-JP1 (*nfxC*, Δ*lasI*)	0.15 ± 0.02
PT918 (*nfxC*, *mexE*)	9.54 ± 0.31
PDO100 (Δ*rhlI*)	$<10^{-3}$

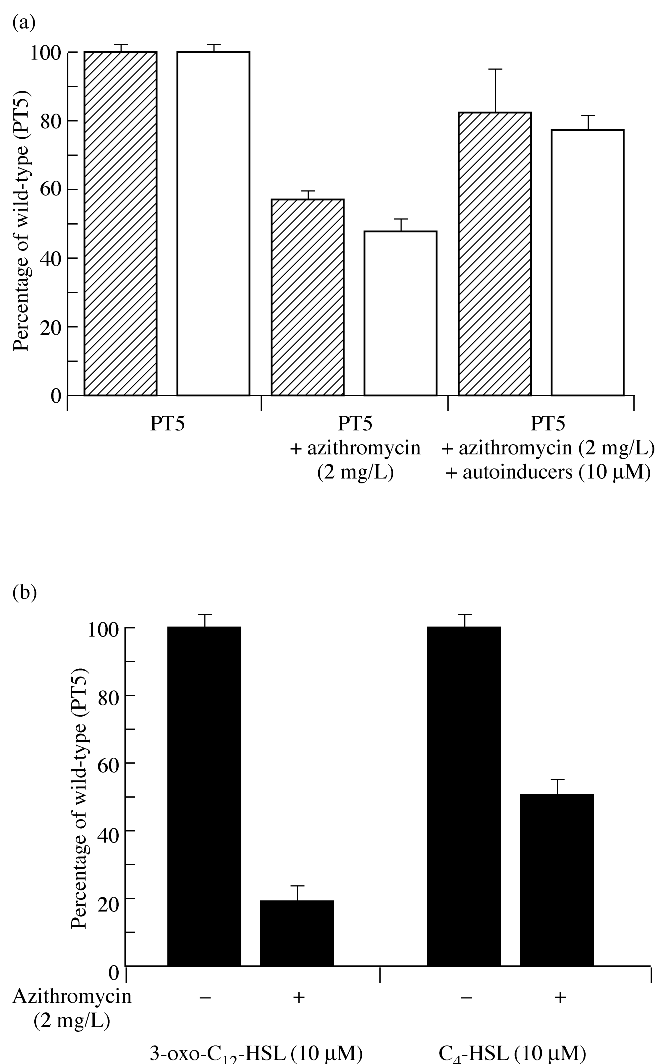

Figure 2. Effect of azithromycin on (a) biofilm formation, determined by Crystal Violet staining (diagonally striped bars) and exopolysaccharide production (white bars) and (b) AHL production.

formation.[36] We have recently shown that 2 mg/L azithromycin inhibits the production of both 3-oxo-C_{12}-HSL and C_4-HSL by *P. aeruginosa.*[15] In view of the above presented results we wondered whether azithromycin inhibits biofilm formation by interfering with autoinducer synthesis. In the presence of 2 mg/L azithromycin, the biofilm formed by strain PT5 was reduced by 45% as quantified by Crystal Violet staining (Figure 2a). This decrease was associated with a 50% reduction in exopolysaccharide production as measured by the total carbohydrate assay. In contrast 2 mg/L azithromycin did not affect biofilm production by mutant PT502 (27% of wild-type in the absence versus 25% in the presence of 2 mg/L azithromycin), suggesting that azithromycin exerts its effect on biofilm formation via inhibition of cell-to-cell signalling.

In order to confirm that azithromycin reduced the production of both AHLs in the biofilm, we directly extracted and measured both AHLs from strain PT5 biofilms grown on PVC strips in the absence and presence of 2 mg/L azithromycin. As expected, both 3-oxo-C_{12}-HSL and C_4-HSL were severely reduced in the presence of the macrolide (Figure 2b). Addition of both AHLs in the presence of azithromycin restored the amount of biofilm formed to 85% of the initial levels observed in the absence of azithromycin as measured by Crystal Violet staining, and to 80% as measured by exopolysaccharide production (Figure 2a). Taken together, these results indicate that 2 mg/L azithromycin inhibits the production of biofilm by wild-type PT5 in our biofilm model at least partially by interfering with the production of the two autoinducers. As 3-oxo-C_{12}-HSL is required for biofilm differentiation,[9] it is likely that azithromycin, by reducing both C_4-HSL and 3-oxo-C_{12}-HSL formation, leads not only to a reduction in the total amount of biofilm formed, but also affects its differentiation. It has been recently described that rhamnolipid surfactant is important for the maintenance of biofilm architecture by maintaining open channels within the biofilm structure.[13] Moreover the *rhl* cell-to-cell signalling system has been suggested to protect *P. aeruginosa* strains growing inside biofilms by controlling the production of nitrite reductase essential for the detoxification from NO produced during growth in anaerobic conditions.[12] Both these functions are dependent on the adequate production of C_4-HSL.[2] It appears therefore that azithromycin by reducing cell-to-cell signal production not only reduces the production of extracellular virulence factors such as elastase,[15] but also interferes with biofilm formation at different levels. This could be particularly useful in clinical situations, such as cystic fibrosis,[4,11] and colonization of intubation devices in which growth in biofilm might play an important role. The recent encouraging results obtained in CF patients treated with azithromycin[37–39] might therefore not only be the result of the suggested anti-inflammatory effect of this macrolide, but also because of its cell-to-cell signalling blocking activity. These results support the need for active research to develop more efficient cell-to-cell signalling blockers that could, according to the results presented here, reduce *P. aeruginosa* biofilm formation on medical devices such as endotracheal intubation devices.

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