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# Role of quorum sensing in UVA-induced biofilm formation in *Pseudomonas aeruginosa*

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**Abstract**

*Pseudomonas aeruginosa*, a versatile bacterium present in terrestrial and aquatic environments and a relevant opportunistic human pathogen, is largely known for the production of robust biofilms. The unique properties of these structures complicate biofilm eradication, because they make the biofilms very resistant to diverse antibacterial agents. Biofilm development and establishment is a complex process regulated by multiple regulatory genetic systems, among them is quorum sensing (QS), a mechanism employed by bacteria to regulate gene transcription in response to population density. In addition, environmental factors such as UVA radiation (400–315 nm) have been linked to biofilm formation. In this work, we further investigate the mechanism underlying the induction of biofilm formation by UVA, analysing the role of QS in this phenomenon. We demonstrate that UVA induces key genes of the Las and Rhl QS systems at the transcriptional level. We also report that *pelA* and *pslA* genes, which are essential for biofilm formation and whose transcription depends in part on QS, are significantly induced under UVA exposure. Finally, the results demonstrate that in a *relA* strain (impaired for ppGpp production), the UVA treatment does not induce biofilm formation or QS genes, suggesting that the increase of biofilm formation due to exposure to UVA in *P. aeruginosa* could rely on a ppGpp-dependent QS induction.

## INTRODUCTION

Biofilms are complex communities of micro-organisms attached to surfaces. These communities are embedded in a matrix of extracellular polymeric substances composed mainly of proteins, DNA and polysaccharides. *Pseudomonas aeruginosa* biofilms have been extensively studied, because of their implication in severe chronic infections [1] and their negative effects in industry [2, 3]. Biofilm formation is known to be a strategy by which bacteria increase their survival, since this way of life makes them more resistant to hostile environments [4–7].

The development of biofilm has been demonstrated to proceed according to an underlying genetic program [8, 9]. Specifically, in *P. aeruginosa*, it is mainly regulated by cyclic diguanosine-5′-monophosphate (c-di-GMP), small RNAs (sRNAs) and the regulatory genetic quorum sensing (QS) system [10]. c-di-GMP is a global intracellular secondary messenger, and current research indicates that c-di-GMP is a general key regulator of the biofilm cycle [11]. It has been reported that high internal levels of c-di-GMP induce the production of adhesins and extracellular matrix components that enable bacteria to form biofilm, whereas low levels down-regulate the synthesis of these matrix components and promote dispersion of biofilm cells [10]. However, sRNAs are conserved post-transcriptional regulators in bacteria [12]. Recent studies on pathogenic bacteria have shown that sRNAs have important effects on biofilm formation and virulence [13, 14].

QS is a mechanism employed by bacteria to regulate gene transcription in response to population density that is mediated by specific signal molecules, called autoinducers, released into the environment [15, 16]. In *P. aeruginosa*, QS includes three main systems: the *N*-acylated homoserine lactone-based Las and Rhl systems, whose signals are *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL), respectively [17], and the 2-alkyl-4-quinolone-based system whose main signal is the *Pseudomonas* quinolone signal (PQS). The Las system comprises the signal synthase LasI, encoded by the gene *lasI*, which produces 3OC12-HSL, and the signal receptor LasR, encoded by the gene *lasR* [18]. The Rhl system comprises

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**Abbreviations:** c-di-GMP, cyclic diguanosine-5′-monophosphate; C4-HSL, *N*-butanoylhomoserine lactone; 3OC12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; qRT-PCR, quantitative real-time PCR; QS, quorum sensing; ROS, reactive oxygen species; sRNA, small RNA.

the signal synthase RhlI, encoded by the gene *rhlI*, which produces C4-HSL, and the signal receptor RhlR, encoded by the gene *rhlR* [17]. When autoinducers reach a given threshold concentration in the cell, they bind to their cognate receptors and expression of certain target genes is activated [19, 20]. PQS provides a link between Las and Rhl; the synthesis of this signal is controlled by the *pqsA–pqsE* operon [21, 22].

The first evidence of the relationship between QS and biofilms was reported in 1998 by Davies *et al.* [23]. They showed that a mutant strain defective in QS was unable to form fully mature biofilms. Later, other authors demonstrated that *N*-acylated homoserine lactone analogues able to inhibit QS hindered biofilm development of *P. aeruginosa* in flow-chambers [24]. Moreover, it is known that QS is important in the regulation of biofilm matrix components. It has been demonstrated that PQS has an essential role in extracellular DNA generation [25]. In addition, QS controls the production of rhamnolipids, an extracellular secondary metabolite crucial to normal biofilm architecture [26]. It has also been suggested that QS positively regulates the expression of *pel* and *psl* operons, which are responsible for the synthesis of the two main matrix polysaccharides, Pel and Psl [27]. Sakuragi and Kolter [28] demonstrated that the Las QS system is involved in *pel* transcription through its role in activating the Rhl QS signalling system.

In addition to being regulated genetically, biofilm formation is modified by environmental factors such as temperature, nutrients, pH, oxidative stress, interaction with the host, antimicrobials and UV radiation [29–33]. Enhancement of biofilm formation in *P. aeruginosa* by moderate doses of UVC radiation (280–100 nm) has been reported, suggesting a relationship between this phenomenon and the increase of the multi-functional RecA protein, a key factor in DNA stability and repair [31]. In nature, this radiation does not reach the atmosphere, because it is completely absorbed by the ozone layer; however, it is commonly employed in germicidal devices. More recently, Pezzoni *et al.* [33], demonstrated that UVA radiation (400–315 nm) induces biofilm formation in *P. aeruginosa*, enabling the biofilm cells to achieve a high level of protection against lethal UVA doses [7]. UVA, the major fraction of solar UV radiation reaching the Earth's surface, is one of the main stress factors that bacteria face in the environment [34–36]. It has been demonstrated that the lethal effects of UVA are produced by oxidative damage caused by reactive oxygen species (ROS) generated by the absorption of the light by endogenous photosensitizers (e.g. flavoproteins, cytochromes and quinones) in the presence of oxygen [37, 38]. The lethal effects of UVA radiation have been used for disinfection purposes in strategies such as SODIS (SOlar DISinfection of natural waters) and photocatalytic treatments [39, 40]. However, exposure to low doses of UVA induces several sublethal effects, such as a transient inhibition of bacterial growth without significant cell death [41], and loss of phage susceptibility [42], as well as adaptive responses related to the expression of genes related to DNA repair and antioxidative defence [43–46].

**Table 1.** Strains and plasmids

| Strain/plasmid | Relevant genotype and/or phenotype | Reference |
|---|---|---|
| ***P. aeruginosa*** | | |
| PAO1 | Wild-type | [92] |
| PAO-JP1 | *lasI*::Tet, derived from PAO1 strain | [93] |
| PDO100 | *rhlI*::Tn*501*-2, derived from PAO1 strain | [94] |
| PAO-JP2 | *lasI*::Tet *rhlI*::Tn*501*-2, derived from PDO100 strain | [93] |
| PW2798 | *pqsA*::Is*lacZ*/hah, derived from PAO1 strain | [95] |
| PW2696 | *relA*::Is*lacZ*/hah, derived from PAO1 strain | [95] |
| PAO1Δ*pel* | *pelA*; polar mutant of the *pel* operon; markerless | [96] |
| PAO1Δ*psl* | *pslBCD*; polar mutant of *psl* operon; markerless | [97] |
| PAO1Δ*pel psl* | Δ*pelApslBCD*; markerless | [96] |
| ***E. coli*** | | |
| DH5α | F-ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44* | Gibco |
| **Plasmids** | | |
| pKDT1.7 | *lasB′–lacZ lasR* | [93] |
| pECP61.5 | *rhlA′–lacZ ptac-rhlR* | [93] |

In order to further the understanding of the mechanism underlying the induction of biofilm formation in *P. aeruginosa* by UVA, we analysed the role of QS in this phenomenon. Approaches involving the use of mutants and analysis of gene expression were employed, and it was concluded that this regulatory genetic system plays a leading role in triggering biofilm formation of *P. aeruginosa* cells exposed to sublethal doses of UVA radiation.

## METHODS

### Bacterial strains, plasmids and culture conditions

Table 1 lists the strains and plasmids used in this study. Plasmids were maintained with 100 µg ampicillin $ml^{-1}$ in *Escherichia coli* or 200 µg carbenicillin $ml^{-1}$ in *P. aeruginosa*. Bacterial cultures were grown at 37 °C with shaking in complete Luria–Bertani (LB) broth (10 g tryptone, 5 g yeast extract and 5 g NaCl made up to 1000 ml with distilled water); for solid medium 15 g agar $l^{-1}$ was added. When required, synthetic *P. aeruginosa* autoinducers (Cayman Chemicals) were added to cultures at final concentrations of 2 µM for 3OC12-HSL and 10 µM for C4-HSL, according to Schuster *et al.* [47].

## Irradiation source

Cell suspensions were irradiated using a bench with two Philips TDL 18W/08 tubes (>95% UVA emission at 365 nm). The incident fluence under the experimental conditions was measured at the surface of the suspensions with a 9811.58 radiometer (Cole-Parmer Instruments). The UVA tubes were mounted on aluminium anodized reflectors to enhance the fluence rate on the section to be irradiated.

## Biofilm formation

Overnight cultures were diluted to $OD_{650}$ 0.05 in LB medium and divided into two 15 ml fractions, each of which was placed in a glass beaker (4.5 cm internal diameter) containing sterile glass slides (20×25×1 mm). The slides were placed vertically at the bottom to allow biofilm development at the air–liquid interface [48]. The beakers with the slides were placed in a multichamber coupled to a thermocycler bath, so that the temperature of the suspensions was maintained at 37 °C. One of the fractions was irradiated from above at a fluence rate of 25 W $m^{-2}$ at the level of the free surface of the suspension, while the other was covered with a black plastic sheet (dark control). The fluence employed in this study may be encountered normally in the environment [49].

## Biofilm analysis

### Concentration of bacterial cells

Biofilm cell concentration was determined by counting the number of c.f.u. per area unit. To do so, slides containing the biofilms were removed at the specified times and washed by letting sterile distilled water (about 10 ml) drop down gently on them to remove unattached cells. The bacterial biomass was then scraped from the glass with a sterile plastic spatula, recovered in 0.5 ml saline solution (0.1 M NaCl in water) and homogenized by vigorous vortexing. Appropriate dilutions of these suspensions were plated on LB solid medium. Plates were incubated in the dark immediately after irradiation and the colonies were counted after incubation for 24 h at 37 °C.

### Total biofilm biomass

Total biofilm biomass was evaluated by staining with crystal violet. Slides carrying biofilms were washed as described above to remove unattached cells. Slides were then stained for 30 min with 0.1% (w/v) aqueous crystal violet solution. The crystal violet attached to the slides was dissolved in a mixture of 96% ethanol and 30% acetic acid (1:1). Absorbance at 575 nm was measured in the resulting solution.

### Isolation and chemical analysis of biofilm matrix

The process of biofilm harvesting and matrix fractionation was based on previous studies [50–52]. Briefly, the slides were removed from the glass beakers and washed once with distilled water. The biofilms were carefully scraped from the glass surfaces and suspended in 0.1 M NaCl. The cells were dispersed by vigorous stirring for 5 min at room temperature and then separated by centrifugation for 30 min at 4 °C. The absence of cells in the supernatant (matrix fraction) was confirmed by plating on solid LB medium. The viable cell number obtained in the cell fraction was similar to that observed in biofilm assays without matrix separation, indicating that no significant cell lysis was generated by the procedure. The protein, DNA and total polysaccharide content was evaluated in the matrix fraction. DNA and proteins were quantified without previous purification. Total polysaccharides were separated by precipitation with ethanol at −20 °C and centrifugation [50], before quantification by the phenol/sulfuric acid method [53], using glucose (Merck) as a standard. Protein content was determined by Lowry's method [54], using bovine albumin (Sigma) as a standard. Extracellular DNA was quantified by evaluating the absorbance at 260/280 nm using a NanoDrop 2000 instrument (Thermo Scientific).

## Autoinducer extraction

Logarithmic ($OD_{650}$ 0.1 and 0.3) and 24 h stationary cultures were centrifuged for 10 min at 10,000 r.p.m. and supernatants were extracted twice in equal volumes of acidified ethyl acetate. The extracts were stored at −20 °C until further use in the bioassays [55, 56].

## Autoinducer bioassays

Autoinducer levels in culture supernatants were determined in bioassays using reporter plasmids carrying *lacZ* fusions dependent on QS signals for their expression, as previously described [55, 57]. The reporter strains were *E. coli* DH5α pKDT1.7 and *P. aeruginosa* PAO-JP2 pECP61.5 (Table 1). The plasmid pKDT1.7 is specific for 3OC12-HSL. It contains a copy of the *lasR* gene as well as a *lasB′–lacZ* fusion, so addition of 3OC12- HSL results in the induction of the *β*-galactosidase enzyme. The plasmid pECP61.5 was assayed in *P. aeruginosa* PAO-JP2, because it was more sensitive than DH5α [57]. This plasmid contains a copy of *rhlR* and a *rhlA′–lacZ* fusion whose expression is specific for C4-HSL. Specificity of reporter plasmids in our experimental conditions was verified using synthetic autoinducers.

### 3OC12-HSL bioassay

An overnight culture of the DH5α pKDT1.7 strain was diluted to an $OD_{650}$ of 0.05 and grown at 37 °C with shaking to an $OD_{650}$ of 0.3. At this time, 5 ml aliquots of the culture were transferred to flasks containing test samples, grown for 90 min at 37 °C and *β*-galactosidase activity was assayed.

### C4-HSL bioassay

An overnight culture of the PAO-JP2 pECP61.5 strain was diluted to an $OD_{650}$ of 0.1 and grown at 37 °C with shaking to an $OD_{650}$ of about 0.3. At this time, 5 ml aliquots of the culture were transferred to flasks containing test samples. Growth was continued overnight and *β*-galactosidase activity was assayed.

## *β*-Galactosidase activity assay

*β*-Galactosidase activity was assayed as described by Miller [58] in cells treated with 5 µl de SDS 0.1% and 10 µl of chloroform per sample. Specific activities are expressed in Miller units with reference to $OD_{650}$.

**Table 2.** Primers

| qRT-PCR primers | Sequence (5′→3′) | Reference |
|---|---|---|
| 16S Fw | AGCTTGCTCCTTGATTCAGC | [46] |
| 16S Rv | AAGGGCCATGATGACTTGAC | [46] |
| *lasI* Fw | TGTTCAAGGAGCGCAAAGG | [98] |
| *lasI* Rv | ATGGCGAAACGGCTGAGT T | [98] |
| *rhlI* Fw | TGCTCTCTGAATCGCTGGAA | [98] |
| *rhlI* Rv | GTTTGCGGATGGTCGAACTG | [98] |
| *lasR* Fw | AGCGACCTTGGATTCTCG AAG | [98] |
| *lasR* Rv | CGAAGAACTCGTGCTGCTTTC | [98] |
| *rhlR* Fw | TTGCTGAGCGTGCT TCC | [98] |
| *rhlR* Rv | AGGATGATGGCGATTTCC C | [98] |
| *pelA* Fw | CCTTCAGCCATCCGTTCTTCT | [65] |
| *pelA* Rv | TCGCGTACGAAGTCGACCTT | [65] |
| *pslA* Fw | AAGATCAAGAAACGCGTGGAAT | [65] |
| *pslA* Rv | TGTAGAGGTCGAACCACACCG | [65] |

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by using a total RNA extraction kit (Ambion-Life Technology). After treatment with DNase I, cDNA was obtained using random hexamers (Promega) and avian myeloblastosis virus reverse transcriptase (Promega), following the manufacturer's instructions. qRT-PCR was performed using a real-time PCR cycler (Rotor-Gene Q; Qiagen) and real-time PCR mix (qPCR mix; Biodynamics). The primers employed are listed in Table 2. The cycling conditions for *lasI*, *rhlI*, *lasR* and *rhlR* genes were as follows: denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 25 s, 58 °C for 15 s and 72 °C for 15 s. The same conditions were used for *pelA* and *pslA* amplification, except that the annealing temperature was 55 °C. The 16S rRNA gene was used as a reference for normalization of the expression levels of target genes under each condition [46]. Relative changes in the expression of individual genes between the treated and control conditions were obtained through the relative standard curve method [59].

### Statistical analysis

The significance of each treatment was evaluated by an unpaired two-tailed Student's *t*-test with confidence levels at >95% (i.e. $P<0.05$ was considered significant). Significance levels indicated in the figures correspond to UVA-treated versus control conditions; details of additional statistical analysis have been included in the text.

## RESULTS

### Role of QS in UVA-induced biofilm formation

In order to analyse the role of QS in the induction of biofilm formation by UVA, we compared biofilms of the wild-type PAO1 and QS mutants; all of them were obtained from growing cultures exposed to sublethal doses of UVA radiation or kept in the dark. The QS mutants comprised PAO1 derivatives deficient for the synthesis of the signals 3OC12-HSL (*lasI* strain), C4-HSL (*rhlI* strain), 3OC12-HSL and C4-HSL (*lasI rhlI* strain), and PQS (*pqsA* strain). Fig. 1a shows the effect of UVA exposure on the number of viable cells in 24 h biofilms. As described previously [33], a significant increase in the viable cell count was observed in PAO1 biofilms formed under UVA radiation ($P<0.05$). On the contrary, the QS-deficient mutants *lasI*, *rhlI* and the double mutant *lasI rhlI* showed no difference in the number of viable cells for 24 h biofilms grown under UVA with regard to their respective biofilms formed in the dark. Compared to the wild-type strain, these mutants showed a reduced capacity ($P<0.05$) to form biofilm both in the dark and under UVA exposure. When the *pqsA* mutant was analysed, behaviour similar to that of the wild-type was observed with regard to the significant induction of biofilm formation under UVA exposure ($P<0.005$). In the dark, *pqsA* biofilms showed lower values of biofilm cells compared to PAO1 ($P<0.05$); however, no significant difference was observed between the number of cells of *pqsA* and PAO1 biofilms developed under UVA radiation.

The total biofilm mass in the control and UVA-treated 24 h biofilms was evaluated by staining with crystal violet, a dye commonly used to visualize and quantify biofilm, which stains not only cells but also any biological material adhered to the surface. Biofilm quantification by crystal violet staining followed the same tendency as the viable cell count, showing a significant increase in the PAO1 ($P<0.005$) and the *pqsA* ($P<0.05$) strains compared to their respective dark controls; conversely, in the other QS mutants, no significant difference was observed due to the treatment (Fig. 1b). In non-exposed cultures, it was possible to observe a significant decrease in the biofilm formation of *lasI rhlI* mutant compared to the PAO1 strain ($P<0.05$), but no significant differences were observed between the biofilms of *lasI, rhlI* and *pqsA* mutants compared to the wild-type (Fig. 1b). No difference was observed between biofilms formed under UVA exposure for *pqsA* and PAO1 strains; on the contrary, significant differences ($P<0.005$) were observed between the biofilms of the other QS mutants and PAO1 under this treatment.

In order to confirm these results, *lasI*, *rhlI* and *lasI rhlI* mutants were grown to stationary phase in the presence of synthetic autoinducers and 24 h biofilms were analysed. As shown in Fig. 1c, d, addition of 3OC12-HSL to the *lasI* strain restored its phenotype at a similar level to that observed in the wild-type with regard to biofilm production in the dark (Fig. 1a, b), as well as the positive effect of the radiation. The same was observed when C4-HSL was added to the culture medium of the *rhlI* strain and both autoinducers were added to the double mutant *lasI rhlI* (Fig. 1c, d).

A very important part of the *P. aeruginosa* biofilm is the matrix. Its final composition depends on the environmental conditions, the age of the biofilm and the study strain [60]. Therefore, the matrices of the wild-type and QS mutants were also analysed.

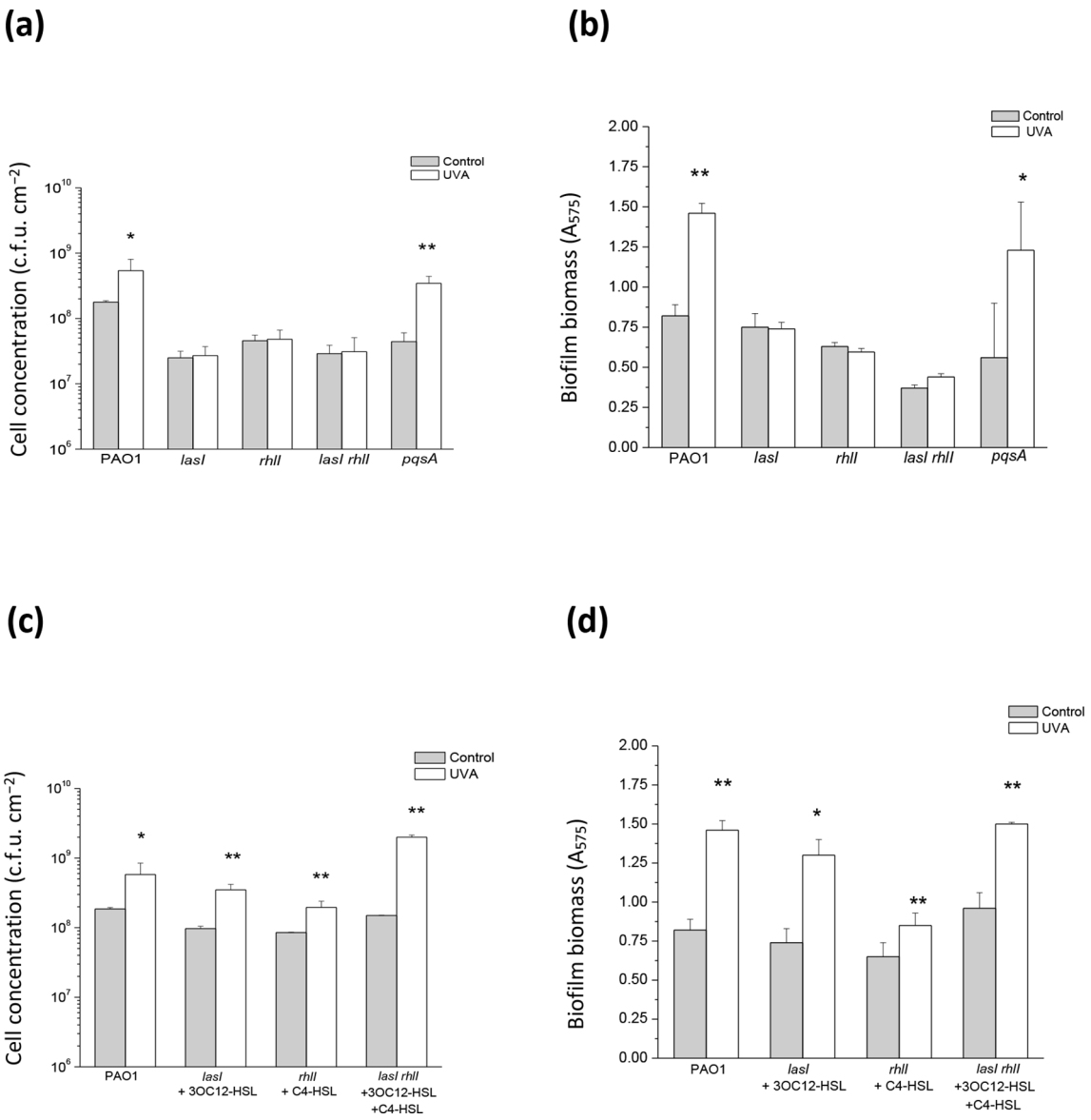



**Fig. 1.** Role of QS in UVA-induced biofilm formation and effect of synthetic autoinducers on biofilm formation of QS-defective strains. Biofilms of PAO1 and QS mutants *lasI*, *rhlI*, *lasI rhlI* and *pqsA* were grown in LB under UVA or in the dark (control) for 24 h. The number of c.f.u. cm$^{-2}$ attached to the slides was quantified by the plate count method (a). Total biofilm biomass was quantified by crystal violet staining followed by measuring the absorbance at 575 nm (b). Biofilms of PAO1 and QS mutants were grown in LB (PAO1 strain) or LB containing 2 µM 3OC12-HSL (*lasI* strain), 10 µM C4-HSL (*rhlI* strain) or both autoinducers (*lasI rhlI* strain), under UVA or in the dark (control). The number of c.f.u.cm-2 (c) and crystal violet staining (d) of biofilms were quantified. Each value is the mean of at least three independent experiments. Error bars indicate the SD. *, $P$<0.05; **, $P$<0.005.

Regardless of whether the biofilm was grown under UVA or in the dark, *lasI*, *rhlI* and *lasI rhlI* strains showed similar matrix composition to the PAO1 strain under the control conditions (Table 3). As previously described [33], matrix biofilms of the PAO1 strain grown under UVA showed a significant increase in the concentration of proteins, DNA and total polysaccharides. In spite of the low percentage of DNA observed in the matrix of the *pqsA* strain, which is expected and characteristic of this mutant [25, 61], a significant increase ($P$<0.05) was observed in all the analysed matrix components in biofilms obtained under UVA, as observed in the PAO1 strain (Table 3).

## Induction of activity of QS autoinducers in response to UVA radiation

Based on the results obtained with the QS mutants, we tested the hypothesis that UVA promotes biofilm formation through activation of the QS system. Although the highest expression of QS genes is achieved at the stationary growth phase, they

**Table 3.** Quantification of matrix components of PAO1 and its QS-deficient derivatives

Matrices of 24 h biofilms grown under UVA or in the dark (control) were evaluated for protein, polysaccharide and DNA content. The results are the means of at least three independent experiments and errors represent the SD of the experiments. *, $P<0.05$; **, $P<0.005$.

| Strain | Protein (μg cm$^{-2}$) | | Polysaccharide (μg cm$^{-2}$) | | DNA (μg cm$^{-2}$) | |
|---|---|---|---|---|---|---|
| | Control | UVA | Control | UVA | Control | UVA |
| PAO1 | 47±15 | 165±10** | 71±15 | 142±44* | 5±2.04 | 37±5* |
| *lasI* | 43±10 | 54±18 | 57±5 | 58.5±4.9 | 6.14±0.75 | 7.1±1.7 |
| *rhlI* | 43±15 | 37±8 | 50±1.5 | 53.3±2.1 | 5.35±1.79 | 4.2±2.3 |
| *lasI rhlI* | 49±8 | 41±12 | 52±5.8 | 50±6.3 | 5.35±0.99 | 4.65±2.6 |
| *pqsA* | 51±6.6 | 86±7.1* | 59±6.7 | 118±17* | 1.5±0.21 | 4±0.4* |

can also be expressed early in the logarithmic phase [47]. Therefore, bacteria were cultured to $OD_{650}$ 0.1, $OD_{650}$ 0.3 and stationary growth phase (24 h) under UVA exposure or in the dark in order to analyse QS induction. First, QS activity was indirectly determined by using transcriptional fusions that respond to autoinducers 3OC12-HSL (*lasB´–lacZ*) or C4-HSL (*rhlB´–lacZ*). As shown in Fig. 2a, a significant difference was observed in *β*-galactosidase activity produced by the *lasB´–lacZ* fusion in the presence of extracts from irradiated PAO1 cultures grown to logarithmic phase with respect to extracts of non-irradiated cells. No induction was observed in extracts of 24 h irradiated cells. In contrast, no significant difference in *β*-galactosidase activity from the *rhlB´–lacZ* fusion was detected among treated and control extracts from logarithmic cultures (Fig. 2b). However, extracts from irradiated stationary cultures had an almost triplicate expression of the *rhlB´–lacZ* fusion compared to control extracts ($P<0.0005$).

## Effect of UVA on *lasR, rhlR, lasI* and *rhlI* transcription

In order to investigate whether the activation of QS by UVA occurs at the transcriptional level, expression assays of genes encoding regulators *lasR* and *rhlR* and autoinducer synthases *lasI* and *rhlI* were performed by qRT-PCR of cultures obtained as described above. Under UVA exposure, the levels of *lasR* mRNA increased significantly in the early logarithmic phase ($OD_{650}$ 0.1) and in the stationary phase (Fig. 3a), while the levels of *rhlR* mRNA significantly increased in the stationary phase (Fig. 3b) of irradiated cells. When the relative expression levels of *lasI* and *rhlI* were analysed, it was observed that UVA radiation significantly increased the levels of *lasI* mRNA in the early logarithmic phase ($OD_{650}$ 0.1) (Fig. 3c), while *rhlI* mRNA levels increased later ($OD_{650}$ 0.3) (Fig. 3d). In the stationary phase, no significant change was observed in the expression of *lasI* and *rhlI* between controls and treated cells (Fig. 3c, d).

## Role of Pel and Psl polysaccharides in UVA-induced biofilm formation

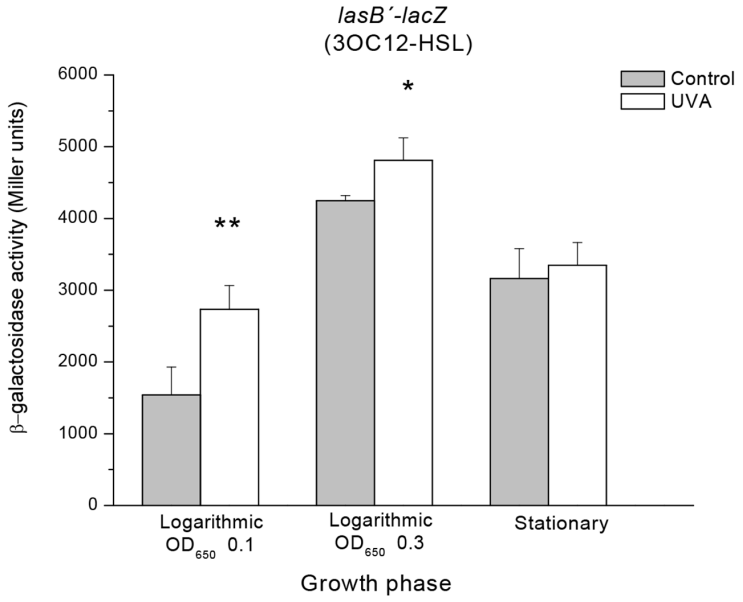
In order to identify QS-dependent factors directly involved in the induction of biofilm formation by UVA, we studied the role of Pel and Psl polysaccharides under our working conditions. These extracellular compounds are essential for biofilm adhesion, protection and structure [62, 63]. The multiple pathways of transcriptional control of *pel* and *psl* operons include QS, which positively regulates their expression [28, 64, 65]. Biofilms of *pel*, *psl* and *pel psl* mutants grown under sublethal UVA doses or in the dark were analysed and compared to the wild-type. When biofilms of polysaccharide mutants were grown under UVA, no difference was observed with regard to their respective controls grown in the dark, both when measured by c.f.u. cm$^{-2}$ (Fig. 4a) or when measured by crystal violet staining (Fig. 4b). As described by Colvin *et al.* [65], *psl* and *pel psl* mutants produced a significantly reduced level of biofilm mass, as measured by c.f.u. cm$^{-2}$ ($P<0.005$) or crystal violet staining ($P<0.05$), compared to PAO1 (Fig. 4a) in the control. A significant decrease ($P<0.05$) was also observed in the biofilm formed by the *pel* mutant compared to PAO1 in the dark (Fig. 4a), but only in c.f.u. cm$^{-2}$. A significant decrease was observed in biofilms of *pel* ($P<0.05$), *psl* ($P<0.005$) and *pel psl* ($P<0.005$) strains compared to PAO1 biofilms obtained under UVA exposure, as measured by c.f.u. cm$^{-2}$. Analysing biofilm formation under UVA by crystal violet staining, this significant difference was similar between the three strains ($P<0.005$) and PAO1.

In order to investigate whether UVA is capable of inducing the expression of *pel* and *psl* operons, we analysed the expression of *pelA* (Fig. 5a) and *pslA* (Fig. 5b) by qRT-PCR. During the early logarithmic phase ($OD_{650}$ 0.1), exposure to UVA radiation significantly increased the expression of both genes; later in the logarithmic phase ($OD_{650}$ 0.3), no significant induction was detected (Fig. 5). There were no significant changes depending on the growth phase for expression of both *pelA* and *pslA* genes under the control conditions.

## Role of ppGpp in the induction of QS by UVA radiation

It has been reported that the QS system is positively regulated by ppGpp [55, 66, 67], a transcriptional regulator related to virulence, resistance to stress factors and persistence [68–70]. In addition, UVA radiation increases ppGpp levels

**(a)**




**(b)**

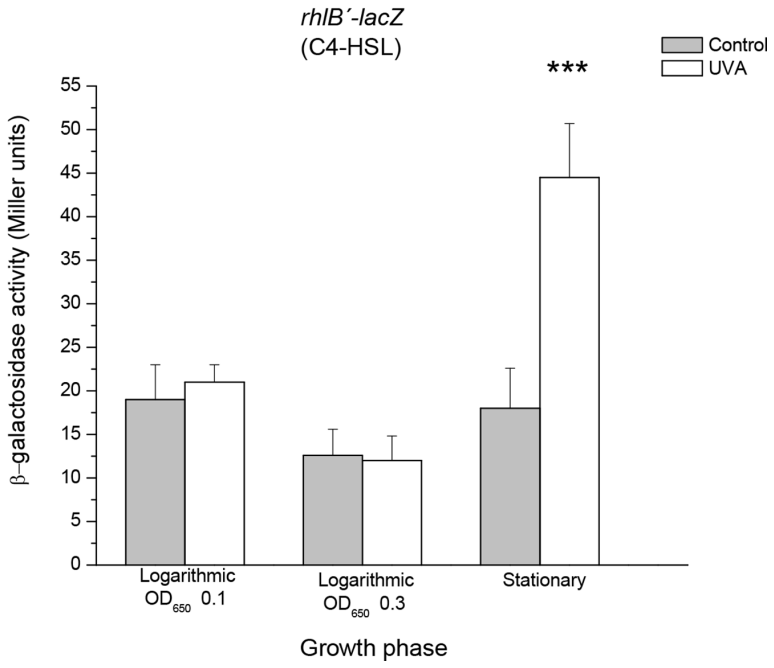



**Fig. 2.** Effect of UVA on the activity of *P. aeruginosa* QS autoinducers. The level of activity of QS autoinducers was determined in supernatants from PAO1 cultures grown under UVA or in the dark (control) to logarithmic ($OD_{650}$ 0.1 and 0.3) or stationary growth phases. Autoinducer activity was determined by measuring the $\beta$-galactosidase activity of reporter strains carrying the transcriptional fusions *lasB´–lacZ* (for 3OC12-HSL) (a) or *rhlB´–lacZ* (for C4-HSL) (b). Each value is the mean of at least three independent experiments. Error bars indicate the SD. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$.

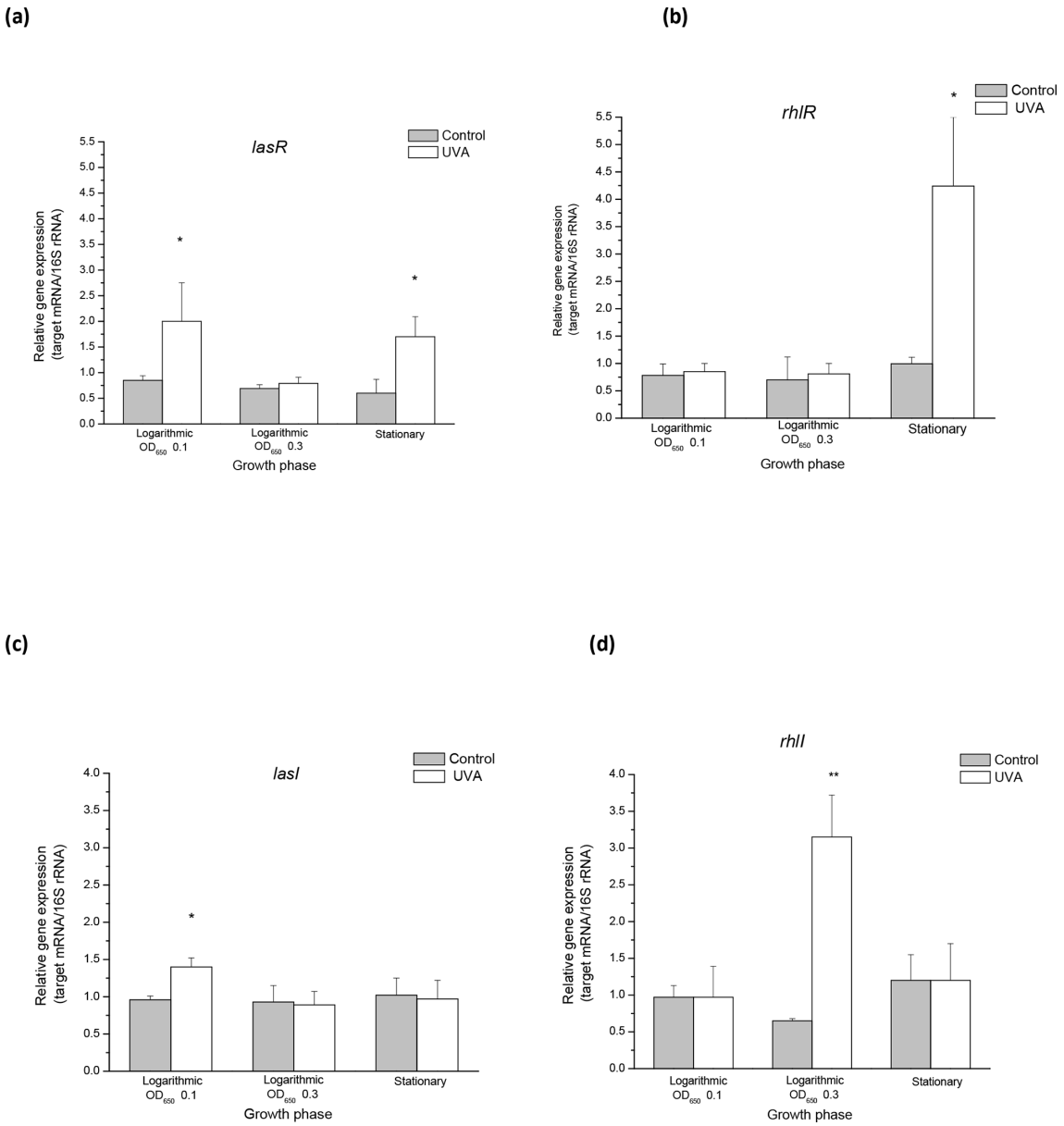



**Fig. 3.** Effect of UVA on the expression of QS genes. PAO1 cultures grown under UVA or in the dark (control) to logarithmic ($OD_{650}$ 0.1 and 0.3) or stationary growth phases were assayed for expression of *lasR* (a), *rhlR* (b), *lasI* (c) and *rhlI* (d) by qRT-PCR. The 16S rRNA gene was used as a reference for normalization for each condition. Each value is the mean of at least three independent experiments. Error bars indicate SD. *, $P<0.05$; **, $P<0.0005$.

by activation of its main synthetase, RelA [71, 72]. Thus, we investigated whether the phenomenon of QS biofilm induction by UVA could be governed by ppGpp. Firstly, we studied the formation of 24 h biofilms of a *relA* strain (impaired for ppGpp production) exposed to sublethal UVA radiation. Fig. 6a shows the effect of UVA on biofilm formation (measured by c.f.u. $cm^{-2}$) of the wild-type and an isogenic *relA* strain. Although a slight increase was observed in the c.f.u. $cm^{-2}$ of the *relA* biofilm grown under UVA compared to its control, this difference was not statistically significant, conversely to that observed in the PAO1 strain ($P<0.05$). A significant increase in the viable cell count of biofilms of the PAO1 strain compared to the *relA* derivative was observed both in the darkness ($P<0.05$) and under UVA ($P<0.05$) (Fig. 6a). No significant induction of biofilm formation by UVA was observed in the *relA* strain, as evaluated by crystal violet staining (Fig. 6b). As seen in the c.f.u. quantification, it was possible to observe a significant increase of biofilm formation in the wild-type compared to the *relA* mutant both in the darkness ($P<0.05$) and under UVA ($P<0.005$) (Fig. 6b).

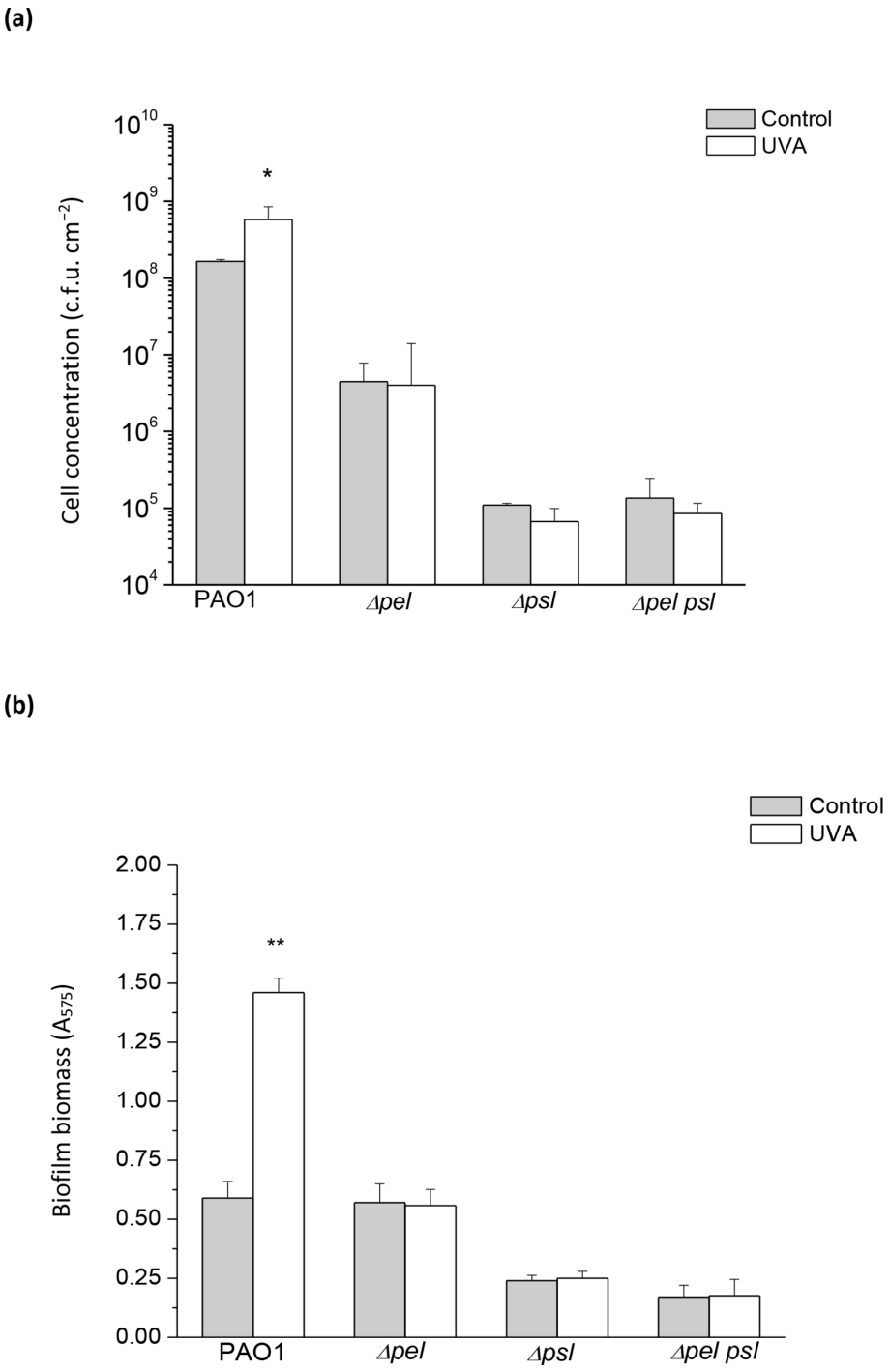



**Fig. 4.** Role of matrix polysaccharides Pel and Psl in UVA-induced biofilm formation. Biofilms of PAO1 and mutants *pel*, *psl* and *pel psl* were grown under UVA or in the dark (control) for 24 h. The number of c.f.u. cm$^{-2}$ attached to the slides was quantified by the plate count method (a). The total biofilm biomass was quantified by crystal violet staining followed by measuring the absorbance at 575 nm (b). Each value is the mean of at least three independent experiments. Error bars indicate SD. *, $P$<0.05; **, $P$<0.005.

Then, we analysed QS activity levels in the supernatants of logarithmic and stationary cultures of a *relA* strain. As shown in Fig. 6c, no difference was observed in $\beta$-galactosidase activity for the expression of the *lasB′–lacZ* fusion between extracts of irradiated and non-irradiated *relA* cells, in any of the analysed growth stages. Similarly, no significant change was observed in $\beta$-galactosidase activity for the expression of the *rhlB′–lacZ* fusion (Fig. 6d). Finally, we investigated by qRT-PCR whether in this strain sublethal UVA exposure was capable of modifying the expression of genes encoding

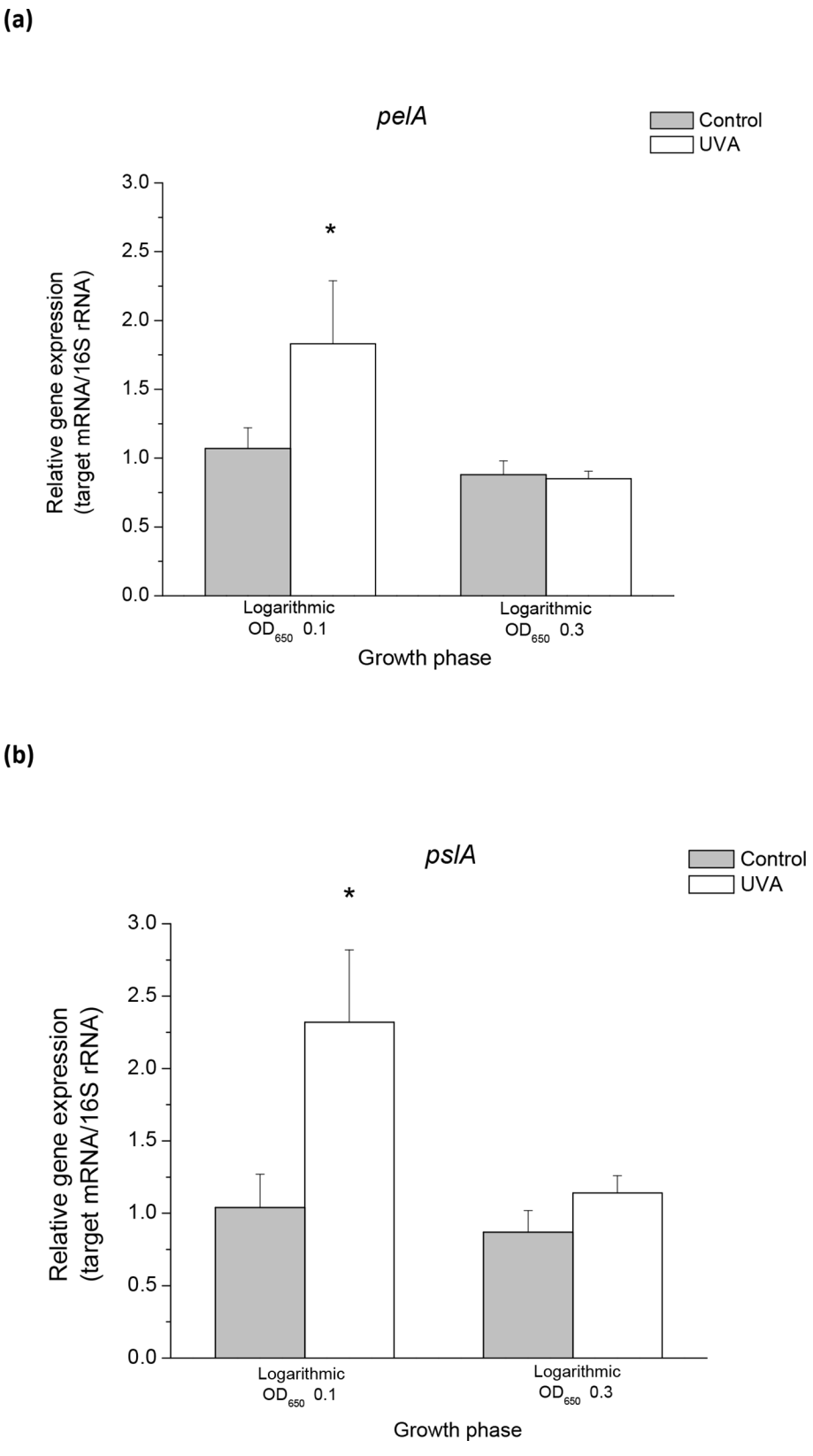



**Fig. 5.** Effect of UVA on expression of *pelA* and *pslA* genes. PAO1 cultures grown under UVA or in the dark (control) to logarithmic ($OD_{650}$ 0.1 and 0.3) or stationary growth phases were assayed for expression of *pelA* (a) and *pslA* (b) genes. The 16S rRNA gene was used as a reference for normalization for each condition. Each value is the mean of at least three independent experiments. Error bars indicate SD. *, $P<0.05$.

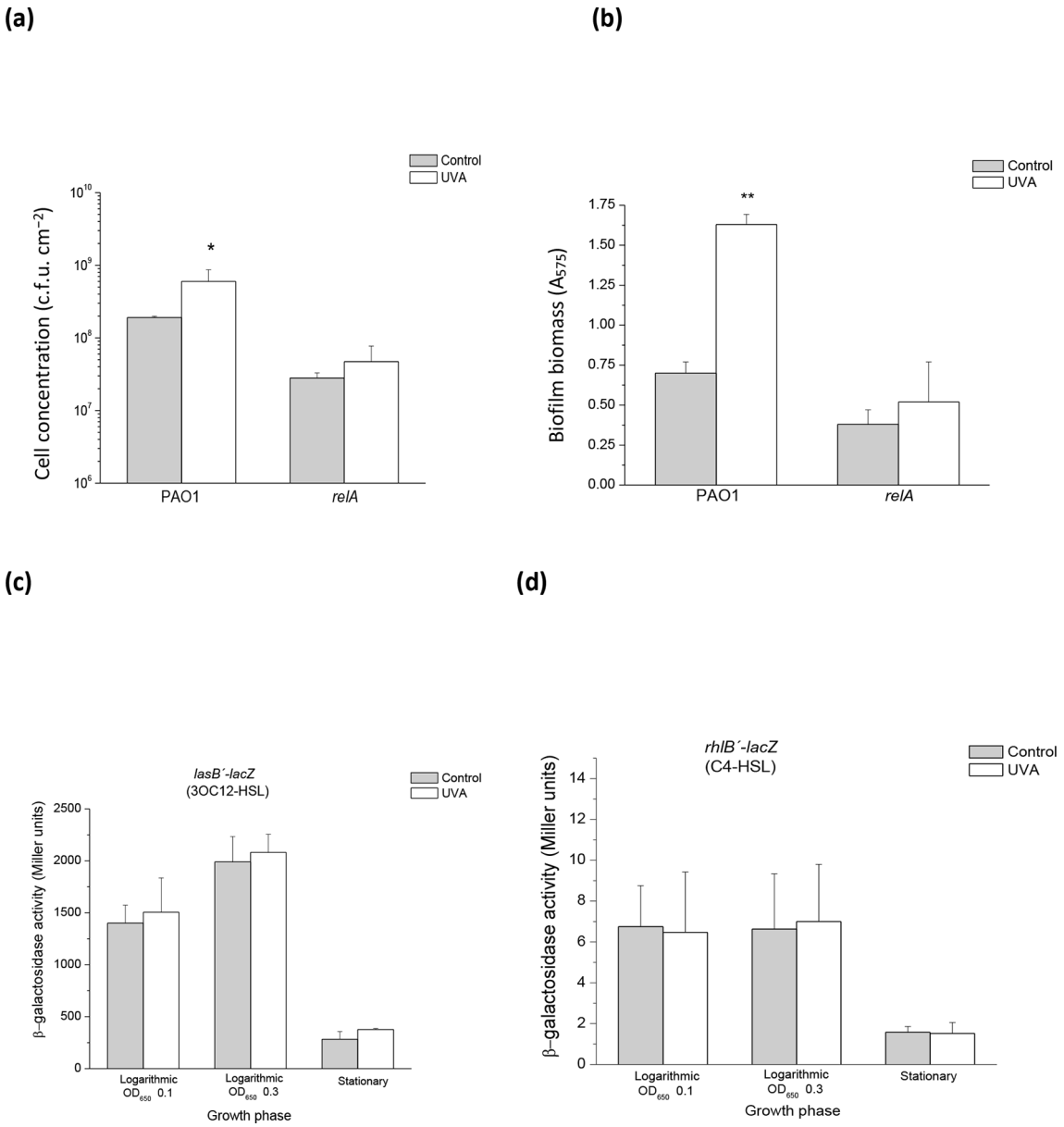



**Fig. 6.** Role of ppGpp in UVA-induced biofilm formation and UVA-dependent activity of *P. aeruginosa* QS autoinducers. Biofilms of PAO1 and *relA* strains were grown under UVA or in the dark (control) for 24 h. The number of c.f.u. $cm^{-2}$ attached to the slides was quantified by the plate count method (a). The total biofilm biomass was quantified by crystal violet staining followed by measuring absorbance at 575 nm (b). The activity of autoinducers was determined in supernatants from cultures of the *relA* strain grown under UVA or in the dark (control) to logarithmic ($OD_{650}$ 0.1 and 0.3) or stationary growth phases. Determination of autoinducer activity was performed by measuring the $\beta$-galactosidase activity of reporter strains carrying the transcriptional fusions *lasB´–lacZ* (for 3OC12-HSL) (c) or *rhlB´–lacZ* (for C4-HSL) (d). Each value is the mean of at least three independent experiments. Error bars indicate SD.*, $P<0.05$; **, $P<0.005$.

QS regulators and autoinducer synthetases. Regardless of the growth phase analysed, the exposure to UVA radiation did not affect the expression of these genes in the *relA* mutant (Fig. 7).

## DISCUSSION

Recently, we demonstrated [33] in three prototypical strains of *P. aeruginosa* that exposure to sublethal UVA doses promotes biofilm formation. *P. aeruginosa* biofilms have been shown to be much more resistant to UVA damage than their planktonic counterparts [7]. UVA-induced biofilm formation, therefore, clearly represents a major defensive response against this stress factor. Possible explanations (not mutually exclusive) for this have been discussed: growth in the biofilm mode enables the protection of the inner cell layers at the expense of the surface cell layers [73]; alginate biofilm matrix

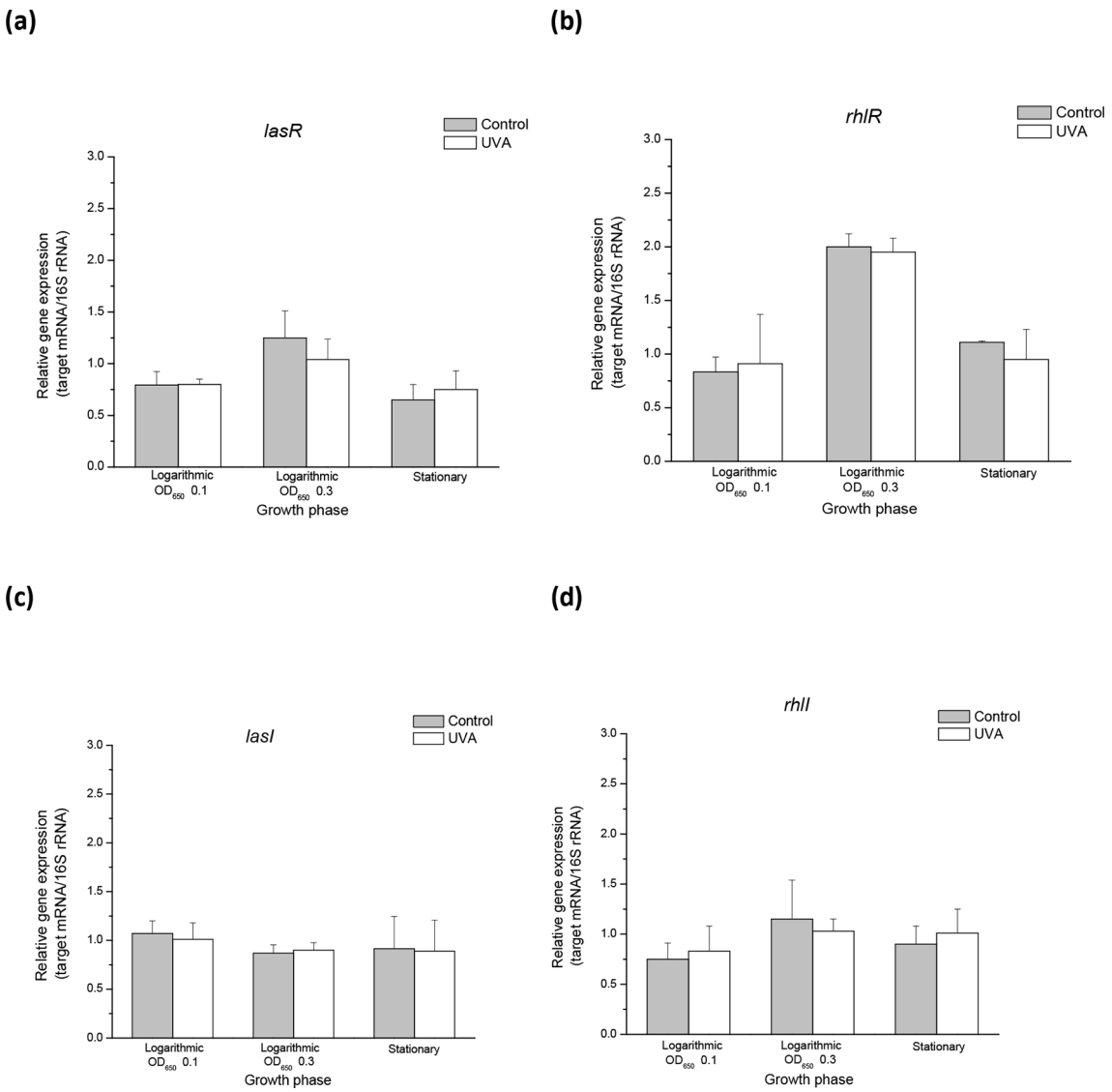



**Fig. 7.** Role of ppGpp in UVA-induced expression of QS genes. Cultures of the *relA* strain grown under UVA or in the dark (control) to logarithmic ($OD_{650}$ 0.1 and 0.3) or stationary growth phases were assayed for expression of *lasR* (a), *rhlR* (b), *lasI* (c) and *rhlI* (d) by qRT-PCR. The 16S rRNA gene was used as a reference for normalization for each condition. Each value is the mean of at least three independent experiments. Error bars indicate SD.

attenuates UV light [4]; changes in the genetic expression of attached cells increases the defences against oxidative stress [74]; accumulation of catalase in the biofilm matrix efficiently counteracts the UVA effect [7].

In the present work, we analysed in depth the genetic mechanisms involved in the phenomenon of UVA-induced biofilm formation in *P. aeruginosa*. Taking into account that QS has been related to biofilm development [23, 75] and UVA radiation has been suggested to induce the production of the QS signal C4-HSL [56], this regulatory system emerged as the obvious candidate to analyse. The activation of QS by UVA was suggested for the first time by Costa *et al.* [56]. Until then, most studies on QS activation had been based on the increase of cell density rather than on environmental factors [76, 77].

In the Costa *et al.* study, *P. aeruginosa* cells suspended in saline solution were exposed to sublethal UVA doses, grown to logarithmic phase in the dark and then assayed for production of QS autoinducers. The authors reported a threefold induction activity of the Rhl signal C4-HSL, but observed no effect on the Las signal 3OC12-HSL [56].

In the current study, the fact that UVA radiation was unable to induce biofilm formation in QS mutants deficient for production of the autoinducers 3OC12-HSL and C4-HSL led us to hypothesize that the two main QS systems of *P. aeruginosa*, Las and Rhl, could be activated by UVA exposure, with the consequent induction of biofilm formation. By using an experimental design that couples UVA exposure with cellular expression, induction of QS genes by UVA is reported here

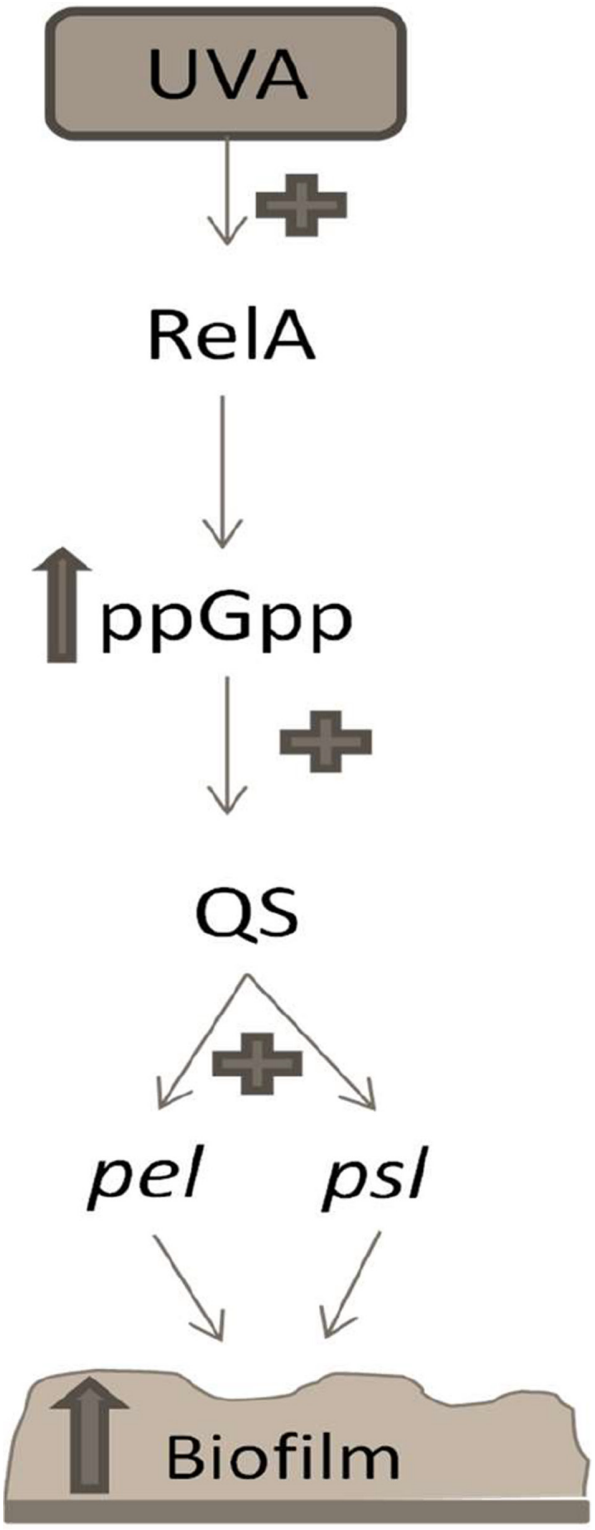



**Fig. 8.** Diagram illustrating the proposed model for the induction of biofilm formation by exposure to low UVA doses in *P. aeruginosa*. Sublethal UVA doses increase the level of ppGpp in a RelA-dependent way, promoting the transcription and activity of QS key genes, which in turn induce *pel* and *psl* genes, essential for biofilm formation. As result of this, biofilm formation is promoted.

for what is believed to be the first time. Results employing transcriptional fusions demonstrated that UVA radiation is able to increase the activity of both autoinducers 3OC12-HSL and C4-HSL. Whilst qRT-PCR analysis revealed that UVA induces transcription of autoinducers and regulator genes at different growth stages. Although a previous study where the timing of *P. aeruginosa* QS gene expression was analysed revealed that most QS genes are induced at the transition from the logarithmic phase to the stationary phase [47], this study demonstrated that *lasR*, *lasI*, *pelA* and *pslA* could be activated at a very early stage under UVA exposure (Figs 3a, c and 5a, b). In the case of *lasR* and *rhlR* regulators, induction at the stationary phase could be seen only under UVA exposure (Fig. 3a, b).

Bacteria growing in biofilms produce extracellular matrices that act as a scaffold, holding and protecting the cells of the biofilm community. Polysaccharides are key components of the biofilm matrix, and contribute to the overall biofilm architecture and resistance of sessile bacteria [6, 78]. As described previously, *P. aeruginosa* synthesize three exopolysaccharides related to biofilm: alginate, Pel and Psl [63]. Non-mucoid strains, such as PAO1, primarily utilize the Pel and Psl polysaccharides for biofilm formation [62], which are both essential structural components of the matrix [65]. Psl is involved in the initial attachment to biotic or abiotic surfaces and in the maturity of biofilm [79]. Pel is important in the formation of a pellicle at the air–liquid interface [48] and also plays an important role in the latest maturity stages of the biofilm [48]. The expression pathways of *pel* and *psl* operons remain to be firmly established, since there are multiple regulators related to the synthesis of these polysaccharides [65]. However, Sakuragi and Kolter [28] reported that, among others, the QS system Las is involved in their genetic regulation. Therefore, we studied the expression of genes encoding Pel and Psl synthesis under conditions of UVA exposure. As seen with QS mutants, a lower capacity to form biofilm was observed in mutants unable to synthesize these polysaccharides, especially in the *psl* strains. No difference was observed due to the UVA treatment, enabling us to hypothesize that these polysaccharides are related to the mechanism studied. Using qRT-PCR, we demonstrated that both *pelA* and *pslA* genes are significantly induced by UVA exposure at the early logarithmic phase ($OD_{650}$ 0.1). Taken as a whole, our results led us to conclude that UVA could promote biofilm formation, at least in part, through activation of *psl* and *pel* operons via QS activation. Other QS-regulated genes, such as those involved in the production of the siderophores pyoverdine and pyochelin, which are important in biofilm formation [80], or rhamnolipids, specifically regulated by the Rhl system and important for the establishment and maintenance of biofilm [81], could also be related to the phenomenon.

In order to understand how UVA could activate QS, we analysed the role of the master transcriptional regulator ppGpp. The RelA enzyme is the main synthetase of ppGpp, largely known for its activation in response to amino acid deprivation [72]. This mechanism, known as the stringent response, was defined as an adaptive mechanism that

enables the bacteria to adapt rapidly to nutritional (amino acids, carbon source) starvation, although other stresses, such as oxidative injuries or antibiotics, can trigger the same response [72, 82]. The stringent response relies on the activity of ppGpp. On the one hand, it represses the transcription of genes involved in RNA and protein biosynthesis with the consequent arrest of bacterial growth, and on the other hand, it induces genes involved in protective functions [83]. It has been demonstrated that non-lethal UVA doses can modify the aminoacylation capacity of certain tRNAs, a situation that mimics amino acid deprivation [84, 85]. The presence of uncharged tRNAs leads to a RelA-dependent increase in the level of ppGpp, resembling the stringent response [71]. This sublethal effect has been investigated by several researchers and the phenomenon was proposed as an adaptive mechanism against the lethal and mutagenic effects of higher doses of UVA and solar irradiation [86]. It was also demonstrated that ppGpp promotes the synthesis of QS signals [66, 67, 87]. Our results, demonstrating that in a *relA* strain, which produces very low ppGpp levels [54], UVA exposure did not significantly increase biofilm formation and did not induce QS genes, suggest that the biofilm induction by UVA may rely on activation on the QS system by ppGpp. This should not dismiss the possibility that UVA could activate the formation of biofilm by another additional route, such as by the increase of ROS levels. It has been proposed that ROS modulate c-di-GMP levels [88], which have been linked to biofilm formation [89]. In this regard, we demonstrated in a previous work that a *pqsA* mutant, deficient for PQS production, is very resistant to UVA radiation compared to the wild-type. This was explained by suggesting that PQS acts as photosensitizer that increases the ROS production and oxidative damage of biological targets under UVA exposure [38]. Our results in this work, demonstrating that a *pqsA* mutant shows a level of UVA mediated-biofilm induction comparable to the wild-type, besides proving that PQS is dispensable in this phenomenon, suggest that ppGpp-dependent induction could be more relevant than ROS in this mechanism, since the exposure of this strain to UVA generates a low level of oxidative stress [38].

In summary, this work suggests that the increase of biofilm formation by exposure to low UVA doses obeys, at least in part, a ppGpp-dependent QS induction. A diagram illustrating the proposed model according to the results obtained in this work is shown in Fig. 8. *P. aeruginosa*, a versatile bacterium present in terrestrial and aquatic environments and an opportunistic human pathogen, produces robust biofilms resistant to diverse antibacterial agents, having enormous impact on industry and human health [2, 3, 90, 91]. In this regard, studies in greater depth on the genetic basis and environmental factors related to biofilm formation are relevant from a practical and ecological standpoint.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Høiby N, Ciofu O, Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol* 2010;11:1663–1674.
2. Willcox MD, Harmis N, Cowell W, Holden T. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. *Biomaterials* 2001;22:3235–3247.
3. Rajasekar A, Anandkumar B, Maruthamuthu S, Ting YP, Rahman P. Characterization of corrosive bacterial consortia isolated from petroleum product-transporting pipelines. *Appl Microbiol Biotechnol* 2010;85:1175–1188.
4. Elasri MO, Miller RV. Study of the response of a biofilm bacterial community to UV radiation. *Appl Environ Microbiol* 1999;65:2025–2031.
5. Hassett DJ, Ma J-F, Elkins JG, McDermott TR, Ochsner UA *et al*. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol Microbiol* 1999;34:1082–1093.
6. Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001;9:34–39.
7. Pezzoni M, Pizarro RA, Costa CS. Protective role of extracellular catalase (KatA) against UVA radiation in *Pseudomonas aeruginosa* biofilms. *J Photochem Photobiol B* 2014;131:53–64.
8. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol* 2000;54:49–79.
9. Sauer K, Cullen MC, Rickard AH, Zeef LAH, Davies DG *et al*. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* 2004;186:7312–7326.
10. Fazli M, Almblad H, Rybtke ML, Givskov M, Eberl L *et al*. Regulation of biofilm formation in *Pseudomonas* and *Burkholderia* species. *Environ Microbiol* 2014;16:1961–1968.
11. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 2009;7:263–273.
12. Gottesman S, Storz G. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol* 2011;3:a003798.
13. Brencic A, Lory S. Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 2009;72:612–632.
14. Michaux C, Verneuil N, Hartke A, Giard J-C. Physiological roles of small RNA molecules. *Microbiology* 2014;160:1007–1019.
15. Fuqua C, Parsek MR, Greenberg EP. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* 2001;35:439–468.
16. Solano C, Echeverz M, Lasa I. Biofilm dispersion and quorum sensing. *Curr Opin Microbiol* 2014;18:96–.
17. Pearson JP, Passador L, Iglewski BH, Greenberg EP. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 1995;92:1490–1494.
18. Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 1993;260:1127–1130.
19. Sifri CD. Quorum sensing: bacteria talk sense. *Clin Infect Dis* 2008;47:1070–1076.
20. Verbeke F, De Craemer S, Debunne N, Janssens Y, Wynendaele E *et al*. Peptides as quorum sensing molecules: measurement

techniques and obtained levels in vitro and in vivo. *Front Neurosci* 2017;12:11–18.

21. Pesci EC, Milbank JBJ, Pearson JP, McKnight S, Kende AS *et al*. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 1999;96:11229–11234.
22. McKnight SL, Iglewski BH, Pesci EC. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas* aeruginosa. *J Bacteriol* 2000;182:2702–2708.
23. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW *et al*. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 1998;280:295–298.
24. Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB *et al*. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* 2002;148:87–102.
25. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS *et al*. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* 2006;59:1114–1128.
26. Pamp SJ, Tolker-Nielsen T. Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol* 2007;189:2531–2539.
27. Gilbert KB, Kim TH, Gupta R, Greenberg E, Schuster M. Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. *Mol Microbiol* 2009;73:1072–1085.
28. Sakuragi Y, Kolter R. Quorum-sensing regulation of the biofilm matrix genes (*pel*) of *Pseudomonas aeruginosa*. *J Bacteriol* 2007;189:5383–5386.
29. Di Bonaventura G, Stepanović S, Picciani C, Pompilio A, Piccolomini R. Effect of environmental factors on biofilm formation by clinical *Stenotrophomonas maltophilia* isolates. *Folia Microbiol* 2007;52:86–90.
30. Garrett TR, Bhakoo M, Zhang Z. Bacterial adhesion and biofilms on surfaces. *Prog Nat Sci* 2008;18:1049–1056.
31. Ben Said M, Khefacha S, Maalej L, Dalu I, Hassen A. Effect of ultraviolet, electromagnetic radiation subtype C (UV-C) dose on biofilm formation by *Pseudomonas aeruginoisa*. *Afr J Microbiol Res* 2011;5:4353–4358.
32. Gambino M, Cappitelli F. Mini-review: biofilm responses to oxidative stress. *Biofouling* 2016;32:167–178.
33. Pezzoni M, Pizarro RA, Costa CS. Exposure to low doses of UVA increases biofilm formation in *Pseudomonas aeruginosa*. *Biofouling* 2018;34:673–684.
34. Webb RB. Lethal and mutagenic effects of near-ultraviolet radiation. *Photochem Photobiol Rev* 1977;2:169–261.
35. Fernández RO, Pizarro RA. Lethal effect induced in *Pseudomonas aeruginosa* exposed to ultraviolet-A radiation. *Photochem Photobiol* 1996;64:334–339.
36. Fernández RO, Pizarro RA. *Pseudomonas aeruginosa* UV-A-induced lethal effect: influence of salts, nutritional stress and pyocyanine. *J Photochem Photobiol B* 1999;50:59–65.
37. Baümler W, Regensburger J, Knak A, Felgenträger A, Maisch T. UVA and endogenous photosensitizers the detection of singlet oxygen by its luminescence. *Photochem Photobiol* 2012;11:107–117.
38. Pezzoni M, Meichtry M, Pizarro RA, Costa CS. Role of the *Pseudomonas* quinolone signal (PQS) in sensitising *Pseudomonas aeruginosa* to UVA radiation. *J Photochem Photobiol B* 2015;142:129–140.
39. Gamage J, Zhang Z. Applications of photocatalytic disinfection. *Int J Photoenergy* 2010;2010:764870
40. McGuigan KG, Conroy RM, Mosler H-J, du Preez M, Ubomba-Jaswa E *et al*. Solar water disinfection (SODIS): a review from bench-top to roof-top. *J Hazard Mater* 2012;235-236:29–46.
41. Jagger J. Near UV radiation effects on microorganisms. *Photochem Photobiol* 1981;34:761–768.
42. Day RS, Muel B. Ultraviolet inactivation of the ability of *Escherichia coli* to support the growth of phage T7: an action spectrum. *Photochem Photobiol* 1974;20:98–102.
43. Berney M, Weilenmann H-U, Egli T. Gene expression of *Escherichia coli* in continuous culture during adaptation to artificial sunlight. *Environ Microbiol* 2006;8:1635–1647.
44. Soule T, Gao Q, Stout V, Garcia-Pichel F. The global response of *Nostoc punctiforme* ATCC 29133 to UVA stress, assessed in a temporal DNA microarray study. *Photochem Photobiol* 2013;89:415–423.
45. Sassoubre LM, Ramsey MM, Gilmore MS, Boehm AB. Transcriptional response of *Enterococcus faecalis* to sunlight. *J Photochem Photobiol B* 2014;130:349–356.
46. Pezzoni M, Tribelli PM, Pizarro RA, López NI, Costa CS. Exposure to low UVA doses increases KatA and KatB catalase activities, and confers cross-protection against subsequent oxidative injuries in *Pseudomonas aeruginosa*. *Microbiology* 2016;162:855–864.
47. Schuster M, Lostroh CP, Ogi T, Greenberg EP. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 2003;185:2066–2079.
48. Friedman L, Kolter R. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* 2004;51:675–690.
49. Hoerter JD, Arnold AA, Kuczynska DA, Shibuya A, Ward CS *et al*. Effects of sublethal UVA irradiation on activity levels of oxidative defense enzymes and protein oxidation in *Escherichia coli*. *J Photochem Photobiol B* 2005;81:171–180.
50. Chang W-S, van de Mortel M, Nielsen L, Nino de Guzman G, Li X *et al*. Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. *J Bacteriol* 2007;189:8290–8299.
51. Tielen P, Rosenau F, Wilhelm S, Jaeger K-E, Flemming H-C *et al*. Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. *Microbiology* 2010;156:2239–2252.
52. Messiaen AS, Nelis H, Coenye T. Investigating the role of matrix components in protection of *Burkholderia cepacia* complex biofilms against tobramycin. *J Cyst Fibros* 2014;13:56–62.
53. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350–356.
54. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265–275.
55. Erickson DL, Lines JL, Pesci EC, Venturi V, Storey DG. *Pseudomonas aeruginosa relA* contributes to virulence in *Drosophila melanogaster*. *Infect Immun* 2004;72:5638–5645.
56. Costa CS, Pezzoni M, Fernández RO, Pizarro RA. Role of the quorum sensing mechanism in the response of *Pseudomonas aeruginosa* to lethal and sublethal UVA irradiation. *Photochem Photobiol* 2010;86:1334–1342.
57. Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD *et al*. *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. *Infect Immun* 2002;70:1783–1790.
58. Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1972.
59. Larionov A, Krause A, Miller W. A standard curve based method for relative real time PCR data processing. *BMC Bioinform* 2005;6:62.
60. Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunol Med Microbiol* 2010;59:253–268.
61. Yang L, Barken KB, Skindersoe ME, Christensen AB, Givskov M *et al*. Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. *Microbiology* 2007;153:1318–1328.
62. Wozniak DJ, Wyckoff TJO, Starkey M, Keyser R, Azadi P *et al*. Alginate is not a significant component of the extracellular

polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci USA* 2003;100:7907–7912.

63. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr Opin Microbiol* 2007;10:644–648.
64. Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A. The *pel* genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. *Microbiology* 2005;151:985–997.
65. Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC *et al*. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ Microbiol* 2012;14:1913–1928.
66. Van Delden C, Comte R, Bally M. Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 2001;183:5376–5384.
67. Schafhauser J, Lepine F, McKay G, Ahlgren HG, Khakimova M *et al*. The stringent response modulates 4-hydroxy-2-alkylquinoline biosynthesis and quorum-sensing hierarchy in *Pseudomonas aeruginosa*. *J Bacteriol* 2014;196:1641–1650.
68. Yan X, Zhao C, Budin-Verneuil A, Hartke A, Rincé A *et al*. The (p)ppGpp synthetase RelA contributes to stress adaptation and virulence in *Enterococcus faecalis* V583. *Microbiology* 2009;155:3226–3237.
69. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS. ppGpp conjures bacterial virulence. *Microbiol Mol Biol Rev* 2010;74:171–199.
70. Schofield WB, Zimmermann-Kogadeeva M, Zimmermann M, Barry NA, Goodman AL. The stringent response determines the ability of a commensal bacterium to survive starvation and to persist in the gut. *Cell Host Microbe* 2018;24:120–132.
71. Ramabhadran TV, Jagger J. Mechanism of growth delay induced in *Escherichia coli* by near ultraviolet radiation. *Proc Natl Acad Sci USA* 1976;73:59–63.
72. Cashel M, Gentry DR, Hernandez VJ, Vinella D. The stringent response. In: Neidhardt FC (ed). *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Washington, DC: American Society for Microbiology; 1996. pp. 1458–1496.
73. Gao Q, Garcia-Pichel F. Microbial ultraviolet sunscreens. *Nat Rev Microbiol* 2011;9:791–802.
74. Polo A, Diamanti MV, Bjarnsholt T, Høiby N, Villa F *et al*. Effects of photoactivated titanium dioxide nanopowders and coating on planktonic and biofilm growth of *Pseudomonas aeruginosa*. *Photochem Photobiol* 2011;87:1387–1394.
75. De Kievit TR, Gillis R, Marx S, Brown C, Iglewski BH. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. *Appl Environ Microbiol* 2001;67:1865–1873.
76. De Kievit TR, Iglewski BH. Bacterial quorum sensing in pathogenic relationships. *Infect Immun* 2000;68:4839–4849.
77. Miller MB, Bassler BL. Quorum sensing in bacteria. *Annu Rev Microbiol* 2001;55:165–199.
78. Branda SS, Vik A, Friedman L, Kolter R. Biofilms: the matrix revisited. *Trends Microbiol* 2005;13:20–26.
79. Ma L, Conover M, Lu H, Parsek MR, Bayles K *et al*. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* 2009;5:e1000354.
80. Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci USA* 2005;102:11076–11081.
81. De Kievit TR. Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* 2009;11:279–288.
82. Moradali MF, Ghods S, Rehm BHA. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Front Cell Infect Microbiol* 2017;7:39.
83. Potrykus K, Cashel M. (p)ppGpp: still magical? *Annu Rev Microbiol* 2008;62:35–51.
84. Favre A, Hajnsdorf E, Thiam K, Caldeira de Araujo A. Mutagenesis and growth delay induced in *Escherichia coli* by near-ultraviolet radiations. *Biochimie* 1985;67:335–342.
85. Blondel MO, Favre A. tRNAPhe and tRNAPro are the near-ultraviolet molecular targets triggering the growth delay effect. *Biochem Biophys Res Commun* 1988;150:979–986.
86. Caldeira de Araujo A, Favre A. Near ultraviolet DNA damage induces the SOS responses in *Escherichia coli*. *EMBO J* 1986;5:175–179.
87. Baysse C, Cullinane M, Dénervaud V, Burrowes E, Dow JM *et al*. Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties. *Microbiology* 2005;151:2529–2542.
88. Strempel N, Nusser M, Neidig A, Brenner-Weiss G, Overhage J. The oxidative stress agent hypochlorite stimulates c-di-GMP synthesis and biofilm formation in *Pseudomonas aeruginosa*. *Front Microbiol* 2017;8:2311.
89. Ha D-G, O'Toole GA. c-di-GMP and its effects on biofilm formation and dispersion: a *Pseudomonas aeruginosa* review. *Microbiol Spectr* 2015;3:MB-0003-2014.
90. Nickel JC, Ruseska I, Wright JB, Costerton JW. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob Agents Chemother* 1985;27:619–624.
91. Mansouri H, Alavi SA, Yari M. A study of *Pseudomonas aeruginosa* bacteria in microbial corrosion. *2nd International Conference on Chemical, Ecology and Environmental Sciences (ICCEES'2012)* 28–29 April; 2012, Singapore.
92. Holloway BW. Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* 1955;13:572–581.
93. Pearson JP, Pesci EC, Iglewski BH. Roles of *Pseudomonas aeruginosa* las and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* 1997;179:5756–5767.
94. Brint JM, Ohman DE. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J Bacteriol* 1995;177:7155–7163.
95. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E *et al*. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 2003;100:14339–14344.
96. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ *et al*. *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol Microbiol* 2010;75:827–842.
97. Kirisits MJ, Prost L, Starkey M, Parsek MR. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* 2005;71:4809–4821.
98. Wang EW, Jung JY, Pashia ME, Nason R, Scholnick S *et al*. Otopathogenic *Pseudomonas aeruginosa* strains as competent biofilm formers. *Arch Otolaryngol Head Neck Surg* 2005;131:983–989.

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