Photochemistry and Photobiology, 2010, 86: 1334–1342

Role of the Quorum Sensing Mechanism in the Response of *Pseudomonas aeruginosa* to Lethal and Sublethal UVA Irradiation

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Received 16 June 2010, accepted 11 August 2010, DOI: 10.1111/j.1751-1097.2010.00800.x

ABSTRACT

The role of quorum sensing (QS) in the response of *Pseudomonas aeruginosa* to UVA radiation was investigated in the PAO1 strain and derivatives defective in the synthesis of the QS signals 3OC12-HSL (*lasI* strain), C4-HSL (*rhlI* strain) or both (*lasI rhlI* strain). Cell viability measurements demonstrated that the double mutant was significantly more sensitive to UVA than single mutants, which in turn showed reduced cell survival with regard to the PAO1 strain. Irradiation under nitrogen atmosphere and chemiluminescence measurements indicated the oxidative nature of the UVA-induced damage. The activity of the antioxidant enzymes catalase and superoxide dismutase was assayed in these strains before and after irradiation, and a strong correlation between catalase levels and UVA sensitivity was observed. When a sublethal UVA dose was applied to PAO1, a growth delay was observed and this mechanism depended on the rhl system. Moreover, low doses of UVA irradiation triplicated the level of C4-HSL in log PAO1 cells. It is concluded that QS is fundamental in the defense against the toxic effects of UVA in *P. aeruginosa*. The induction of the QS system by UVA independently of cell density could function as an adaptative strategy to withstand this hostile environmental condition.

INTRODUCTION

One of the more important stressing agents present in the environment is solar UVA radiation (320–400 nm), representing the major fraction of UV radiation reaching the Earth's surface. Lethal and mutagenic effects of UVA radiation have been extensively investigated in bacteria (1). It has been well established that high doses of UVA lead to lethal effects mainly through reactive oxygen species which produce oxidative damage to proteins and lipids with the consequent loss of cell viability (2,3). On the other hand, exposure to low UVA fluences induces several sublethal effects including a transient inhibition of bacterial growth without significant cell death (4), loss in phage susceptibility (5) and inhibition of tryptophanase induction (6). Bacterial effects of UVA radiation have been studied in diverse organisms including *Escherichia coli* (1), *Enterobacter cloacae* (7), *Bacillus subtilis* (8) and *Pseudomonas aeruginosa* (9).

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P. aeruginosa is a versatile bacteria present in diverse terrestrial and aquatic environments and an important pathogen of humans, insects and plants. Fernández and Pizarro (9) demonstrated that exposure of midexponential cells of *P. aeruginosa* to UVA irradiation produces cell death due in part to an oxidative damage causing inhibition of respiration and transport systems, as well as direct damage to membrane-bound enzymes succinate dehydrogenase and lactate dehydrogenase. However, protective factors such as the presence of salts in the plating media or nutritional stress allow *P. aeruginosa* cells to increase survival to lethal UVA exposure (10).

P. aeruginosa possesses several antioxidative strategies for defense against reactive oxygen species generated by its strong aerobic metabolism, including two superoxide dismutases (SODs), MnSOD and FeSOD, encoded by *sodA* and *sodB,* respectively (11), and two catalases, encoded by *katA* and *katB* (12,13). Environmental factors such as H_2O_2 or iron are known to influence the expression of these genes (13–15); in addition, genetic regulation by the quorum sensing (QS) mechanism controls the expression of *katA*, *sodA* and *sodB* (16).

QS is a mechanism employed by bacteria to regulate gene transcription in response to population size mediated by diffusible signal molecules called autoinducers released to the environment (17). The core of the QS system involves two signaling systems, las (QS I) and rhl (QS II), that function as regulators of hundreds of genes. The las system consists of the signal synthase LasI, which produces *N*-3-oxododecanoyl-L-homoserine lactone (3OC12-HSL), and the signal receptor LasR (18). The rhl system consists of the signal synthase RhlI, which determines the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL), and the receptor RhlR (19). When these receptors bind their cognate signals, gene expression of certain target genes is activated. The two QS systems are arranged in a hierarchical way, as the LasR–LasI system activates transcription of *rhlR* and *rhlI* genes (20). However, a recent work demonstrated that the QS hierarchy is more complex and depends on the growth phase (21). Some QS-dependent genes are las or rhl specific, but most of them show increased responses to both signals; most of the QS genes are maximally expressed in the stationary growth phase (22).

The aim of the present study was to investigate the role of the QS system in the response of *P. aeruginosa* to UVA radiation. In this study, we demonstrate that the QS mechanism is essential in the tolerance of *P. aeruginosa*

stationary phase cells to lethal UVA fluences. A dose-dependent mechanism of growth delay as response to UVA sublethal exposure, not described yet in *P. aeruginosa*, is dependent on the C4-HSL signal. Finally, we demonstrated that UVA radiation activates the expression of the rhl system by the premature induction of C4-HSL synthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. Table 1 lists strains and plasmids used in this study. Plasmids were maintained with 100 μg mL^{-1} ampicillin in *E. coli* or 200 μg mL^{-1} carbenicillin in *P. aeruginosa*.

Bacterial cultures were grown at 37°C with shaking in complete Luria-Bertani (LB) broth (23) until stationary phase of growth was reached; for solid medium 15 g L^{-1} agar 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.* (22).

DNA techniques. Plasmid DNA was purified and manipulated by using standard techniques (24). Plasmids were transferred into *E. coli* (24) or *P. aeruginosa* (25) by transformation.

Irradiation source. Cell suspensions were irradiated with a bench with two Philips TDL 18W/08 tubes (more than 95% of the UVA emission at 365 nm) mounted about 18 cm from the suspension surface. The incident fluence in our experimental conditions was measured at the surface of the suspension with a 9811.58 Cole-Parmer Radiometer (Cole-Parmer Instruments Co., Chicago, IL). The UVA tubes are mounted on aluminum anodized reflectors enhancing the fluence rate on the section to be irradiated.

Irradiation procedure. Stationary cultures grown in LB medium were washed once and suspended in saline solution (NaCl 0.1 M) at OD_{650} of 0.4. The suspension was divided into two fractions of 50 mL and placed in two glass beakers (diameter of the exposed surface 10 cm) open to air. One of these fractions was irradiated from above at a fluence rate of 20 W m^{-2} at the level of the free surface of the suspension while the other fraction remained in the dark. Both suspensions were maintained in an ice bath under slow magnetic stirring during the entire procedure.

When the cells were irradiated in nitrogen atmosphere, 13 mL suspensions were prepared as indicated above and placed in glass beakers of 4.5 cm internal diameter. Suspensions were bubbled with the corresponding gas (air or nitrogen) for 10 min prior to irradiation. The beakers were covered with a thin plastic wrapper (fluence of 20 W m^{-2} at the level of the free surface of the suspension) and the internal atmosphere was maintained supplying the same gas during the irradiation. Control samples were treated in the same way but remained in the dark.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Pseudomonas aeruginosa		
PAO1	Wild type	B.W. Holloway
PAO-JP1	*lasI*::Tet, derived from PAO1 strain	Pearson *et al.* (59)
PDO100	*rhlI*::Tn*501-2*, derived from PAO1 strain	Brint and Ohman (36)
PAO-JP2	*lasI*::Tet *rhlI*::Tn*501-2*, derived from PDO100 strain	Pearson *et al.* (59)
PW8190	*katA*::Is*lacZ*/hah, derived from PAO1 strain	Washington Genome Center (60)
Escherichia coli		
DH5α	F$^-$ Φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	Gibco
Plasmids		
pKDT1.7	*lasB′–lacZ lasR*	Pearson *et al.* (59)
pECP61.5	*rhlA′–lacZ ptac-rhlR*	Pearson *et al.* (59)

Survival curves and growth delay measurements. In order to measure cell viability, samples of the suspensions were taken during irradiation at the indicated times and plated on LB solid medium after dilution. Plates were incubated in the dark immediately after irradiation for avoiding light-induced DNA repair and the colonies were counted after 24–48 h at 37°C.

Growth delay was assayed by taking samples of the irradiated and control suspensions at different times. Samples were centrifuged, suspended to an OD_{650} of about 0.1 in prewarmed LB broth, and incubated at 37°C with shaking in a thermostatic water bath. Growth was followed by measuring the OD_{650} as a function of time. Growth delay was measured as a horizontal displacement of the growth curve, according to Jagger (26).

Chemiluminescence assay. Photoemissive species were followed by means of a liquid scintillation system in the "out of coincidence" mode (27). For this purpose, during the UVA treatment, 5 mL aliquots were transferred to the scintillation system, equipped with photomultipliers sensitive in the blue region up to 600–650 nm (Tri-Carb, Model 1500; Packard Instruments Co.).

Autoinducer extraction. Suspensions of *P. aeruginosa* PAO1 strain were irradiated for 60 or 120 min at a fluence rate of 20 W m^{-2} as described above. Samples of the irradiated and control suspensions were centrifuged, suspended to an OD_{650} of 0.1 in prewarmed LB broth, and incubated at 37°C with shaking in a thermostatic water bath. When irradiated and control cultures reached an OD_{650} of 0.5, 40 mL supernatants were extracted twice in equal volume of acidified ethyl acetate and stored at −20°C until further use in the bioassays (28).

Autoinducer bioassays. Autoinducer levels in culture supernatants were determined in bioassays using reporter plasmids carrying *lacZ* fusions dependent on QS signals for its expression, as previously described (28,29). The reporter strains were *E. coli* DH5α pKDT1.7 and *P. aeruginosa* PAO-JP2 pECP61.5 (Table 1). The plasmid pKDT1.7 in *E. coli* 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 (29); this plasmid contains a copy of *rhlR* and an *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 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 with test samples and grown for 90 min at 37°C and β-galactosidase was assayed.

C4-HSL bioassay: An overnight culture of PAO-JP2 pECP61.5 strain was diluted to an OD_{650} of 0.1 and then 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 was assayed.

β-galactosidase activity assay. β-galactosidase activity was assayed as described by Miller (30) in cells treated with SDS and chloroform. Specific activities are expressed in Miller units referred to OD_{650}.

Catalase and superoxide dismutase activity assays. For catalase activity, cell extracts were prepared from 10 mL of control and irradiated suspensions harvested by centrifugation at 10 000 ***g*** for 10 min at 4°C. Cells were suspended in 5 mL of ice-cold 50 mM potassium phosphate buffer pH 7, sonicated in an ice-water bath and clarified by centrifugation at 13 000 ***g*** for 10 min at 4°C. Catalase activity was monitored by following the decomposition of 10 mM H_2O_2 at 240 nm according to Aebi (31). One unit of activity was that which decomposes 1 μmol of H_2O_2 per min per mg of protein.

For SOD activity, cell extracts were obtained as described for the catalase assay but cells were suspended in 50 mM Tris buffer pH 7.8. The SOD activity was measured using a SOD assay kit (Cayman Chemicals). One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Protein content was determined by Lowry's method (32).

Quantification of pyocyanin. Pyocyanin was measured according to the method of Essar *et al*. (33). Supernatants from 3 mL of overnight cultures of PAO-JP2 pECP61.5 strain grown in presence of test samples for β-galactosidase assays were extracted with 1.8 mL of chloroform. The chloroform layer was then extracted with 0.6 mL of 0.2 M HCl, and the absorbance of the HCl layer was measured at 520 nm.

RESULTS

Response of QS-deficient mutants to lethal UVA fluences

Although the molecular mechanisms involved in bacterial cell damage caused by UVA are complex, results of several researchers indicate that the toxic effects are mediated mainly by reactive oxygen species. Taking into account that some QS regulated genes are involved directly in the antioxidative defense pathways in *P. aeruginosa*, an experimental design was carried out in order to evaluate the involvement of the QS mechanism in the bacterial response to this stressing agent. This was accomplished by studying the response to UVA radiation of the wild type PAO1 and its derivatives deficient in the synthesis of the autoinducers 3OC12-HSL (PAO-JP1 strain), C4-HSL (PDO100 strain) or both (PAO-JP2 strain) (Table 1).

Suspensions of stationary phase cells of PAO1, PAO-JP1, PDO100 and PAO-JP2 strains were exposed to 20 W m^{-2} for 180 min and the number of viable cells was determined as indicated above. As shown in Fig. 1, when bacterial suspensions were exposed for the first 120 min, the PDO100 strain was more sensitive to UVA radiation than both the wild type PAO1 and PAO-JP1 strains. At higher dose exposures, cell viability of PAO-JP1 decreased to values similar to those found in PDO100. The irradiated double mutant PAO-JP2 showed a lower survival compared to the wild type and the single mutants under identical irradiation conditions. To demonstrate that both autoinducers are required for optimal cell viability following UVA radiation, *lasI*, *rhlI* and *lasI rhlI* mutants were grown to stationary phase in presence of synthetic autoinducers. As shown in Fig. 2a, addition of 3OC12-HSL to the PAO-JP1 growth media restored its colony-forming ability to about the level found in the irradiated wild type PAO1 as well as addition of C4-HSL to the preirradiation culture medium reduced the lethal effect of UVA in PDO100 (Fig. 2b). Addition of either 3OC12-HSL or C4-HSL to the culture medium of PAO-JP2 strain slightly restored cell viability after 180 min of irradiation. Conversely, when both compounds were added together to the growth medium, irradiated PAO-JP2 reached a survival level similar to that of the wild type (Fig. 2c).

Oxidative nature of UVA lethal effect

In order to determine if the lethal effect of UVA irradiation on QS defective strains has an oxidative component, the influence of oxygen on its UVA response was assayed. As shown in Fig. 3, irradiation in a nitrogen atmosphere significantly restored the cell viability of all the assayed strains following 180 min of irradiation, especially in the most sensitive strain, the double mutant PAO-JP2.

In order to evaluate the radioinduced oxidative stress in QS defective strains, the ultraweak chemiluminescence procedure

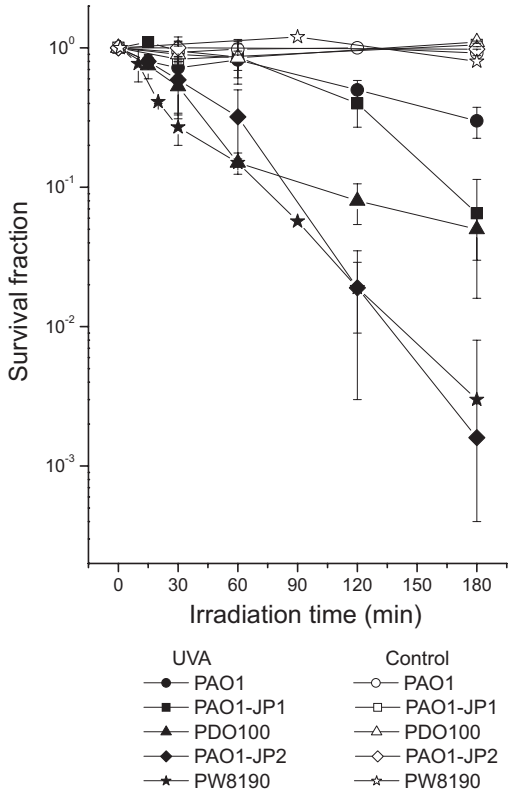

Figure 1. Survival curves of the wild type PAO1 and its QS defective and *katA* derivatives under UVA radiation. Suspensions of PAO1 (wild type), PAO-JP1 (*lasI*), PDO100 (*rhlI*), PAO-JP2 (*lasI rhlI*) and PW8190 (*katA*) strains were exposed to a fluence of 20 W m^{-2} or kept in the dark. Aliquots were withdrawn at different times and plated to determine survival. Error bars represent the standard deviations of counts of at least three independent experiments.

was employed. As shown in Fig. 4, an increase of chemiluminescence values was observed in QS defective mutants in comparison with the wild type PAO1 strain during the UVA exposure. PAO-JP2 cell suspension exhibited a greater increase in light emitted by reactive photoemissive species, in keeping with its greater sensitivity to UVA radiation. Control cell suspensions maintained in the dark did not show important changes of chemiluminescence values.

Catalase and SOD activity of QS-deficient mutants

In order to investigate if the greater sensitivity to UVA radiation of QS mutants correlates with a low level of antioxidative enzymes, we measured catalase and SOD activity in control and irradiated cells. As shown in Fig. 5a, catalase activity showed the highest level in the wild type, while the single mutants *lasI* and *rhlI* displayed a reduction in the catalase activity, about 55% of the activity measured in the PAO1 strain. Furthermore, a marked decrease in the enzyme activity was observed in the double mutant *lasI rhlI*. In fact, only about 10% of the value found in the wild type was detected. As shown in Fig. 5b, a reduction in total SOD activity was seen in *las* mutants PAO-JP1 and PAO-JP2, which presented 32% and 16% of the activity detected in the PAO1 strain, respectively. Conversely, the *rhlI* mutant PDO100 showed a level of SOD activity similar to the wild type. After 180 min of UVA irradiation (total fluence 216 kJ m^{-2}), catalase activity was

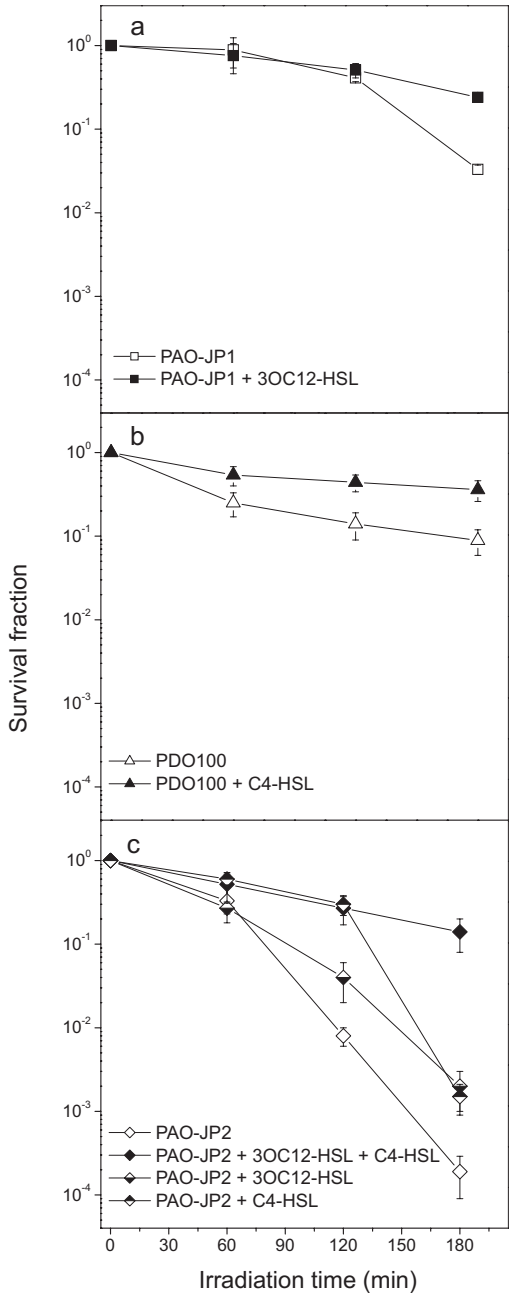

Figure 2. Effect of synthetic autoinducers on survival to UVA of QS defective strains. PAO-JP1 (a), PDO100 (b) and PAO-JP2 (c) strains were grown to stationary phase in plain LB or LB containing 2 μM 3OC12-HSL (PAO-JP1 and PAO-JP2), 10 μM C4-HSL (PDO100 and PAO-JP2) or both (PAO-JP2). Cell suspensions were exposed to 20 W m^{-2} of UVA radiation and aliquots were withdrawn at different times and plated to determine survival fraction. Error bars represent the standard deviations of counts of at least three independent experiments.

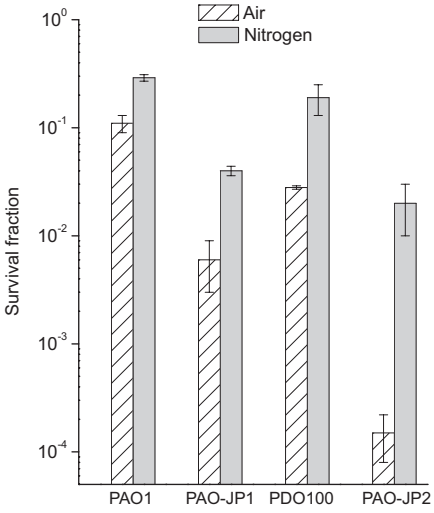

Figure 3. Effect of irradiation in a nitrogen atmosphere on UVA response of the wild type PAO1 and its QS defective derivatives. Suspensions of PAO1 (wild type), PAO-JP1 (*lasI*), PDO100 (*rhlI*) and PAO-JP2 (*lasI rhlI*) strains were exposed for 180 min to 20 W m^{-2} UVA in air or nitrogen as described in Materials and Methods. Aliquots were withdrawn and plated to determine survival. Error bars represent the standard deviations of counts of at least three independent experiments.

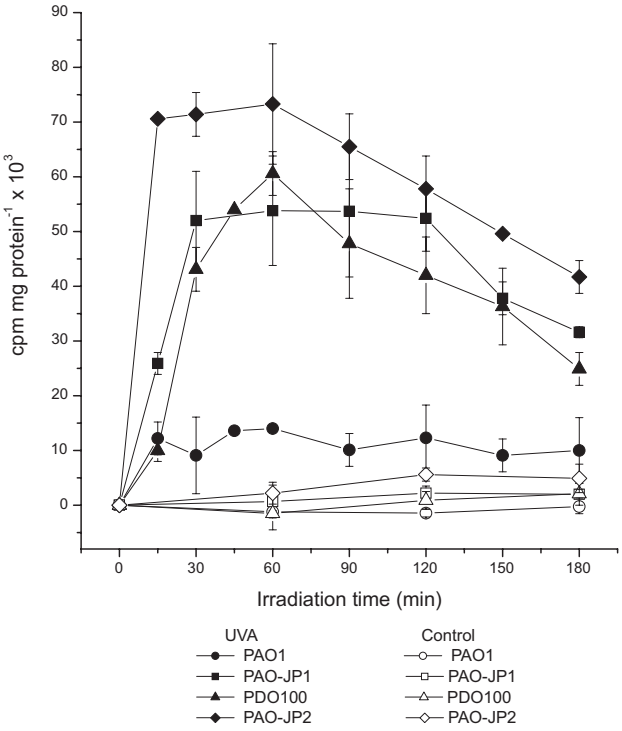

Figure 4. Chemiluminescence measurements of the wild type PAO1 and its QS defective derivatives under UVA radiation. Suspensions of PAO1 (wild type), PAO-JP1 (*lasI*), PDO100 (*rhlI*) and PAO-JP2 (*lasI rhlI*) strains were exposed to 20 W m^{-2} UVA or kept in the dark. Aliquots were withdrawn at different times and chemiluminescence measured as indicated in the text. Error bars represent the standard deviations of counts of at least three independent experiments.

greatly reduced in all the assayed strains (Fig. 5a), whereas SOD activity was almost not modified by the same treatment (Fig. 5b).

To test the importance of catalase in protecting cells against UVA, a catalase-deficient *katA* derivative of the PAO1 strain (PW8190, catalase activity 2.8 U mg^{-1}) was irradiated as previously described. As shown in Fig. 1, the strain PW8190 exhibited a marked sensitivity to UVA in comparison with the wild type and showed a survival level similar to PAO-JP2.

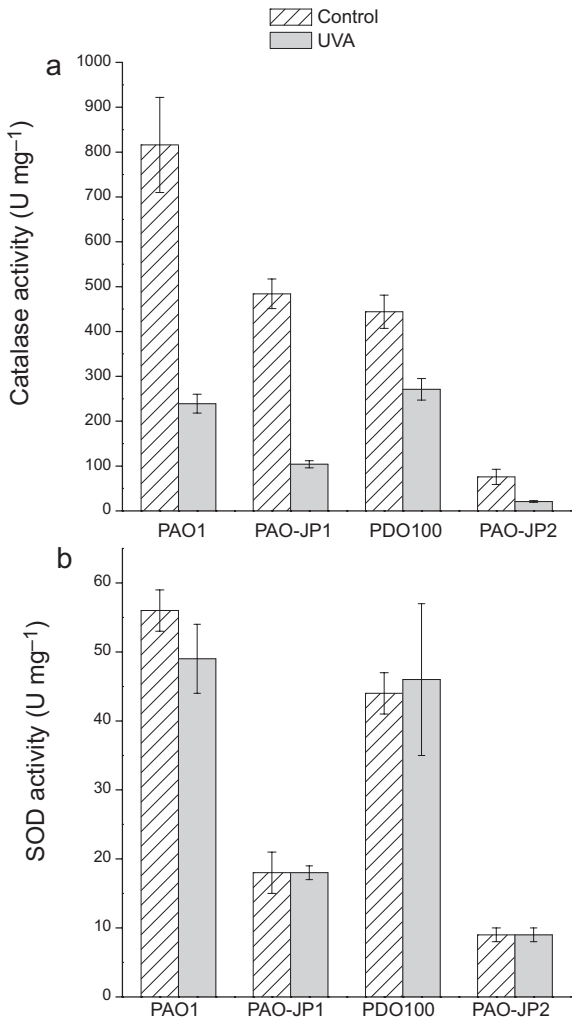

Figure 5. Catalase and SOD activity of wild type PAO1 and its QS defective derivatives. PAO1 (wild type), PAO-JP1 (*lasI*), PDO100 (*rhlI*) and PAO-JP2 (*lasI rhlI*) strains were grown to stationary phase and irradiated for 180 min at a fluence rate of 20 W m^{-2}. Cell free extracts of control and irradiated cells were assayed for catalase activity (a) and SOD activity (b) as indicated in Materials and Methods. Results are average of at least three experiments; error bars represent the standard deviation.

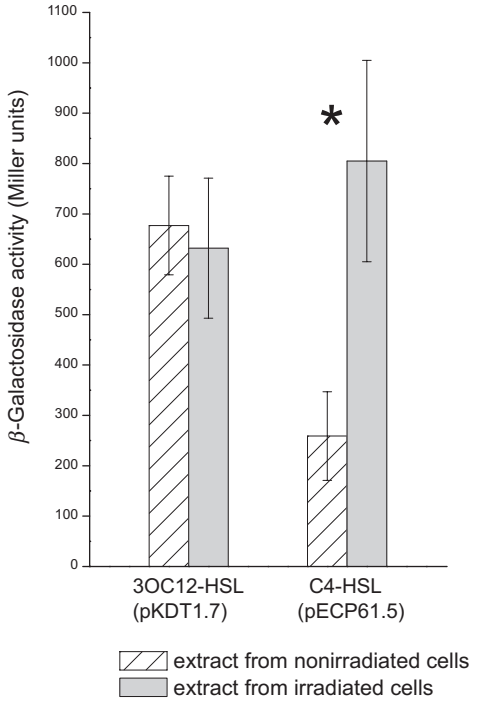

Figure 6. Effect of UVA on the production of *Pseudomonas aeruginosa* autoinducers. The level of autoinducers was determined in supernatants of PAO1 exponential cells (DO = 0.5) previously exposed to 144 kJ m^{-2} UVA or maintained in the dark, as described in Materials and Methods. Determination of autoinducers level was performed measuring β-galactosidase activity of reporter strains DH5α pKDT1.7 (for 3OC12-HSL) and PAO-JP2 pECP61.5 (for C4-HSL). Five milliliters cultures of these strains were assayed with 5 μL extract from control cells or 5 μL extract from irradiated cells. β-galactosidase activity is expressed in Miller units; all the assays were performed in duplicate and the results are the average of at least three experiments; error bars indicate the standard deviation. β-galactosidase activity of reporter strains treated with extract from control or irradiated cells was compared using a Student's *t*-test; the asterisk represents statistically significant differences ($P < 0.01$).

Production of QS autoinducers upon irradiation with UVA

Besides cell density, other factors related to stress conditions such as overproduction of the stringent response effector ppGpp or alterations at the membrane structure level, are capable of activating the synthesis of QS signals (34,35). In order to investigate if UVA radiation could also induce the early expression of QS signals, the level of autoinducers in supernatants of exponential PAO1 cells previously irradiated or maintained in the dark was determined as described in Materials and Methods. As shown in Fig. 6, no difference in β-galactosidase activity for the expression of the *lasB′–lacZ* fusion was detected among extracts from irradiated (120 min at a fluence rate of 20 W m^{-2}) and nonirradiated PAO1 cells. However, extracts from PAO1 cells previously exposed to the same radiation triplicated the expression of the *rhlB′–lacZ* fusion in comparison with the control extract. Extracts obtained after irradiation for 60 min at the same fluence rate produced the same effect with both reporter plasmids, but the induction of *rhlB′–lacZ* duplicated compared to nonirradiated cells (data not shown). The induction of the rhl system by UVA irradiation was confirmed by determination of pyocyanin level in cultures of PAO-JP2 pECP61.5 (*rhlB′–lacZ*) strain assayed for β-galactosidase. Genes involved in the biosynthesis of pyocyanin, a blue pigment related to virulence in *P. aeruginosa*, are specifically activated by the RhlR/C4-HSL system (36). Pyocyanin is absent in the PAO-JP2 pECP61.5 strain because it does not produce C4-HSL. However, when this strain grows in the presence of autoinducer extracts, pyocyanin synthesis should occur and correlate with C4-HSL concentration. An increase in the pyocyanin level was observed when the PAO-JP2 pECP61.5 strain was grown in the presence of extract of irradiated PAO1 (A_{520} 0.192 ± 0.03) compared to the extract of nonirradiated cells (A_{520} 0.144 ± 0.012), suggesting a higher concentration of C4-HSL autoinducer.

Response of QS-deficient mutants to sublethal UVA fluences

The influence of QS in the sublethal growth delay phenomenon was assessed by exposing stationary phase cells of the wild type

PAO1 and its QS defective derivatives to different sublethal UVA total fluences (Fig. 7). In the case of the PAO1 strain, a dose-dependent growth delay was observed (growth delay lasting 19, 37 and 78 min for total fluences of 18, 36 and 72 kJ m^{-2}, respectively), indicating that this response is present in *P. aeruginosa*. No important differences among the four strains were seen at 18 kJ m^{-2} (Fig. 7a), but when UVA irradiation increased to 36 kJ m^{-2}, two groups of strains could be clearly differentiated: PAO1/PAO-JP1 (growth delay lasting 37 and 33 min, respectively), and PDO100/PAO-JP2 (growth delay lasting 66 and 59 min, respectively) (Fig. 7b). When a total fluence of 72 kJ m^{-2} was assayed, the growth delay of PAO-JP1 increased to 79 min, a value similar to that observed in the irradiated PAO1 strain (Fig. 7c); this fluence was not assayed in PDO100 and PAO-JP2 strains because lethal effects appeared (see Fig. 1, 60 min).

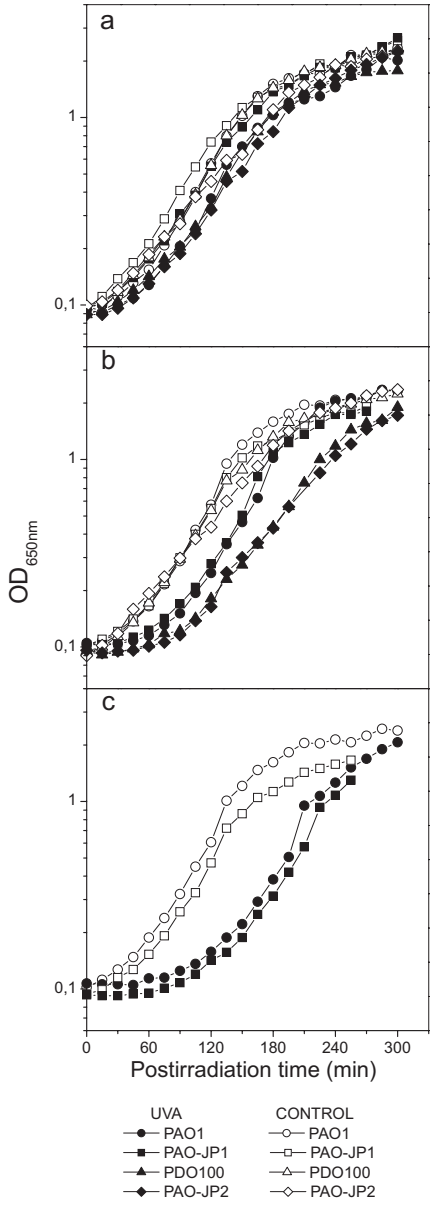

Figure 7. Growth delay induced by sublethal UVA fluences of the wild type PAO1 and its QS defective derivatives. Suspensions of PAO1 (wild type), PAO-JP1 (*lasI*), PDO100 (*rhlI*) and PAO-JP2 (*lasI rhlI*) strains were exposed to a fluence rate of 20 W m^{-2} for 15, 30 or 60 min, corresponding to a total fluence of 18 (a), 36 (b) or 72 (c) kJ m^{-2}, respectively. Control cells were maintained in the dark. Immediately after irradiation, control and irradiated cells were suspended in prewarmed LB broth and incubated at 37°C as indicated in the text. Growth was followed by OD_{650} measurements. A representative experiment is shown; all experiments were carried out in triplicate.

DISCUSSION

The aim of the present study was to evaluate the participation of the QS system in the response of *P. aeruginosa* to UVA radiation; mutants defective in their major intercellular signals, 3OC12-HSL and C4-HSL, were employed. Taking into account that QS has been found to be triggered at high cell densities, we focused our study on cells at the stationary growth phase.

Although at lower fluences only C4-HSL seems to have an important role in neutralizing UVA toxicity, the absence of both QS autoinducers results in high sensitivity (about 3–4 log reduction in the number of viable cells) after 180 min of low-intensity UVA radiation (20 W m^{-2}), while the failure of either rhl or las signals individually only produced 1 log reduction following the same exposure (Fig. 1). Our data suggest that both QS systems are essential to maintain optimal cell viability after UVA irradiation. If only las system was relevant in UVA response, PAO1 and PDO100 should be similar and if only rhl system was important, PDO100 and PAO-JP2 should have a similar behavior. Furthermore, only the addition of both synthetic QS signals to the preirradiation culture of *lasI rhlI* mutant restored its colony-forming ability to values similar to those determined in the irradiated wild type PAO1 (Fig. 2), confirming the importance of both QS systems on the UVA response. It is well known that the LasR/3OC12-HSL system initiates the QS regulatory cascade, activating the transcription of *rhlR*, among other regulators (20). This hierarchical scheme was revisited in a recent work where it is demonstrated that the rhl regulon is only delayed but functional in the absence of the las system, allowing the expression of rhl-dependent virulence determinants during the late stationary phase (21). In agreement with this model, the fact that PAO-JP1 and PAO-JP2 exhibit different UVA responses at the stationary phase of growth suggests that the RhlR regulon can be activated in the absence of the las system in the PAO-JP1 strain and regulate the UVA response.

It has been well established that the lethal effects of UVA radiation are oxygen dependent (2,3). Furthermore, a very important protection against the toxic UVA effects was observed when the irradiation of bacterial suspensions of *P. aeruginosa* was carried out under nitrogen atmosphere (9), demonstrating the involvement of an oxidative component in the UVA-induced damage. In our experimental conditions, irradiated QS defective mutants exhibited an increase in cell viability when suspensions were exposed to UVA under reduced oxygen tension (nitrogen atmosphere), suggesting the involvement of QS systems in the defense against the bacterial UVA-induced oxidative damage. This assumption was confirmed by monitoring the generation of active oxygen species during irradiation by the chemiluminescence method. The procedure was employed in the study of photon emission

related to oxidative stress in microorganisms (37) and a very close relationship was found between the increase of chemiluminescence levels and the UVA-induced damage in *E. cloacae*, *P. aeruginosa* and *E. coli* (7,38). Ultraweak chemiluminescence in the visible region has been attributed to photon emission by excited carbonyl groups and singlet O_2 dimers arising from the decomposition of membrane lipid peroxides (37), which in turn are associated with an increase in reactive oxygen species generated by UVA radiation (2). A very important increase in chemiluminescence during the exposure of QS mutants to sublethal doses of UVA radiation is reported, suggesting elevated lipid peroxidation in these strains. Here again the results obtained demonstrate the fundamental contribution of the QS mechanism against the oxidative UVA-induced damage.

Toxic effects of UVA radiation are mainly due to the action of reactive oxygen species, including superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen. SODs (FeSOD and MnSOD) and catalases are enzymes with an essential role in preventing accumulation of some of these toxic compounds. SODs catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide, which in turn is decomposed to water and oxygen by catalases.

Besides activating genes involved in virulence and biofilm formation (36,39), QS induces the transcription of genes implicated in the defense against oxidative stress, including those coding for catalase KatA and SODs MnSOD and FeSOD, as reported by Hassett *et al.* (16). In agreement with that report, our results indicate that catalase levels of stationary phase cells are regulated by both QS signals, whereas SOD activity depends strongly on the presence of the 3OC12-HSL autoinducer (Fig. 5). In this study, we show a correlation between UVA sensitivity following 180 min irradiation and the catalase content in QS mutants before the irradiation procedure; however, this correlation is not very clear as UVA irradiation reduced catalase activity in the PAO1 and PDO100 strains to comparable levels and UVA response was different. SOD activity seems not to have a relevant role in UVA protection, as PAO-JP1 and PDO100 strains, with similar cell viability levels after 180 min irradiation show differences in total SOD activity.

Controversial results about the importance of catalase in the protection against UVA in *E. coli* were reported (40–42). The increased radiosensitivity of a *katA* derivative of the PAO1 strain, deficient in the main catalase KatA, demonstrates the important role of catalase activity against UVA-induced damage in *P. aeruginosa* (Fig. 1). Moreover catalase has been found to inhibit bacterial photon emission, indicating that hydrogen peroxide is involved in the production of chemiluminescence (43). Our data allow us to propose that the greater sensitivity to lethal UVA doses found in QS mutants could be ascribed at least in part to lower levels of catalase activity, perhaps with a minor role for the SOD enzyme. The UVA protection by catalase could be related to the decomposition of hydrogen peroxide to water and oxygen, impairing the production of the highly toxic hydroxyl radicals by the Fenton and the Haber-Weiss reactions. As previously reported (44,45), catalase activity underwent a marked reduction after UVA radiation in all the assayed strains whereas SOD activity was not modified by the same irradiation conditions. Exposure of catalase to UVA produces important structural and functional changes: its spectral absorbance and isolelectric point are modified, aggregation is induced and its enzymatic activity is substantially reduced (45). The 365 nm irradiation of catalase leads to the formation of catalytically inactive intermediates with different spectra and this formation may explain its inactivation by radiation (46). Another possible explanation for the protective role of catalase is that this enzyme may protect other targets by absorbing the energy of UVA radiation.

Besides catalase and SOD activity, mutations impairing the synthesis of autoinducers affect other bacterial properties, such as the content of the *Pseudomonas* quinolone signal (PQS) (47). PQS is a third intercellular signal produced by *P. aeruginosa* which has been related to oxidative stress and cell fitness (48). PQS production is induced by the las system and repressed by the rhl system. Thus, while PAO-JP1 and PAO-JP2 strains do not produce PQS, the PDO100 strain produces six-fold more PQS than the wild type PAO1 (49). The content of PQS reported for these strains does not correlate with the UVA response observed in this study, indicating that possibly PQS is not involved in the resistance mechanism to this agent despite its connection with oxidative stress. However we suggest that, in addition to the level of antioxidant enzymes, other factors such as the presence of more sensitive UVA target/s, changes at the membrane structure level or deficiencies in repair/protection systems, should not be dismissed as a potential cause of the UVA sensitivity of QS mutants.

In this study we present evidence for a sublethal UVA-induced growth delay in *P. aeruginosa* when stationary phase cells were employed. Growth delay has been proposed as a mechanism related to bacterial protection against lethal and mutagenic effects of UVA or solar irradiation (50). In *E. coli*, the radioinduced growth delay phenomenon was attributed to UVA absorption by the chromophore 4-thiouridine, a tRNA base that crosslinks with a neighboring cytidine (51,52) losing its charge capacity. The presence of uncharged tRNAs by UVA action leads to a *relA*-dependent increase in the level of ppGpp (53), the main effector of the stringent response, that acts as an amplifier of the growth lag (54). In *P. aeruginosa*, besides its functions in nutritional stress (55), ppGpp has been related to QS and virulence (28,34). Fernández and Pizarro reported ppGpp accumulation in exponential cells of *P. aeruginosa* as a consequence of UVA irradiation (10). However, the growth delay mechanism was not detected (9). Data obtained in our laboratory suggest that the stringent response does not appear to contribute to the growth delay amplification of stationary phase cells of *P. aeruginosa*, as a PAO1 *relA* derivative, which is defective in the main route of ppGpp synthesis, exhibits the same growth delay as the isogenic *relA*$^+$ strain (data not shown), according to the report of Thiam and Favre in *E. coli* (54).

In addition to the stringent response, previous studies have reported that oxidative disturbance of bacterial membranes induced by UVA exposure contributes to increase the UVA-dependent growth delay in *E. coli* (38,56). According to results reported here, the QS system acts against oxidative damage in irradiated bacteria, and it could be proposed that the difference found in the extent of growth delay between the wild type PAO1 strain and *rhlI* mutants (PDO100 and PAO-JP2 strains) at 36 kJ m^{-2} (Fig. 7b) might be ascribed to an increased sensitivity of these strains to oxidative damage,

suggesting that the rhl system appears to be important in this response.

UVA radiation exposure at 20 W m^{-2} for 120 min was able to increase C4-HSL level in PAO1 exponential cells by about three-fold when compared with nonirradiated controls. Premature synthesis of *P. aeruginosa* QS signals independent of cell density has been reported by several workers. Van Delden *et al.* (34) demonstrated that both *lasR* and *rhlR* gene expression and autoinducer synthesis are activated in log cells by overexpression of the *relA* gene. Furthermore, in low magnesium conditions, a *relA* dependent enhanced production of 3OC12-HSL was reported by Erickson *et al.* (28). In a later study, Baysee *et al.* (35) demonstrated that inactivation of the *lptA* gene, which alters the fatty acid profile of phospholipids with the consequent modification of the membrane properties, results in a premature production of the QS signals C4-HSL and C6-HSL. Activation of QS by UVA independently of cell density could act as an adaptative mechanism against the toxic effects of radiation by inducing the transcription of genes involved in protective functions. Regulation of gene transcription by UVA irradiation has been reported in *E. coli*, and some of the induced genes are those involved in protective functions against oxidative stress and in DNA repair (57,58). In our experimental conditions, only C4-HSL induction was detected, whereas 3OC12-HSL levels remained similar in both control and irradiated cells. However, taking into account that the wild type strain begins to produce 3OC12-HSL at low cell density (34), the bioassay employed herein to detect this signal might not be able to detect such differences. Further studies are necessary to determine if early induction of C4-HSL by UVA irradiation correlates with an increase in the expression and/or activity of antioxidant systems.

In summary, this work reports the importance of the QS mechanism in response to UVA irradiation in *P. aeruginosa* at the stationary phase of growth. This finding might be explained on the basis of its importance in the transcription of genes related to antioxidant defense, although it cannot be excluded that less resistant UVA targets or deficiencies in other repair/protection systems are present in QS mutants. A growth delay mechanism as response to sublethal UVA exposure is present in this organism and the rhl (QSII) system seems to be involved in this response. In addition, the activation of expression of the rhl system by UVA radiation is reported. Given the great interest in environmental factors as modulators of global mechanisms of gene expression, this may be a promising subject for further studies.

Acknowledgements—The excellent technical assistance of Ms. P. Pereyra Schuth is gratefully acknowledged. We thank Dr. E.P. Greenberg for kindly providing the QS mutants and plasmids, and Dr. Cristian Navntoft for the design and construction of the irradiation source. This research was supported in part by a grant (PICT 38241) from Agencia Nacional de Promoción Científica y Tecnológica (Argentina).

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