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Exposure to low UVA doses increases KatA and KatB catalase activities, and confers cross-protection against subsequent oxidative injuries in *Pseudomonas aeruginosa*

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Solar UVA radiation is one of the main environmental stress factors for *Pseudomonas aeruginosa*. Exposure to high UVA doses produces lethal effects by the action of the reactive oxygen species (ROS) it generates. *P. aeruginosa* has several enzymes, including KatA and KatB catalases, which provide detoxification of ROS. We have previously demonstrated that KatA is essential in defending *P. aeruginosa* against high UVA doses. In order to analyse the mechanisms involved in the adaptation of this micro-organism to UVA, we investigated the effect of exposure to low UVA doses on KatA and KatB activities, and the physiological consequences. Exposure to UVA induced total catalase activity; assays with non-denaturing polyacrylamide gels showed that both KatA and KatB activities were increased by radiation. This regulation occurred at the transcriptional level and depended, at least partly, on the increase in H_2O_2 levels. We demonstrated that exposure to low UVA produced a protective effect against subsequent lethal doses of UVA, sodium hypochlorite and H_2O_2. Protection against lethal UVA depends on *katA*, whilst protection against sodium hypochlorite depends on *katB*, demonstrating that different mechanisms are involved in the defence against these oxidative agents, although both genes can be involved in the global cellular response. Conversely, protection against lethal doses of H_2O_2 could depend on induction of both genes and/or (an)other defensive factor(s). A better understanding of the adaptive response of *P. aeruginosa* to UVA is relevant from an ecological standpoint and for improving disinfection strategies that employ UVA or solar irradiation.

INTRODUCTION

Bacteria respond to changes in the environment by regulating their gene expression in order to adapt to stressful conditions. The study of the mechanisms involved in bacterial adaptation to stress factors is a fundamental task in bacterial physiology. Solar UVA radiation (400–315 nm), the major fraction of UV radiation reaching the Earth's surface, represents one of the main environmental stress factors for bacteria. Several lines of research have demonstrated that lethal effects of UVA are due to oxidative damage of proteins, lipids and DNA by the action of reactive oxygen species (ROS) (Chamberlain & Moss, 1987; Hu & Tappel, 1992; Girard *et al.*, 2011). ROS are generated by the absorption of UVA by endogenous photosensitizers, such as flavoproteins, cytochromes and quinones, and include singlet oxygen, superoxide anions, hydroxyl radicals and H_2O_2 (Bäumler *et al.*, 2012; Pezzoni *et al.*, 2015). In order to investigate the mechanisms involved in the adaptation of bacteria to UVA, the global transcriptomic response of bacteria exposed to low doses of UVA was analysed in micro-organisms such as *Shewanella oneidensis*, *Escherichia coli*, *Nostoc punctiforme* and *Enterococcus faecalis* (Qiu *et al.*, 2005; Berney *et al.*, 2006a; Soule *et al.*, 2013; Sassoubre *et al.*, 2014). These studies revealed that activation of genes coding for enzymes responsible for ROS detoxification and DNA repair is a common response to UVA exposure. In the case of *Pseudomonas aeruginosa*, activation of genes involved in DNA repair, such as *recA* and *din*, by UVA has been reported (Kidambi *et al.*, 1996). In addition,

Abbreviations: qRT, quantitative real-time; ROS, reactive oxygen species; SOD, superoxide dismutase; SODIS, solar disinfection of drinking water.

a protective effect of exposure to sublethal UVA against the action of subsequent lethal doses was found to be dependent on *relA*, the main gene responsible for the synthesis of the transcriptional regulator ppGpp (Pezzoni *et al.*, 2012). However, there is no information about the induction by UVA of genes involved in detoxification of ROS; thus, mechanisms associated to this adaptive stress response remain unknown.

P. aeruginosa is a bacterium present in terrestrial and aquatic environments, and an opportunistic human pathogen able to produce severe complications in immunocompromised individuals, patients with burn wounds and people suffering cystic fibrosis. It has multiple enzymic systems for defence against ROS. These include two superoxide dismutases (Fe-SOD and Mn-SOD) (Hassett *et al.*, 1993) to decompose the superoxide anion to H_2O_2 and O_2, and four alkyl hydroperoxidases (AhpA, AhpB, AhpCF and Ohr) (Ochsner *et al.*, 2000) and three monofunctional catalases (KatA, KatB and KatC) (Brown *et al.*, 1995; Ma *et al.*, 1999; Mossialos *et al.*, 2006) to decompose peroxides to H_2O and O_2. KatA is the main catalase and has unique characteristics: it is unusually stable, and essential to H_2O_2 and UVA resistance, osmoprotection and virulence (Hassett *et al.*, 2000; Costa *et al.*, 2010; Pezzoni *et al.*, 2014; Lee *et al.*, 2005). KatA is highly expressed during all growth phases, but it is induced at the stationary growth phase and by increased levels of H_2O_2 (Brown *et al.*, 1995; Heo *et al.*, 2010). KatB is only detected in the presence of H_2O_2 or paraquat and is only partially involved in resistance to oxidative stress (Brown *et al.*, 1995; Lee *et al.*, 2005). However, KatC is induced by high temperature and dispensable in tolerance to H_2O_2 (Mossialos *et al.*, 2006).

In order to better understand how *P. aeruginosa* deals with high UVA doses, we investigated whether exposure to sublethal doses of UVA is able to modulate KatA and KatB activities, and the effect of this photostress on the response to subsequent oxidative injuries. Taking into account the relevance of *P. aeruginosa* as a pathogen, the results presented in this study should be considered in disinfection strategies that employ UVA or solar irradiation as killing agents, such as photocatalytic treatments or solar disinfection of drinking water (SODIS).

METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains used in this work were PAO1 (WT), and its isogenic derivatives PW8190 (*katA*::Is*lacZ*/hah) and PW8769 (*katB*::Is*lacZ*/hah); transposon Is*lacZ*/hah is inserted in-frame in both mutants so that they can be used as reporter strains in expression assays by measuring β-galactosidase activity. Mutants were obtained from the University of Washington Genome Center (Jacobs *et al.*, 2003). Bacterial cultures were routinely grown at 37 °C with shaking in complete LB broth (10 g tryptone, 5 g yeast extract and 5 g NaCl bring the volume up to 1000 ml in distilled water); for solid medium, agar was added at 15 g l^{-1}. Kanamycin (150 µg ml^{-1}) was added to primary cultures of complemented *katA* p*katA* and *katB* p*katB* strains; all other cultures were obtained in the absence of antibiotics.

Irradiation source. Cell suspensions were irradiated using a bench with two Philips TDL 18W/08 tubes (>95 % of UVA emission at 365 nm). The incident fluence under our 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 anodised reflectors to enhance the fluence rate on the section to be irradiated.

Growth under sublethal UVA irradiation. Mid-exponential cultures (OD_{650} 0.3) were diluted to OD_{650} 0.05 in LB medium and divided into two 30 ml fractions, each of which was placed in a glass beaker (4.5 cm internal diameter). The beakers 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, whilst the other fraction was covered with a black plastic sheet (dark control). The cell suspensions were stirred continuously with a magnetic bar. Cell growth of irradiated and control suspensions was followed by measuring OD_{650}. The fluence employed may be encountered normally in the environment (Hoerter *et al.*, 2005).

Chemiluminescence assays. Production of photoemissive species was followed by means of a liquid scintillation system in the out-of-coincidence mode (Cadenas & Sies, 1984). For this purpose, 5 ml aliquots were taken during bacterial growth and quickly transferred to the scintillation system, equipped with photomultipliers sensitive in the blue region up to 600–650 nm (Tri-Carb 1500; Packard Instruments). Chemiluminescence values were expressed as c.p.m. per OD_{650} unit.

Total catalase activity assay. Cultures grown under UVA or in the dark were harvested at OD_{650} 0.3 by centrifugation, suspended in ice-cold 50 mM potassium phosphate buffer, pH 7, sonicated in an ice-water bath and clarified by centrifugation at 12 000 ***g*** for 10 min at 4 °C. Catalase activity was monitored in the supernatant by following the decomposition of 10 mM H_2O_2 according to Aebi (1984). One unit of activity was that which decomposed 1 µmol H_2O_2 min^{-1} (mg protein)$^{-1}$. Protein content was determined by the Lowry method (Lowry *et al.*, 1951).

Catalase native PAGE analysis. KatA and KatB activities were analysed in non-denaturing polyacrylamide gels, according to the method of Wayne & Diaz (1986). Briefly, 10 µg protein per sample from extracts obtained for total catalase activity assays was loaded in 5 % non-denaturing polyacrylamide gels and run at 53 V/15 mA. Gels were soaked in distilled water for 5 min, followed by 10 min incubation in 4 mM H_2O_2 at room temperature. The H_2O_2 solution was replaced by distilled water and incubated for an additional 5 min. The distilled water was replaced by a solution containing 1 % (w/v) ferric chloride and 1 % (w/v) potassium ferricyanide. When the gel turned dark green, the ferric chloride/potassium ferricyanide solution was replaced with distilled water and the gel was photographed.

***β*-Galactosidase assays.** Strains PW8190 (*katA–lacZ*) and PW8769 (*katB–lacZ*) were grown under UVA or in the dark to OD_{650} 0.3 (either in LB or in LB in the presence of ROS scavengers), and β-galactosidase activity was measured in cells treated with SDS and chloroform (Miller, 1972). Specific activities were expressed in Miller units referred to OD_{650}. The scavengers used were: 1 mg bovine catalase ml^{-1} or 1.2 % sodium pyruvate for H_2O_2 (McDonald *et al.*, 1983; Khaengraeng & Reed, 2005), 1 mg mannitol ml^{-1} for hydroxyl radical (Cai *et al.*, 2014) and 1 % DMSO for superoxide anion (McDonald *et al.*, 1983).

Quantitative real-time (qRT)-PCR. Total RNA from the PAO1 strain grown under UVA or in the dark (OD_{650} 0.3) was extracted by

using a Total RNA Extraction kit (RBC Biosciences). 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 LightCycler (DNA Engine; MJ Research) and Real-Time PCR Mix (EvaGreen qPCR Mix Plus, no Rox). For quantification of *katA* mRNA, assays were performed using the primers katAleft (5′-ATGCGTTTCTACACCGAGCA-3′) and katAright (5′-ATGGTCAACTGATGCAGCGA-3′), designed for this study on the basis of the genome sequence of the PAO1 strain (Stover *et al.*, 2000). For quantification of *katB* mRNA, two sets of primers were employed: sense (5′-GAGCAGAACTTCAAGCAGAC-3′) and antisense (5′-CTCTCGTCGTCGGTGATC-3′), which have been employed previously (Chang *et al.*, 2005; Small *et al.*, 2007), and katBleft (5′-GGTTTCGCCACCAAGTTCTA-3′) and katBright (5′-CGTGGGAGAAGAAATCGAAG-3′), designed for this study. The 16S rRNA gene was used as reference for normalization of expression levels of target genes in each condition by employing the primers 5′-AGCTTGCTCCTTGATTCAGC-3′ and 5′-AAGGGCCATGATGACTTGAC-3′. The cycling conditions for *katA* and 16S rRNA genes were as follows: denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 25 s, 62 °C for 15 s and 72 °C for 15 s, with fluorescence acquisition at 80 °C in single mode. The same conditions were used for *katB* amplification, except for the annealing temperature at 56.5 °C. Relative changes in the expression of individual genes between the treated and control conditions were obtained through the relative standard curve method (Larionov *et al.*, 2005).

Construction of complementing plasmids. Complementing plasmid p*katA* was described in a previous study (Pezzoni *et al.*, 2014). In order to complement the *katB* mutation, plasmid p*katB* was constructed as follows. Genomic DNA of PAO1 was amplified by PCR by using Q5 High-Fidelity DNA Polymerase (New England Biolabs) and the primers leftkatB (5′-GGCAGTCTAATGCAGCCTTC-3′) and rightkatB (5′-AAGCCTGCCAGAATCCAAC-3′), designed for this study. The PCR amplification product (2190 bp), containing the full-length fragment of *katB* and its promoter region (Brown *et al.*, 1995), was ligated into pGEM-T Easy vector (Promega) by previous A-tailing of the PCR product with *Taq* DNA Polymerase (New England Biolabs). The resulting plasmid was introduced by transformation into *E. coli* DH5α (Bethesda Research Laboratories) and selection for ampicillin resistance (100 μg ml^{-1}). The *Eco*RI fragment containing the *katB* gene was removed from this plasmid and cloned into the *Eco*RI site of the broad-host-range cloning vector pBBR1MCS-2 (Kovach *et al.*, 1995). The new resulting plasmid p*katB* was introduced into the *katB* strain by transformation and selection for kanamycin resistance (150 μg ml^{-1}).

Growth under sublethal UVA radiation, and subsequent exposure to lethal doses of UVA, sodium hypochlorite and H_2O_2

UVA sensitivity. Mid-exponential cultures grown under UVA or in the dark (OD_{650} 0.3) were centrifuged and suspended in saline solution (0.1 M NaCl) at OD_{650} 0.4. The suspensions were divided into two 30 ml fractions, which were each placed in a glass beaker open to the air. One of these fractions was irradiated from above at a fluence rate of 20 W m^{-2} for 180 min (total dose 216 kJ m^{-2}), whilst the other fraction remained in the dark. This fluence rate was lower than that used to generate a sublethal stress level (25 W m^{-2}) because, unlike LB medium (employed in experiments of sublethal UVA exposure), saline solution does not absorb radiation at 365 nm (data not shown). Both suspensions were maintained at 20 °C by using a thermocycler bath and under slow magnetic stirring throughout the entire procedure. Samples were taken from both fractions, diluted in saline solution and plated on LB solid medium to assess cell viability. Plates were incubated in the dark to prevent light-induced DNA repair and the colonies were counted after 24–48 h at 37 °C. Survival was expressed as a fraction of the c.f.u. ml^{-1} at time 0.

Sodium hypochlorite sensitivity. To assay sensitivity to sodium hypochlorite, 50 μl cell culture grown under UVA or in the dark (OD_{650} 0.3) was diluted in molten LB (0.75 % agar) and layered on LB plates. Sterile filter paper discs saturated with 8 μl 0.6 % sodium hypochlorite were then placed on the layer. Sensitivity was recorded as the diameter of growth inhibition after 24 h growth at 37 °C.

H_2O_2 sensitivity. To assay sensitivity to H_2O_2, 5 μl serial dilutions of cell cultures grown under UVA or in the dark (OD_{650} 0.3) were spotted onto LB agar or LB agar containing 200 μM H_2O_2. Plates were incubated 24–48 h at 37 °C and photographed.

Statistical analysis. The significance of each treatment was evaluated by Student's *t*-test with confidence levels at >95 % (i.e. $P<0.05$ was considered as significant).

RESULTS

Effect of sublethal UVA doses on cell growth and oxidative damage in *P. aeruginosa*

In order to analyse whether low UVA doses were able to induce catalase activity and the physiological consequences of this phenomenon, we first analysed the growth and the extent of oxidative damage of the WT PAO1 and deficient catalase derivatives Δ*katA* and Δ*katB* exposed to different UVA fluence rates (results not shown). A condition that did not alter cell viability significantly but produced certain oxidative damage was employed throughout this study (Fig. 1). When strains were cultured in complete medium under UVA delivered at a fluence rate of 25 W m^{-2}, they suffered a growth delay compared with control cultures maintained in the dark (Fig. 1a); viable cell count was not affected by the treatment (data not shown). The difference in growth between control and UVA-treated cultures of catalase mutants was significant ($P<0.05$) throughout the experiment (*katA* strain) or between 100 and 240 min (*katB* strain). In the case of the WT, although a growth delay was observed in the UVA-exposed culture compared with the control, this difference was not significant. To evaluate *in vivo* whether the growth delay could be associated to oxidative damage, the ultraweak chemiluminescence procedure was employed (Tilbury & Quickenden, 1988; Pizarro, 1995). A strong peak of light production was observed in all three strains at the beginning of the exposure, indicating oxidative damage (Fig. 1b), but no difference was observed between strains (Fig. 1b).

Induction of KatA and KatB activities by exposure to low UVA doses

Total and individual catalase activities were analysed in the WT and its derivatives *katA* and *katB* grown under UVA or in the dark. Cells were analysed at the exponential growth phase to avoid the induction of *katA* expression at the stationary growth phase in order to highlight the effect of the radiation (Brown *et al.*, 1995). Fig. 2(a) shows that exposure to UVA significantly induced total catalase activity ($P<0.05$) in the three strains compared with

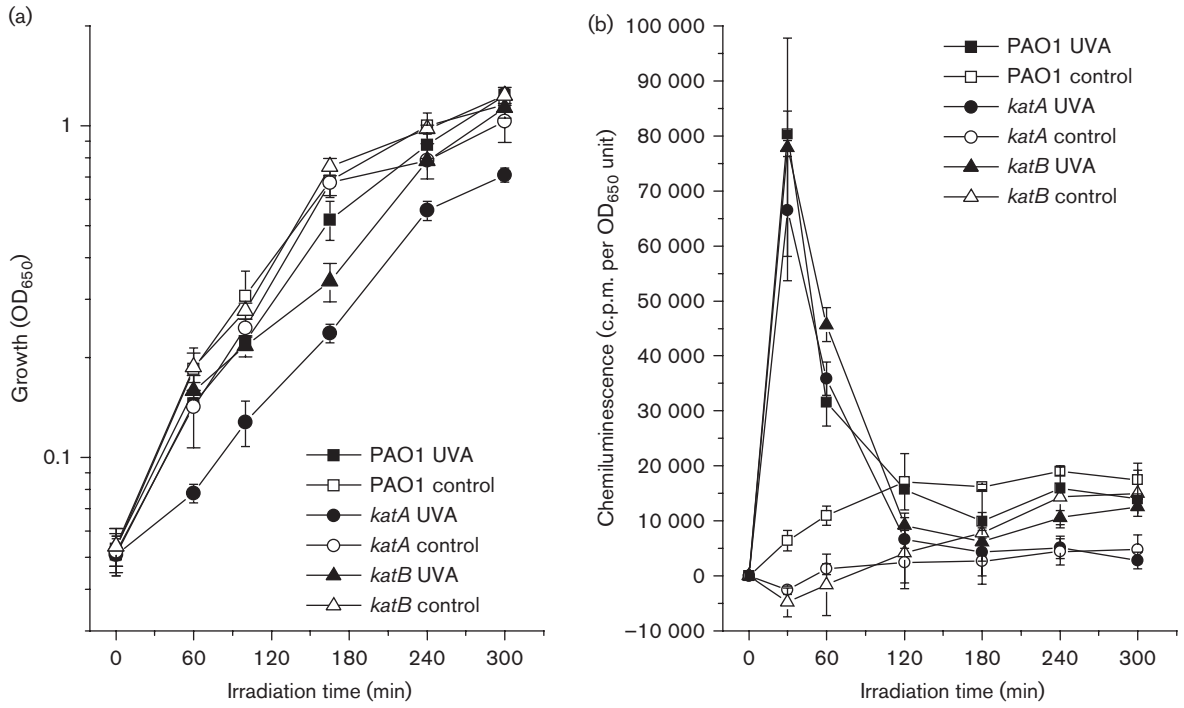

Fig. 1. Effect of exposure to sublethal UVA doses (fluence rate 25 W m^{-2}) on the (a) growth and (b) chemiluminescence values of WT PAO1 and its catalase mutants *katA* and *katB*. Control cells were grown under similar conditions but kept in the dark. Data are presented as mean ± SE of at least three independent assays.

control cells. UVA exposure increased total catalase activity ~25 % in the WT and the *katB* strain; this activity could be detected in the *katA* strain only under UVA. Comparison between irradiated *katA* and *katB* strains, which only produce KatB or KatA, respectively, demonstrated that both catalases were induced by the treatment, with KatB activity being substantially lower (about sixfold) than KatA activity. We then analysed KatA and KatB activities in non-denaturing polyacrylamide gels by making use of their different mobility when they are run in the native form. KatA is a heteromultimer consisting of two 56 kDa monomers and one 45 kDa monomer (Ma *et al.*, 1999); KatB is a tetrameric enzyme composed by four 57 kDa monomers (Brown *et al.*, 1995). Electrophoresis in non-denaturing gels confirmed that growth under UVA increased both KatA and KatB activities, and that KatB activity was not detected under non-inducing conditions (Fig. 2b). These results suggested that exposure of *P. aeruginosa* to sublethal UVA radiation produces a significant increase in KatA and KatB activities – a possible defensive mechanism against subsequent oxidative injuries.

Induction of *katA* and *katB* transcription by exposure to low UVA doses: role of H_2O_2

To understand the mechanism underlying the higher KatA and KatB activities by exposure to UVA radiation, transcription levels of *katA* and *katB* were analysed by qRT-PCR in irradiated and control cultures of the WT. The level of *katA* mRNA increased significantly by about fivefold ($P<0.05$) under UVA radiation compared with the control assay, whereas no increase in *katB* mRNA was observed due to the treatment (Fig. 3). In addition to the *katB* primers successfully employed by other authors (Chang *et al.*, 2005; Small *et al.*, 2007), another set of primers designed for this work was tested to perform qRT-PCR assays, with the same result.

It was reported that transcription of *katA* and *katB* genes is induced in response to increased H_2O_2 levels by activation of the transcriptional regulator OxyR (Ochsner *et al.*, 2000; Heo *et al.*, 2010). The role of H_2O_2, superoxide anion and hydroxyl radical in the regulation of *katA* and *katB* by UVA was then analysed by using the reporter strains *katA–lacZ* and *katB–lacZ* and specific ROS scavengers. Fig. 4(a) shows that the production of β-galactosidase of the strain carrying the *katA* fusion increased significantly ($P<0.005$) by about threefold in irradiated cultures compared with control cultures in plain LB. The presence in the culture medium of the H_2O_2 scavengers catalase and sodium pyruvate inhibited the production of β-galactosidase in cultures grown under UVA; on the contrary, the presence of DMSO (superoxide anion scavenger) or mannitol (hydroxyl radical scavenger) did not modify significantly the value observed in the culture without scavengers (Fig. 4a). In the case of the *katB* fusion, a

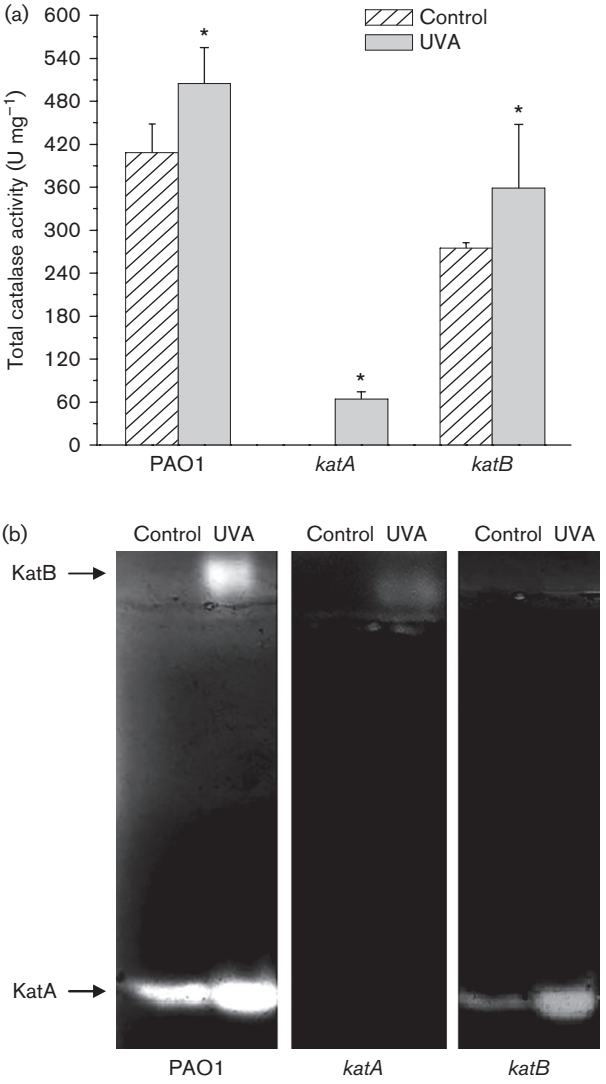

Fig. 2. Extracts from the PAO1 strain and the *katA* and *katB* mutants grown under sublethal UVA doses or kept in the dark (control) were assayed for (a) total catalase activity and (b) catalase activity in non-denaturing gels. (a) Data are presented as mean ± SE of at least three independent assays of total catalase activity. *$P<0.05$. (b) Representative images of non-denaturing polyacrylamide gels stained for catalase activity.

significant twofold induction ($P<0.05$) was seen in irradiated cultures compared with the control condition (Fig. 4b). As seen for *katA*, both H_2O_2 scavengers inhibited expression of β-galactosidase of irradiated cultures compared with cultures grown under UVA without scavengers or in the presence of DMSO or mannitol (Fig. 4b).

In summary, our results indicated that regulation of KatA and KatB activities by exposure to sublethal UVA occurs at the transcriptional level and depends, at least in part, on the increase of endogenous H_2O_2 concentration.

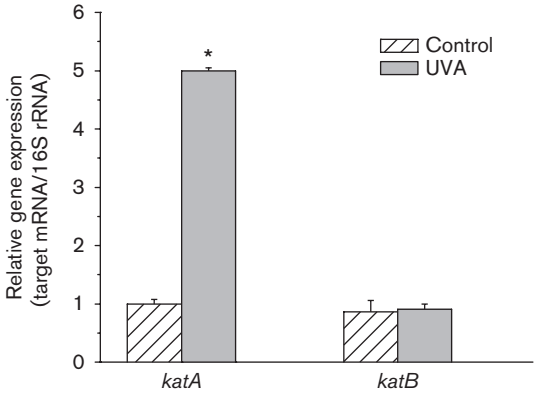

Fig. 3. qRT-PCR of *katA* and *katB* genes from the PAO1 strain grown under sublethal UVA radiation or kept in the dark (control); the 16S rRNA gene was used as reference for normalization in each condition. Data are presented as mean ± SE of at least three independent experiments with three replicates. *$P<0.05$.

Protection against subsequent oxidative stress factors by exposure to sublethal UVA doses: role of *katA* and *katB*

To evaluate whether exposure to low UVA doses can trigger cross-protection against subsequent oxidative injuries, the WT was grown under low UVA doses or in the dark and then challenged with lethal doses of UVA, sodium hypochlorite or H_2O_2. Catalase-deficient derivatives were submitted to the same treatments to evaluate the role of *katA* and *katB* in this phenomenon.

Fig. 5(a) shows that growth under sublethal UVA significantly increased the viability of the WT ($P<0.005$) and the *katB* strain ($P<0.05$) challenged with lethal UVA doses; this protection was not observed in the *katA* strain, demonstrating that this phenomenon depends on the *katA* gene. Growth under sublethal UVA significantly increased the resistance of the WT and the *katA* strains against sodium hypochlorite ($P<0.05$); on the contrary, the treatment did not affect the response of the *katB* strain, indicating that protection depends on *katB* induction (Fig. 5b). Finally, growth under UVA induced protection to H_2O_2 in the three strains in spite of their different sensitivity levels (Fig. 5c), suggesting that both genes are important but not essential, at least individually, for the UVA-mediated H_2O_2 protection. Complementation assays with plasmids carrying the WT *katA* and *katB* alleles confirmed the roles of *katA* and *katB* in these phenotypes (Figs 5a–c). Taken as a whole, these results indicated that exposure to low UVA doses confers tolerance to subsequent lethal doses of different oxidative agents by mechanisms that involve induction of *katA* and *katB* genes.

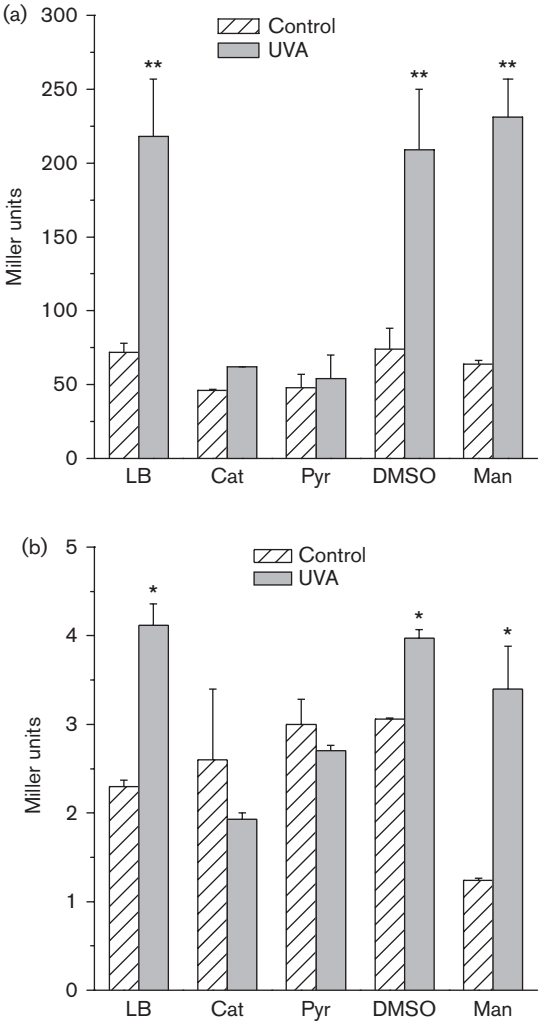

Fig. 4. Reporter strains (a) *katA–lacZ* and (b) *katB–lacZ* were grown under sublethal UVA radiation or in the dark (control), in plain LB and in LB in the presence of catalase (Cat), sodium pyruvate (Pyr), DMSO or mannitol (Man). Samples were taken at OD_{650} 0.3 and β-galactosidase activity was quantified. Data are presented as mean ± SE of at least three independent assays. *$P<0.05$; **$P<0.005$.

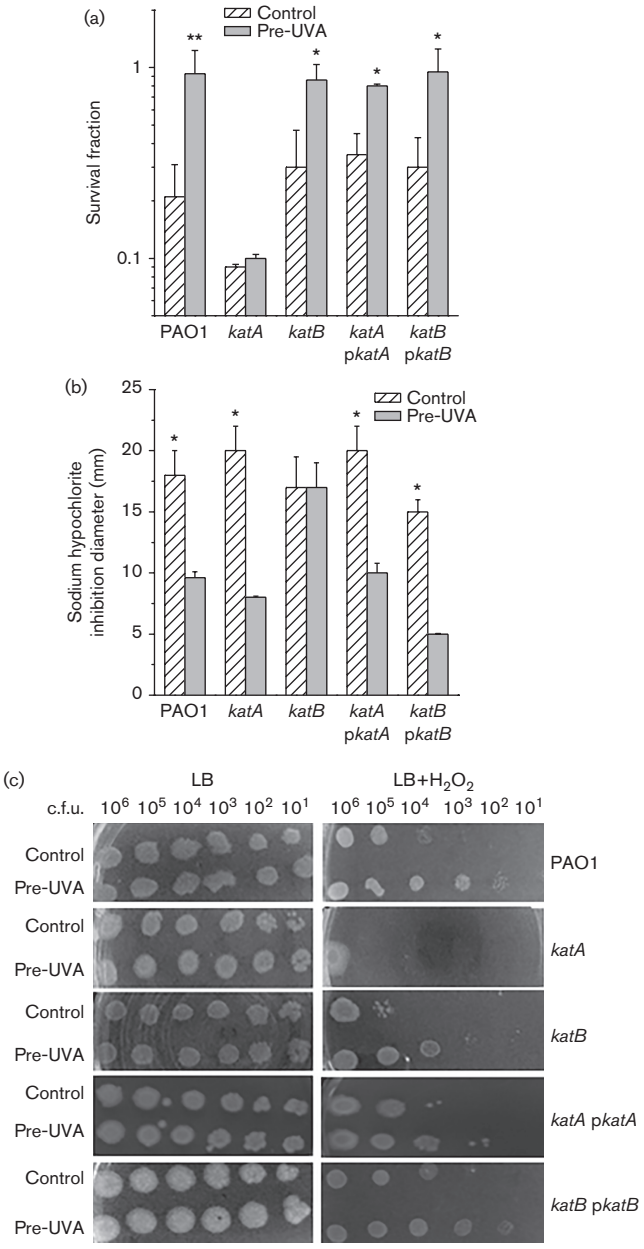

Fig. 5. The PAO1 strain, the *katA* and *katB* mutants, and their complemented derivatives *katB* p*katB* and *katB* p*katB*, were grown under sublethal UVA (pre-UVA) or in the dark (control) and then exposed to lethal doses of (a) UVA, (b) sodium hypochlorite or (c) H_2O_2. (a) The total UVA dose employed for lethality assays was 216 kJ m^{-2} (fluence rate of 20 W m^{-2} for 180 min). After exposure, samples were taken to assess cell viability. (b) Sodium hypochlorite sensitivity was assessed by a diffusion assay by placing 8 µl 0.6 % bleach onto filter paper discs. Data are presented as mean ± SE of at least three independent experiments. *$P<0.05$; **$P<0.005$. (c) H_2O_2 sensitivity was measured by spotting serial dilutions of cell suspensions onto LB plates and LB plates containing 200 µM H_2O_2. Images of representative experiments are shown.

DISCUSSION

In the environment, *P. aeruginosa* can be exposed to low UVA doses that are unable to affect its cell viability significantly. However, ROS levels high enough to induce the expression of defensive systems may be reached. ROS include the oxidizing agent H_2O_2, which is also generated by aberrant electron flow during aerobic respiration or by phagocytic cells during infections. To defend itself from H_2O_2, *P. aeruginosa* decomposes it into H_2O and O_2, largely by the action of KatA and KatB catalases. In this work, we demonstrate for the first time to the best of our knowledge that the genes coding for both enzymes are

activated during its adaptation to sublethal UVA radiation and that this induction is accompanied by an increase in the corresponding enzymic activities. The extent of the growth delay produced by exposure to low doses of radiation could be a good indicator of response to UVA: the longer the growth delay (*katA* > *katB* > WT), the greater the degree of sensitivity to the radiation. Although the treatment did not produce significant cell death, chemiluminescence peaks, attributed to photon emission by excited carbonyl groups and singlet O_2 dimers arising from the decomposition of membrane lipid peroxides (Tilbury & Quickenden, 1988), indicate that cells suffered oxidative damage. Previous results demonstrating the essential role of KatA in the protection against UVA in planktonic cells and biofilms of *P. aeruginosa* (Costa *et al.*, 2010; Pezzoni *et al.*, 2014) indicate that induction of the *katA* gene by the radiation could constitute an adaptive mechanism to face with higher UVA doses. The essential role of KatA may be associated with the unique properties of this enzyme: it has normally high activity levels (Brown *et al.*, 1995), is resistant to several agents (Hassett *et al.*, 2000), active in the extracellular medium (Hassett *et al.*, 2000) and has high affinity for NADPH, a strong reducing agent (Su *et al.*, 2014). Although, as observed previously (Pezzoni *et al.*, 2014), *katB* has been demonstrated not to be relevant in the defence against lethal UVA doses, its induction could contribute in this regard under other experimental conditions.

Unlike *P. aeruginosa*, catalase has a minor role in protection against lethal doses of UVA in other bacteria (Sammartano *et al.*, 1986; Eisenstark & Perrot, 1987; Kramer & Ames, 1987). However, other components of the antioxidative response as well as genes involved in DNA repair participate in the defence against UVA. Mutant strains of *Salmonella enterica* lacking alkyl hydroperoxide reductase (*ahp*) or defective in glutathione synthesis (*ghs*) have high UVA sensitivity (Kramer & Ames, 1987), and a mutant strain of *E. coli* defective for both Fe-SOD and Mn-SOD has greater sensitivity to UVA compared with the WT strain (Hoerter *et al.*, 1989). In addition, mutant strains of *E. coli* deficient in the excision repair system (*uvr*) are highly sensitive to UVA (Webb & Brown, 1976; Shennan *et al.*, 1996), and similar results were obtained in strains of *Salmonella* and *E. coli* defective for RecA, the protein responsible for expression of genes involved in the SOS response (Eisenstark, 1970; Webb & Brown, 1976). The RpoS protein, the alternative sigma transcription factor involved in general stress responses during the stationary growth phase, is also involved in UVA defence both in enterobacteria and *Pseudomonas syringae* (Miller *et al.*, 2001; Maatouk *et al.*, 2004; Berney *et al.*, 2006b). Studies of global gene expression during UVA adaptation in bacteria such as *E. coli*, *Shewanella oneidensis* and *Enterococcus faecalis* showed activation of the genes coding for antioxidant enzymes *ahpCF*, *ahpC* and *sodA* (Qiu *et al.*, 2005; Berney *et al.*, 2006a; Sassoubre *et al.*, 2014); however, induction of genes coding for main catalases was not a common element in these studies. Microarray data of *E. coli* adapted to UVA revealed no effect on *katG* and repression of *katE*, the genes coding for HPI and HPII catalases, respectively (Berney *et al.*, 2006a). This information is consistent with adaptive studies demonstrating a slight increase in HPI activity and a decrease in HPII activity by growth under sublethal UVA doses (Hoerter *et al.*, 2005). In the case of *Shewanella oneidensis*, some minor catalase genes seem to be induced by UVA (Qiu *et al.*, 2005), but the gene coding for the only functional catalase, KatB (Jiang *et al.*, 2014), is not affected by the treatment. Thus, to the best of our knowledge, the induction of catalase by UVA and its relevant role in defence against lethal doses of UVA appears to be unique to *P. aeruginosa*.

The results presented in this study demonstrate that both *katA* and *katB* genes are induced by UVA exposure, but their behaviour was somewhat different. Whilst *katA* clearly showed induction by the two techniques used (fivefold by qRT-PCR and threefold by using a transcriptional fusion), a twofold activation of *katB* was seen only with the corresponding reporter strain. The absence of induction of *katB* by the qRT-PCR assay could be explained by the fact that this analysis was based on one time point; as expression can vary temporally, it is possible that a minor *katB* mRNA peak occurred at a different time and it could not be detected. On the contrary, the reporter assay involves all of the previous history of the cells during UVA exposure before sampling; as a consequence, this method is probably more sensitive to show *katB* activation by UVA compared with qRT-PCR.

Although significant H_2O_2 levels have never been measured in micro-organisms exposed to UVA (Hartman, 1986; Kramer & Ames, 1987), our data employing ROS scavengers and reporter strains support the hypothesis that H_2O_2 is a major product of exposure to lethal UVA doses (Tyrrell, 1985; Hartman, 1986; Kramer & Ames, 1987; Khaengraeng & Reed, 2005). In addition, it is demonstrated that induction of *katA* and *katB* depends, at least in part, on the H_2O_2 levels generated by UVA exposure, suggesting an OxyR-mediated response. OxyR is a central regulator of the oxidative stress response in *P. aeruginosa*. When it is oxidized by H_2O_2, it undergoes a conformational change and acquires DNA-binding capacity, allowing transcription of genes involved in oxidative stress defence, such as *katA*, *katB*, *ahpB* and *ahpCF*, amongst others (Wei *et al.*, 2012).

This study demonstrates that the effect of UVA on KatA and KatB activities is accompanied by cross-protection phenomena. A *relA*-dependent protection against high UVA doses by pre-exposure to low UVA doses has been described previously for *P. aeruginosa* (Pezzoni *et al.*, 2012). Under the conditions employed in our work, we demonstrated that this adaptive response depends on *katA* activation. In addition, induction of protection to the routinely used antimicrobial sodium hypochlorite by UVA exposure was demonstrated for the first time, to the

best of our knowledge. In this case, the phenomenon depends on *katB* induction. A previous study analysing the transcriptomic response of *P. aeruginosa* to sodium hypochlorite-induced oxidative stress revealed an increase in *katB* expression in the presence of this agent (Small *et al.*, 2007). This finding and our results demonstrate the importance of *katB* gene in the response to bleach. Thus, this is the first report of a predominant role of KatB over KatA in defence against a stress factor. This result is quite surprising as a previous study comparing the roles of *katA* and *katB* in oxidative stress, osmotic stress and virulence demonstrated that *katB* always plays a secondary role compared with *katA* (Lee *et al.*, 2005). Why *katB* is more relevant than *katA* in the defence against sodium hypochlorite is an open question and an interesting subject for further studies. It has been demonstrated that hypochlorous acid produces a rapid inactivation of catalase by modification of its haem group (Mashino & Fridovich, 1988; Krych-Madej & Gebicka, 2015). Thus, a mechanism that might explain the predominant role of KatB in the defence against bleach is a higher sensitivity of KatA to direct inhibition by this agent, in spite of its unusual high stability (Hassett *et al.*, 2000). We also observed protection against lethal H_2O_2 levels by previous UVA exposure – a cross-protection phenomenon described in *E. coli* (Tyrrell, 1985). In this case, protection does not depend on KatA or KatB, at least individually. It is also possible that other inducible antioxidative factors, e.g. the OxyR-dependent alkylkhydroperoxide reductases AhpB and/or AhpCF, could be induced by UVA exposure and to contribute to H_2O_2 resistance.

Different disinfection strategies, including SODIS and photocatalytic treatments (Gamage & Zhang, 2010; McGuigan *et al.*, 2012), have been developed by taking advantage of the lethal effects produced by UV radiation. The results presented in this paper are relevant to improving them. SODIS consists of placing drinking water in transparent plastic or glass bottles, which are exposed to the sun. The germicidal effect is based on the combined effect of thermal heating by sunlight and UV radiation, mainly UVA. In photocatalytic treatments, the UVA light is applied in the presence of a photocatalyst, mainly TiO_2, enhancing the bactericidal effect. This is a valuable disinfection technique against a wide range of harmful micro-organisms and represents a viable alternative to traditional disinfection methods such as chlorination, which can produce harmful products. On the basis of our results, it is clear that it is necessary to be sure that the doses of radiation are strong enough to produce irreversible cell death in order to avoid cross-protection against other antibacterial agents.

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