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Differential Roles of OxyR-Controlled Antioxidant Enzymes Alkyl Hydroperoxide Reductase (AhpCF) and Catalase (KatB) in Protection of *Pseudomonas aeruginosa* Against Hydrogen Peroxide in Biofilm Versus Planktonic Culture

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Abstract

The role of the *Pseudomonas aeruginosa* OxyR-controlled antioxidants alkyl hydroperoxide reductase CF (AhpCF) and catalase B (KatB) was evaluated in biofilm versus planktonic culture upon exposure to H_2O_2. AhpCF was found to be critical for survival of biofilm bacteria while KatB was more important for survival of planktonic free-swimming organisms.

Keywords

Pseudomonas aeruginosa; Hydrogen Peroxide; OxyR; Biofilms; Alkylhydroperoxide Reductase; Catalase

1. Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen of humans, predominantly immunocompromised individuals suffering from burn [1], cancer chemotherapy [2], organ transplantation [3] various pneumonias [4]) or patients afflicted with the autosomal genetic disorder, cystic fibrosis (CF) [5, 6]. During CF airway infection, neutrophil titers rise nearly 1500-fold and remain so during the course of the disease [7]. The infection process triggers activation of a respiratory burst, creating an oxidative stress for resident bacteria that predominantly include *P. aeruginosa*. One potentially toxic product of the respiratory burst is hydrogen peroxide (H_2O_2, [8, 9]. Within the phagolysosomal vacuole, H_2O_2 levels can reach concentrations as high as 100 mM [10], a concentration that easily kills *P. aeruginosa* in both planktonic culture and in biofilms, the latter of which are highly organized, typically adherent, antibiotic-resistant communities [11–15]. In fact, stimulated neutrophils release even significant antimicrobial concentrations of H_2O_2 (~12 μM) in the extracellular milieu [16].

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The major response of *P. aeruginosa* to H_2O_2 is governed by the global transactivator, OxyR [17, 18]. This response is generally assumed to be predominantly a defensive strategy because the three major OxyR-regulated genes encode the cytoplasmic antioxidant enzymes catalase B (KatB), **a**lkyl**h**ydro**p**eroxide reductase **CF** (AhpCF), and the periplasmic AhpB [17, 18]. In addition, an ankyrin-like protein AnkB (encoded by the *ankB* gene), part of a two-gene operon with *katB*, also contributes to H_2O_2 resistance by an unappreciated mechanism. An *oxyR* mutant possesses some very distinct phenotypes, the most unusual is an inability to form single colonies on aerobic but not anaerobic rich media (e.g., Luria-Bertani agar) [17, 19]. When *oxyR* mutant bacteria are streaked on aerobic Luria agar plates, *oxyR* mutant bacteria are incapable of growing as isolated colonies because the medium actually generates H_2O_2. In fact, we have shown that $<10^7$ *oxyR* mutant bacteria cannot grow in aerobic L-broth unless catalase is provided or cultures are grown in the absence of oxygen. This mutant is also impaired virulence properties in mouse and *Drosophila melanogaster* infection models [20], and is more susceptible to killing by human neutrophils [20]. Still, the most remarkable phenotype of a *P. aeruginosa oxyR* mutant is an exquisite sensitivity to H_2O_2, despite the fact that the organism possesses near wild-type catalase activity [17, 18]. In this study, we evaluated the potentially different roles of OxyR-controlled antioxidant enzymes in planktonic versus biofilm resistance to the important biocide, H_2O_2.

2. Materials and Methods

2.1. Bacterial growth conditions

Planktonic free-living *P. aeruginosa* were routinely grown aerobically in either trypticase soy broth (TSB) or Luria-Bertani broth (LB). Media were supplemented with 200 μg ml^{-1} carbenicillin when plasmid maintenance was required. Planktonic cultures were grown at 37°C with shaking at 300 rpm for 18 h. For culture of biofilm cells, aerobic, stationary phase bacteria were diluted 1:50 into 1% TSB and 0.2 ml was used to inoculate Stovall flow chambers (Stovall Life Sciences, Greensboro, NC). After a 1 h incubation at room temperature, media flow was initiated at a rate of 0.17 ml min^{-1} and biofilms were allowed to develop for 3 days. For enumeration of colony forming units (CFU) on agar surfaces, media were solidified with 1.5% Bacto agar.

2.2. H_2O_2 susceptibility measurements

All bacteria were grown aerobically in TSB for 18 h at 37°C. Cells were diluted to a final $O.D._{600\ nm}$ of 0.01 in 5 ml of TSB broth containing 0.8% low-melting-point agarose (SeaPlaque). Suspensions were distributed evenly on 15 × 100 mm TSB agar plates and allowed to solidify. Filter paper disks (7 mm) impregnated with 10 μl of 8.8 M H_2O_2 were placed on the top-agar surface and the plates were incubated at 37°C for 24 h. Mature, 3 day-old biofilms were treated with different concentrations of H_2O_2 for 30 min before staining with a viability stain composed of SYTO 9 (green fluorescence) and propidium iodine (red fluorescence) (*Bac*Light, InVitrogen). Biofilm images were obtained using an LSM 510 confocal microscope. The live/dead ratios of the bacteria embedded within biofilms were calculated using the 3-dimensional reconstruction component for LSM (V. 1.4.2) software.

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2.3. Catalase assays

Planktonic and biofilm bacteria were harvested by centrifugation at 4°C. The cell pellets were then resuspended in ice cold 50 mM potassium phosphate, pH 7.0 (KP_i) and disrupted by intermittent sonication on ice. Catalase activity in cell extracts was determined by following the decomposition of 19.5 mM H_2O_2 in KP_i at 240 nm using a Spectronics Genesys 5 spectrophotometer [11, 12, 19]. The Bradford protein assay was used to estimate protein concentration with bovine serum albumin (fraction V) serving as the standard [21]. The definition of one unit (U) of activity is defined as that which decomposes 1 μmol of H_2O_2 min^{-1} mg $protein^{-1}$.

2.4. H_2O_2 minimum inhibitory concentration (MIC) measurements

Aerobic stationary phase cultures were diluted 5000-fold into 5 ml of fresh TSB containing increasing concentrations of H_2O_2. The bacteria were then grown under aerobic conditions for an additional 18 h on a roller wheel at 70 rpm after which the MICs were recorded.

2.5. ß-galactosidase assays

For planktonic cultures, stationary phase aerobic bacteria grown in TSB were used while mature, 3 day-old biofilm bacteria were collected from flow cells by scraping the biofilms into ice cold Z-buffer [22]. Samples were then assayed for ß-galactosidase activity [22]. All assays were performed at least in triplicate, and the values recorded as the mean +/− standard error.

3. Results and Discussion

3.1. H_2O_2 susceptibility of P. aeruginosa oxyR mutant bacteria in planktonic culture: role of KatB, AhpB and AhpCF.

Planktonic *oxyR* mutant bacteria harboring plasmids expressing either *katB*, *ahpB* or *ahpCF* were first grown in TSB to stationary phase and H_2O_2 sensitivity profiles were measured. Fig. 1A demonstrates that provision of the catalase, KatB (lane 5), helped protect the *oxyR* mutant against H_2O_2 more effectively than the alkyl hydroperoxide reductase, AhpCF (lane 6). The periplasmic enzyme, AhpB, provided no protection compared to the aforementioned enzymes (lane 4). Moreover, MIC measurements clearly showed that KatB is more critical for protection of planktonic bacteria against this biocide (Fig. 1B, lanes 4 vs. 5,6) while *oxyR* mutant bacteria are >400-fold more sensitive to H_2O_2 in planktonic culture than their wild-type counterparts (Fig.1B, lanes 2 vs. 1).

We next examined the role of OxyR-regulated antioxidant enzymes KatB, AhpCF and AhpB in biofilm susceptibility to H_2O_2. Aerobically grown, stationary phase bacteria were diluted 50-fold in 1% TSB and used as an inoculant for cultivation of mature biofilms for 3 days in a flow-through cultivation system [19]. We have previously shown that *oxyR* mutant organisms, that are devoid of KatB, AhpB and AhpCF activity, are very sensitive to H_2O_2 in the low millimolar range during biofilm culture [19]. We performed additional biofilm experiments, comparing the H_2O_2 sensitivity profiles of *oxyR* mutant bacteria harboring stable plasmids containing *katB*, *ahpB* or *ahpCF*, respectively. In contrast to wild-type bacteria, the vast majority (~88%) of *oxyR* mutant bacteria were easily killed by a brief 30

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min treatment of mature biofilms with 70 mM H_2O_2 (Fig. 2), a level needed to cause significant killing of highly refractory *P. aeruginosa* in biofilm [19]. We have previously shown that such high concentrations of H_2O_2 can trigger mechanical dispersion of mature biofilms due to vigorous oxygen bubbling mediated by the very high catalase activity of *P. aeruginosa* [19, 23], thus explaining the somewhat contorted structure of H_2O_2-treated biofilms (lower panels, Fig. 2). The *oxyR* mutant expressing AhpB was still very sensitive to H_2O_2, and comparable in sensitivity with *oxyR* mutant bacteria (Fig. 2). In contrast, provision of AhpCF restored biofilm resistance to H_2O_2, in fact, at levels even greater than wild-type bacteria (4.14 ± 1.35 live/dead ratio of treated cells). Surprisingly, and in contrast to planktonic bacteria, the *oxyR* mutant expressing KatB was nearly as sensitive to H_2O_2 as the *oxyR* mutant.

3.2. Protection of wild-type P. aeruginosa biofilms from H_2O_2: role of KatB vs. AhpCF.

We next elected to determine whether the protective effect of AhpCF in *oxyR* mutant bacteria could also be observed in wild-type bacteria. To test this hypothesis, we constructed isogenic single *ahpCF* and *katB* mutants in wild-type strain PAO1 and monitored biofilm susceptibility to H_2O_2. Fig. 3B (in contrast to untreated bacteria in Fig. 3A) demonstrates that the *ahpCF* mutant in biofilms is more sensitive to H_2O_2 relative to an isogenic *katB* mutant. Moreover, the ratio of live versus dead bacteria was nearly 6 times greater in the *katB* mutant relative to *ahpCF* mutant bacteria after exposure to 70 mM H_2O_2 (Fig. 3C).

3.3. Differential expression of the ahpCF and katB genes in planktonic vs. biofilm bacteria treated with H_2O_2

To help explain why AhpCF provided better protection of biofilm bacteria against H_2O_2 while KatB provided better protection of planktonic organisms, we tested the hypothesis that the expression of *katB* and *ahpCF* differ during these two distinct growth modes by constructing *lacZ* reporter fusions to both genes. For planktonic culture, we used stationary phase organisms. Although unattached to surfaces like biofilm bacteria, such organisms represent a metabolically less active form than exponentially growing, mid-log phase cells and, as such, could arguably represent biofilm-like cells. This is due to their significantly reduced metabolic activity and antibiotic resistance properties [24]. We discovered that *katB* expression is upregulated at least 4.1-fold when organisms are exposed to 5 to 25 mM H_2O_2 in planktonic culture but only moderately by 5 mM H_2O_2 in biofilms (Fig. 4A vs. B). In contrast, *ahpCF* expression was inducible by a factor of ~1.6-fold in biofilms while there was no significant induction in planktonic culture (Fig. 4D vs. C).

3.4. Catalase-like activity of AhpCF

Finally, because provision of *ahpCF in trans* afforded protection of biofilm bacteria from H_2O_2 exposure, we formulated a hypothesis that AhpCF in *P. aeruginosa* possesses the capacity to degrade H_2O_2 in addition to organic peroxides. This hypothesis is not unprecedented, for AhpCF in the related organism *Xanthomonas campestris* (AhpC 86%, AhpF 76% identical to *P. aeruginosa* AhpCF) was not only inducible in a *katA* mutant, but possessed catalase activity that rescued the H_2O_2-sensitivity phenotype of the mutant [25]. Similarly, in *Escherichia coli*, AhpCF is important for scavenging low concentrations of endogenously generated H_2O_2 while catalases KatE and KatG provide protection at higher

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concentrations [26]. This is likely due to the recently discovered fact that AhpCF in the related organism *Salmonella typhimurium* is able to utilize H_2O_2 as a substrate with a K_M ~100-fold less than that of organic hydroperoxides [27]. To test the hypothesis that AhpCF could function catalatically, we transformed *lac*-based plasmids harboring constitutively expressed *ahpCF* into an *oxyR katA* mutant, that possesses no detectable catalase activity [17, 18], and measured catalase activity in planktonic culture. We discovered that the *oxyR katA* double mutant harboring *ahpCF* possessed catalase activity albeit at low levels (0.9 ± 0.1 U mg^{-1}). The low catalase activity is likely due to the fact that all AhpCF proteins examined thus far requires NADH as a cofactor [27, 28]. As mentioned above, the *X. campestris* AhpCF is inducible in a *katA* mutant. This is similar to our results demonstrating that expression of *ahpC* was inducible in biofilm bacteria but not in plantonic organisms (Fig. 4C and D). Therefore, we postulated whether catalase levels under both conditions might be inversely correlative with *ahpCF* expression. To test this, we measured catalase activity in both planktonic vs. biofilm culture. We found that all biofilm bacteria harbored lower catalase activity compared to their planktonic counterparts, even though it was not significantly different in *oxyR* mutant bacteria relative to wild-type bacteria (Fig. 5). This is consistent with the fact that mature biofilm bacteria are essentially starved for iron, as evidenced by very low catalase and increased Mn-containing superoxide dismutase activities [29]. Thus, lower expression of catalase in biofilms mediated by alternations in overall iron metabolism relative to planktonic bacteria may be one factor that allows for increased expression of AhpCF in response to H_2O_2.

4. Conclusions

In this study, we have discovered that OxyR-regulated gene products in the important opportunistic pathogen *P. aeruginosa* differ in levels of protection conferred when grown in sessile, organized communities known as biofilms versus planktonic, free-swimming culture. The alkyl hydroperoxidase, AhpCF, was found to be critical for optimal resistance to H_2O_2 in biofilms, while the 240 kDa tetrameric catalase, KatB, is more critical for protection of planktonic bacteria against this biocide. This was, in part, due to differences in the transcriptional response to H_2O_2 in biofilms; transcription of *ahpCF* is increased in biofilm culture while that of *katB* is more upregulated in planktonic organisms. Moreover, AhpCF is capable of detoxifying H_2O_2 more effectively in biofilms, when organisms have less catalase than their planktonic counterparts. Future studies are designed to better understand the molecular basis for differential regulation of OxyR-controlled genes in planktonic vs. biofilm bacteria.

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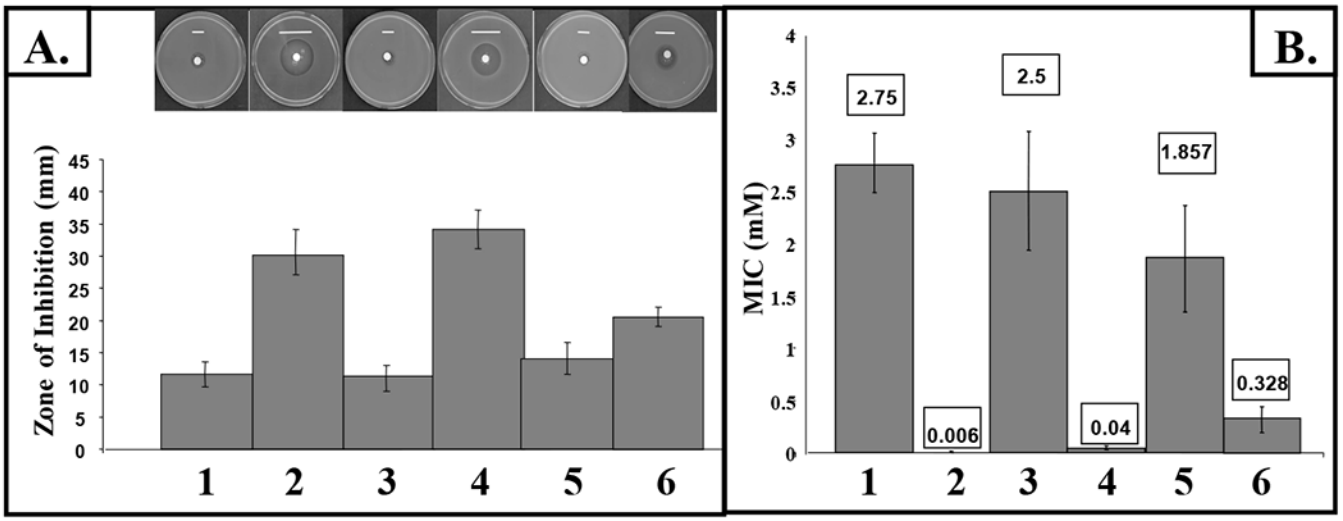

Fig. 1. Sensitivity of *P. aeruginosa* compared with mutants strains to H_2O_2.

(A) Equivalent titers of aerobic stationary phase bacteria grown in TSB broth were added to molten top-agarose and poured onto TSB agar plates. Filter paper disks (7 mm) impregnated with 8.8 M H_2O_2 were then placed on the solidified top agarose. Small white bars, the precise width of the killing zone, are placed just above each zone for ease of viewing. **(B)** Approximately 10^6 stationary phase bacteria were sub-cultured into fresh TSB containing increasing concentration of H_2O_2 after which the MICs were determined 24 hr post-exposure. Lane 1, wild-type; lane 2, *oxyR*; lane 3, *oxyR*-p*oxyR*; lane 4, *oxyR* + p*ahpB*; lane 5, *oxyR* + p*katB*; lane 6, *oxyR* + p*ahpCF*.

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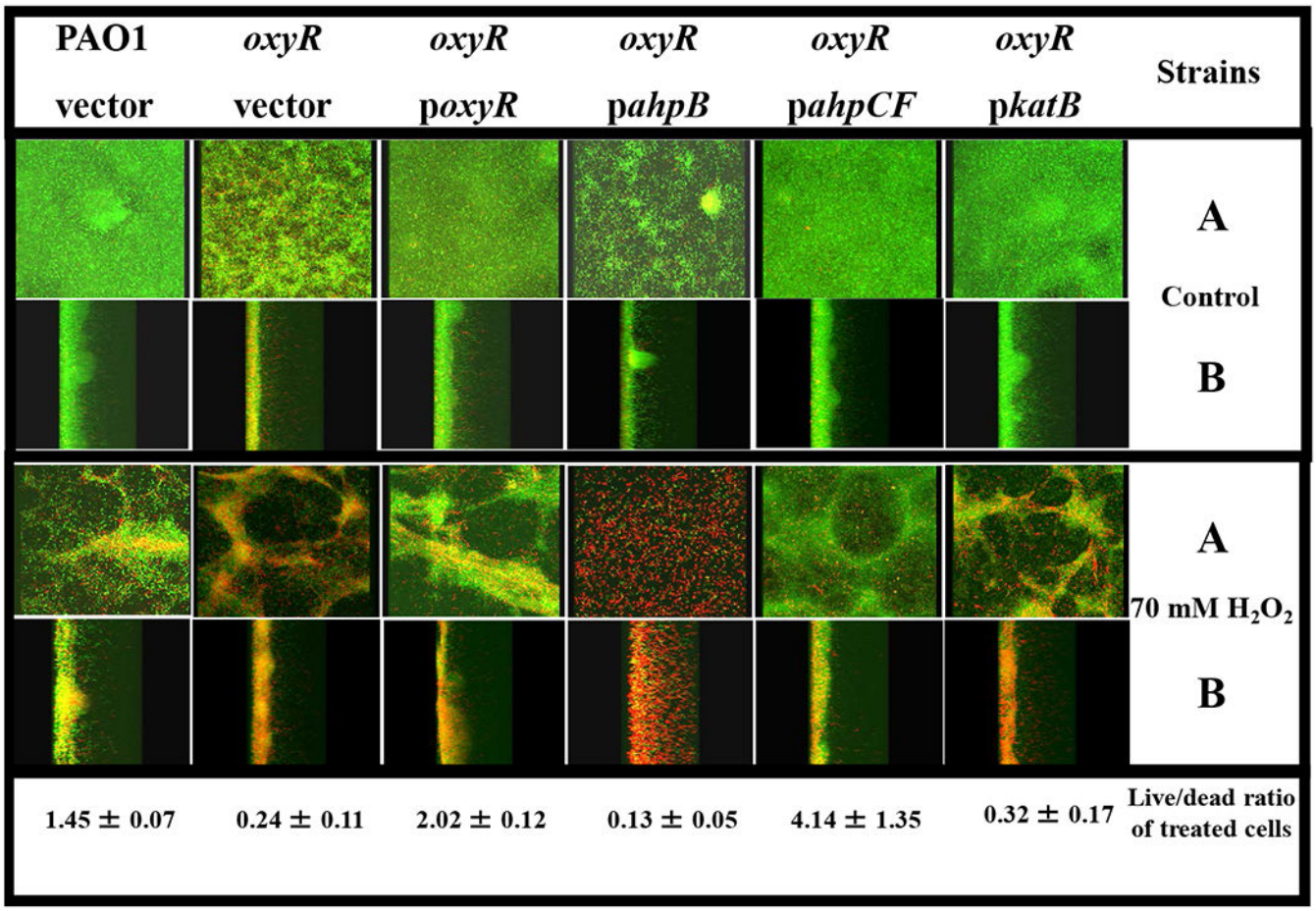

Fig. 2. Confocal laser scanning microscopic images of H_2O_2-treated biofilms.

Biofilms were grown in confocal "friendly" flow chambers as previously described by Lequette and Greenberg [30]. Briefly, bacteria were grown aerobically in L-broth at 37°C until the stationary growth phase, diluted 1:50 into 1% TSB and a 0.2 ml suspension used to inoculate flow cells (Stovall Life Sciences, Inc., Greensboro, NC). Flow was initiated at a rate of 0.17 ml min^{-1} after the bacteria were allowed to attach for 1 hr. After a 3 day incubation at room temperature (~23°C), biofilms were treated with 70 mM H_2O_2 for 30 min. The biofilms were then stained with a live/dead viability stain composed of SYTO 9 and propidium iodine (Molecular Probes, Inc., Eugene, OR). Biofilm images were obtained using an LSM 510 confocal microscope (Carl Zeiss, Inc., Germany). The excitation and emission wavelengths for green fluorescence were 488 nm and 500 nm, while those for red fluorescence were 490 nm and 635 nm, respectively. All biofilm experiments were repeated at least 3 times. The live/dead ratios of the biofilms were calculated using the 3D for LSM (V.1.4.2) software (Carl Zeiss, Inc., Germany). The panels labeled A are top/bottom images while those in B are saggital images. The top third panel depicts the strains used in these studies. The live to dead ratio of treated bacteria is given on the bottom panel for each strain.

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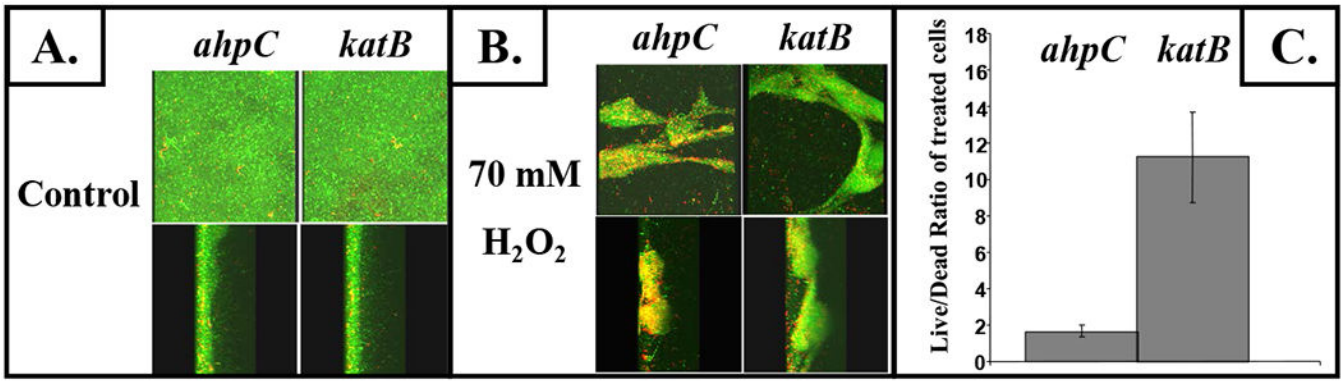

Fig. 3. Sensitivity of *P. aeruginosa ahpCF* versus *katB* mutants to H_2O_2 in biofilms.

The same conditions used in Fig. 2 were used to grown *ahpC* and *katB* mutant biofilms. Confocal laser scanning microscopic images of top and saggital views of mature 3-day old biofilms were obtained in **(A)** untreated bacteria and **(B)** after treatment with 70 mM of H_2O_2 for 30 min followed by viability staining. **(C)** The live/dead ratios of the treated biofilms were calculated using the 3D for LSM (V.1.4.2) software (Carl Zeiss, Inc., Germany).

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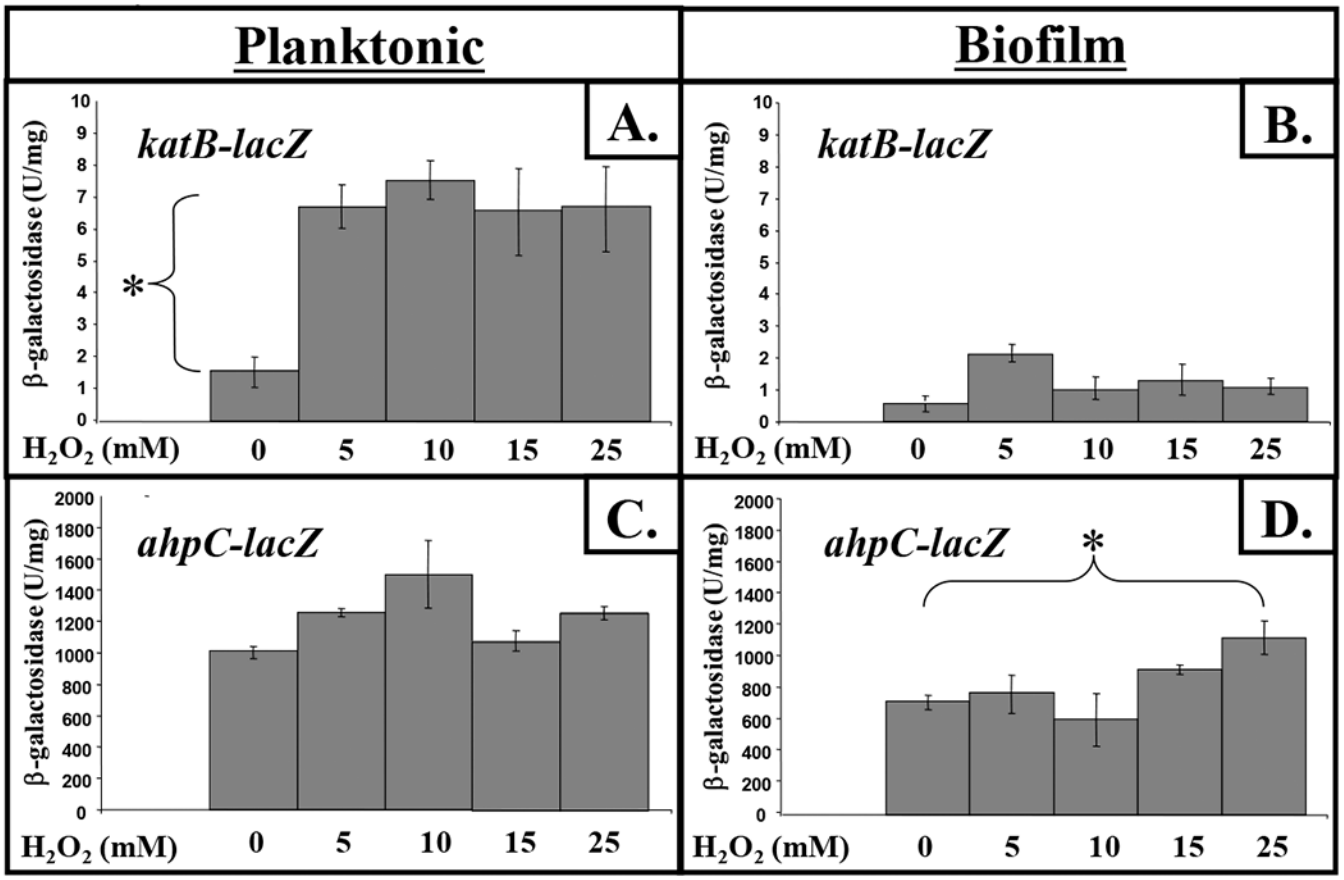

Fig. 4. Expression of *katB* and *ahpCF* in plantonic vs. biofilm culture in response to various concentrations of H_2O_2.

Planktonic, stationary phase bacteria or mature, 3-day old biofilm organisms were exposed to various concentrations of H_2O_2 for 30 min after which β–galactosidase activity was determined in cell extracts (n=3).

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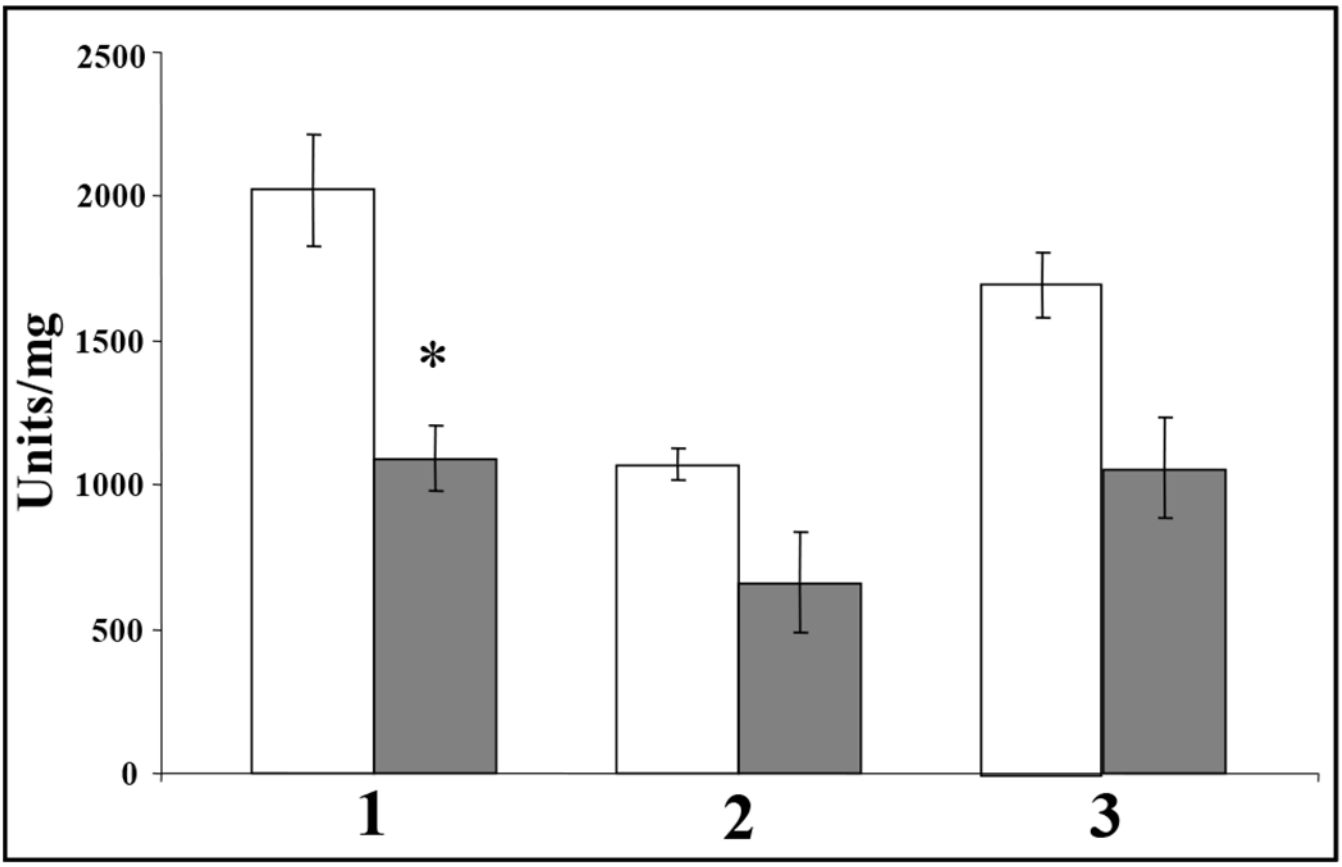

Fig. 5. Catalase activity in plantonic vs. biofilm culture.

All bacteria were harvested from either stationary phase planktonic (white bars) or 3-day old biofilm (grey bars) culture in either TSB or 1% TSB, respectively. Cell extracts were assayed for catalase activity as described in the materials and methods. Lane 1, wild-type; lane 2, *oxyR*; lane 3, *oxyR*-p*oxyR.*