Protective Role of Catalase in *Pseudomonas aeruginosa* Biofilm Resistance to Hydrogen Peroxide

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Received 21 June 1999/Accepted 9 August 1999

The role of the two known catalases in *Pseudomonas aeruginosa* in protecting planktonic and biofilm cells against hydrogen peroxide (H$_2$O$_2$) was investigated. Planktonic cultures and biofilms formed by the wild-type strain PA01 and the katA and katB catalase mutants were compared for their susceptibility to H$_2$O$_2$. Over the course of 1 h, wild-type cell viability decreased steadily in planktonic cells exposed to a single dose of 50 mM H$_2$O$_2$, whereas biofilm cell viability remained at approximately 90% when cells were exposed to a flowing stream of 50 mM H$_2$O$_2$. The katB mutant, lacking the H$_2$O$_2$-inducible catalase KatB, was similar to the wild-type strain with respect to H$_2$O$_2$ resistance. The katA mutant possessed undetectable catalase activity. Planktonic katA mutant cultures were hypersusceptible to a single dose of 50 mM H$_2$O$_2$, while biofilms displayed a 10-fold reduction in the number of culturable cells after a 1-h exposure to 50 mM H$_2$O$_2$. Catalase activity assays, activity stains in nondenaturing polyacrylamide gels, and lacZ reporter genes were used to characterize the oxidative stress responses of planktonic cultures and biofilms. Enzyme assays and catalase activity bands in nondenaturing polyacrylamide gels showed significant KatB biofilm induction occurred in biofilms after a 20-min exposure to H$_2$O$_2$, suggesting that biofilms were capable of a rapid adaptive response to the oxidant. Reporter gene data obtained with a katB::lacZ transcriptional reporter strain confirmed katB induction and that the increase in total cellular catalase activity was attributable to KatB. Biofilms upregulated the reporter in the constant presence of 50 mM H$_2$O$_2$, while planktonic cells were overwhelmed by a single 50 mM dose and were unable to make detectable levels of β-galactosidase. The results of this study demonstrated the following: the constitutively expressed KatA catalase is important for resistance of planktonic and biofilm *P. aeruginosa* to H$_2$O$_2$, particularly at high H$_2$O$_2$ concentrations; KatB is induced in both planktonic and biofilm cells in response to H$_2$O$_2$ insult, but plays a relatively small role in biofilm resistance; and KatB is important to either planktonic cells or biofilm cells for acquired antioxidant resistance when initial levels of H$_2$O$_2$ are sublethal.

Bacterial biofilms profoundly affect industrial systems by promoting material fouling and loss of pump-and-pipe system efficiency. Biofilms also impact human health by causing persistent infections stemming from bacterial accumulations on tooth surfaces and medical implants. Biofilm formation, especially by gram-negative bacteria, is thought to be an effective survival strategy for bacteria against environmental challenges such as desiccation (9) or nutrient limitation (28). The importance of biofilm formation to the survival of microbial populations is perhaps best demonstrated by the significant biofilm resistance to antimicrobial agents (1, 6, 21). Biofilm resistance to antimicrobials is much greater than planktonic organisms, and it is this recalcitrance to a broad spectrum of such compounds that makes biofilm control difficult. An understanding of the primary mechanisms responsible for the reduced susceptibility of biofilms to antimicrobial agents will aid in the successful eradication of problem biofilms in the future.

Biofilm structural characteristics have been assessed for their contribution to biofilm resistance to antimicrobial agents. The retarded penetration of antibiotics into microbial aggregates due to exopolysaccharide encapsulation has been implicated in biofilm resistance (27, 36). Also, reactions between strongly oxidizing biocides such as hypochlorous acid and biofilm constituents, and the neutralization that results, have been shown to provide some protection against killing (8). Undoubtedly, reaction and diffusional barriers to antimicrobial penetration established by the complex exopolysaccharide matrices surrounding biofilm organisms provide some degree of protection. However, it has been suggested that reaction-diffusion limitation alone cannot totally account for biofilm resistance to many antimicrobial agents (6, 35).

It has recently been shown that physiological heterogeneity exists throughout the depth of a biofilm (20), with physiological variation perhaps occurring as a function of oxygen availability (38). Physiological heterogeneity is likely to contribute to the reduced susceptibility of biofilms to antimicrobial agents, especially when growth-dependent antibiotics (i.e., β-lactams) are administered. Also, portions of a biofilm experiencing nutrient limitation may be more resistant to antimicrobial agents due to differential stationary-phase gene regulation, which is well known to render microorganisms more resistant to many adverse environmental conditions (23, 26).

In addition to reaction-diffusion limitation and low physiological activity, physiological adaptation to antimicrobial agents may also be important. Specific physiological responses to antimicrobial agents, especially oxidative stress responses against bactericidal reactive oxygen intermediates (ROIs),
have been well characterized in bacteria (see references 11 and 12 for reviews). ROIs include the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), and the powerfully oxidizing hydroxyl radical (OH$^-$). ROIs primarily result from the partial, univalent reduction of oxygen by aberrant electron flow during electron transport in aerobic metabolism (15). Bacteria may also encounter extracellular fluxes of ROIs from phagocytic cells during infection of animals or humans or when ROIs are employed as disinfectants. Although adaptive responses against oxidative stress caused by these ROIs have been extensively studied with planktonic cells (11, 12), comparatively little is known about biofilm responses to bioicide attack. Biofilms have been shown to become increasingly resistant to repeated doses of antibiotics (14) or non-specific oxidizing biocides such as monochloramine (30), but the basis for this apparent acquired resistance is currently unknown.

In this study, a previously described model system for examining biofilm resistance mechanisms (17) was employed to assess the significance of catalase expression in the protection of Pseudomonas aeruginosa biofilms against the oxidizing biocide H$_2$O$_2$. P. aeruginosa expresses several catalases that catalyze the disproportionation of H$_2$O$_2$, leaving oxygen and water. The primary housekeeping catalase, KatA, is expressed constitutively throughout the growth cycle, with increased expression at the onset of the stationary phase. katB expression is repressed during aerobic growth but is highly inducible upon exposure to H$_2$O$_2$ (7). Another catalase, KatC, has been observed in P. aeruginosa at very low levels, but the importance and regulation of this enzyme is currently unknown (unpublished data). In this study, the importance of catalase for the protection of P. aeruginosa biofilms was examined by comparing the wild-type strain with KatA$^-$ and KatB$^-$ mutants for their susceptibility to H$_2$O$_2$. Spectrophotometric enzyme assays, activity stains in nondenaturing polyacrylamide gels, and reporter gene experiments were employed to characterize the oxidative stress response in biofilms and planktonic cells.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Planktonic cells were cultured in Pseudomonas Basal Medium (PBM) medium (2) that contained 1 g of glucose per liter as a carbon source. Iron in the form of FeCl$_3$ was added to a final concentration of 0.05 mg/liter (2) that contained 1 g of glucose per liter as a carbon source. Iron in the form of FeCl$_3$ was added to a final concentration of 0.05 mg/liter (2).

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristic</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5a</td>
<td>lac Z M15 recA1 hsdRI supE44</td>
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</tr>
<tr>
<td>SM10</td>
<td>thi thr leu tona lacY supE recA Muc</td>
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</tr>
<tr>
<td>HB101</td>
<td>recA thi pro leu hsdRM3</td>
<td>4</td>
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<tr>
<td>P. aeruginosa PAO1</td>
<td>Wild type</td>
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<tr>
<td>PAO1 katA</td>
<td>PAO1 KatA:Gm</td>
<td>24</td>
</tr>
<tr>
<td>PAO1 katB</td>
<td>PAO1 KatB:Gm</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 katB:lacZ</td>
<td>PAO1 katB reporter</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 lacZ</td>
<td>PAO1 lacZ mutant</td>
<td>This study</td>
</tr>
<tr>
<td>plBluescript (KS+)</td>
<td>Extended polylinker, PUC derivation</td>
<td>Strategene</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km', oriT helper</td>
<td>13</td>
</tr>
<tr>
<td>pZ1918</td>
<td>Ap', pUC19/18 with 3.2-kb lacZ</td>
<td>31</td>
</tr>
<tr>
<td>pMini-CTX</td>
<td>Tc', amp site-specific integration vector</td>
<td>18</td>
</tr>
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<td>pFLP2</td>
<td>Ap', Suc', broad-host-range FLP expression vector</td>
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<td>pJFM12</td>
<td>pBluescript with ~3.6-kb EcoRI-EcoRV that contains katA</td>
<td>24</td>
</tr>
<tr>
<td>pJFM13</td>
<td>pEX100T with blunted ~3.6-kb EcoRI-EcoRV from pJFM12</td>
<td>24</td>
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<tr>
<td>pJFM14</td>
<td>pJFM13 with 850-bp Gm' cassette in Smal site of katB</td>
<td>24</td>
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<tr>
<td>pSM2B</td>
<td>pBluescript with 5.4-kb EcoRI fragment of P. aeruginosa containing katB</td>
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<td>pEX100T</td>
<td>Gene replacement vector</td>
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<td>pJE26</td>
<td>Ap', XhoI-HindIII katB fragment in pQF50 forming a katB-lacZ transcriptional fusion</td>
<td>This study</td>
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<td>pGJ020</td>
<td>Tc', pMini-CTX containing lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pGJ03</td>
<td>Tc', pGJE20 containing KatB promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pUCGm</td>
<td>Ap', Gm', pUC19 with 850-bp Gm' cassette</td>
<td>32</td>
</tr>
</tbody>
</table>

* Ap', ampicillin resistance; Km', kanamycin resistance; Suc', sucrose resistance.

**H$_2$O$_2$ treatment.** (i) Planktonic cultures. The wild-type strain P. aeruginosa PAO1 and the katA and katB mutant strains were cultured as described above for broth culture. Stationary-phase cells were harvested and diluted to a cell density of approximately 3.0 $\times$ 10$^7$ CFU - ml$^{-1}$ in PBM medium that did not contain catalase. To determine initial cell viability prior to H$_2$O$_2$ treatment, an aliquot of cells from each flask was serially diluted in phosphate-buffered saline (PBS) (pH 7.2) that contained 0.2% sodium thiosulfate (w/vol) (used in the H$_2$O$_2$ treatments to neutralize H$_2$O$_2$) and then plated on R2A agar (Difco). For H$_2$O$_2$ treatments, H$_2$O$_2$ (Sigma) was added to a final concentration of 50 mM and each flask was immediately returned to shaking at 25°C. At 20-min intervals for a period of 1 h, culture aliquots were serially diluted in the H$_2$O$_2$-neutralizing PBS buffer. Appropriate dilutions were plated on duplicate R2A agar plates and were incubated overnight at 37°C. Following incubation, CFUs were enumerated and H$_2$O$_2$ treatment effects were expressed as the percentage of surviving cells relative to the initial cell viability. In all survival estimates (including biofilm experiments described below), we assumed that CFUs represented viable cells that either were undamaged by the H$_2$O$_2$ treatment or suffered damage but were capable of repair, replication, and colony formation.

(ii) Biofilms. P. aeruginosa PAO1 and katA and katB mutant biofilms were generated with a drip-flow reactor under the conditions described above. Following the 72-h growth period, a biofilm sample was harvested as an untreated control (no H$_2$O$_2$) and then for the remaining biofilms the culture medium inflow was switched to PBM medium that included 50 mM H$_2$O$_2$. The PBM-H$_2$O$_2$ medium was allowed to flow over the biofilms at a rate of 50 ml - h$^{-1}$ for a period of 1 h. For catalase and reporter gene induction experiments, biofilm slides were harvested at 20-min intervals by aseptically scraping each biofilm into 50 ml of PBS (pH 7.2) containing 0.2% sodium thiosulfate to neutralize H$_2$O$_2$. Biofilm biomass was then homogenized by using a P10/35 Brinkman homogenizer (Brinkman Instruments, Westbury, N.Y.) for 15 s at setting 4. Biofilm cell...
viability was determined by diluting homogenized biofilm suspensions and enumerated CFUs on R2A agar as described for the planktonic culture H2O2 treatment experiments. In addition to the viable cell count, the total number of cells released from the biofilm was determined using nonspecific staining and epifluorescence microscopy. Cells from appropriate dilutions were collected on black polycarbonate filters (Poretics, Livermore, Calif.) and stained with 4’6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, Oreg.). Stained cells were visualized with a BH-2 microscope (Olympus, Lake Success, N.Y.) with UV epifluorescent illumination. In order to verify the accuracy and precision of the percent cell viability estimates for H2O2-treated biofilms, untreated control biofilms were also processed and measured for viable counts and total direct counts by the same procedures. All experiments were repeated at least three times.

**Catalase and reporter gene induction assays and native polycarylamide gel electrophoresis (PAGE) analysis.** To induce catalase and the katB::lacZ reporter gene, stationary-phase planktonic cultures were subjected to a 2 mM pulse of H2O2 every 10 min for a period of 1 h, while biofilms were treated with a constant stream of 50 mM H2O2 as described above. Planktonic culture samples and whole biofilms were collected at 20-min intervals during the 1-h oxidative stress treatment period. The residual H2O2 in the biofilm homogenates and planktonic culture samples was neutralized with 0.2% sodium thiosulfate, and chloramphenicol (300 mg liter\(^{-1}\)) was added to arrest protein synthesis. Cell-free extracts for planktonic and biofilm cells were prepared using methods previously described (17). Briefly, treated and control planktonic cultures and biofilm homogenates were centrifuged at 8,000 × g for 5 min at 4°C, washed twice with 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.2), and then resuspended in 0.5 ml of potassium phosphate buffer and transferred to an Eppendorf tube forsonication. Cell extracts were generated by disrupting the cells with two 30-s pulses with a Fisher Scientific model 550 sonicator at power setting 1.8. The sonicate was then centrifuged at 13,000 × g for 10 min at 4°C to remove unbroken cells and cell debris, and the supernatant was then transferred to a fresh tube. Total protein concentration was determined by the method of Bradford (5) with bovine serum albumin fraction V as a standard.

Specific catalase activities for planktonic and biofilm cell-free extracts were determined as previously described (3, 7, 17). A specific catalase activity unit is defined as 1.0 mol of H2O2 degraded · min\(^{-1}·\) mg of total protein \(^{-1}\). Catalase expression was also analyzed by nonadenaturing PAGE according to the method described by Hassett et al. (17). Briefly, cell-free extracts (15 μg of protein/well) were electrophoresed through vertical 5% continuous polyacrylamide gels (made in 0.375 M Tris, pH 8.8) for approximately 10 h at a 10-mA constant current. Gels were stained for catalase activity as previously described (7, 37).

For reporter gene assays, biofilms, and planktonic cells were harvested as described above, but instead of preparing cell-free extracts, biofilm homogenates and planktonic cultures were washed twice in PBS (pH 7.2) and resuspended in Z-buffer (pH 7.2) (25). Resuspended cells were then assayed for β-galactosidase activity by the method described by Miller (25).

**DNA manipulation, cloning, and reporter strain construction.** Routine protocols for plasmid and chromosomal DNA purifications were obtained from Z-buffer (pH 7.2) (25). Resuspended cells were then assayed for catalase activity as previously described (7, 37).

**RESULTS**

Catalase activity in katA and katB mutants. Constitutive and induced catalase activity in *P. aeruginosa* PAO1 and isogenic catalase mutants was determined for stationary-phase cells grown under specific conditions. Stationary-phase specific catalase activity levels in PAO1 were typically about 400 U (Table 2). After repeated doses of low levels of H2O2, total catalase levels increased by roughly 50%. No catalase activity was observed in untreated stationary-phase cultures of the *katB* mutant, nor was activity detectable in equivalent *katA* mutant cultures challenged with repeated doses of H2O2. In the latter case, the lack of the constitutively expressed KatA catalase resulted in almost complete killing within minutes (see below), and thus the cells were not able to induce *katB*. Catalase levels in uninduced *katB* mutant cells were similar to levels in cells of strain PAO1 but did not increase when cultures were treated with H2O2. The results of these studies established the range of catalase activity to be expected with each strain grown in a minimal defined medium and verified that the phenotype matched the mutations introduced into *P. aeruginosa* PAO1.

**H2O2 sensitivity of planktonic cells.** In order to assess the importance of catalase in the protection of planktonic cells from H2O2, each strain was subjected to a single dose of 50 mM H2O2. Reductions in cell viability were determined at 20-min intervals for a period of 1 h. The strains were variably sensitive to the H2O2 treatment (Fig. 1). With a full complement of catalase activity, PAO1 displayed a 3.5 log\(_{10}\) reduction in viability during the 1-h sampling period. The *katB* mutant was slightly more sensitive to H2O2, with a mean log\(_{10}\) reduction of 5.0 after 1 h, though this result was not statistically

**Statistical analysis.** When comparing sample means, a Student’s t test was performed and statistical significance was inferred when *P* ≤ 0.05.

**BamHI site in the multicloning site of mini-CTX to create pGJE02. A 580-bp XhoI-BamHI fragment containing the katB promoter was then subcloned from pMBB (7) into the corresponding sites of the polylinker in pGJE02 to create pGJE03. This places the katB promoter upstream of a katB::lacZ transcriptional fusion. The pGJE03 construct carrying the katB::lacZ fusion was introduced into PAO1 by triparental conjugal mating with *E. coli* DH5α as the donor and *E. coli* HB101(pKK2203) as the helper strain. Tc resistant colonies on PIA containing TC (100 mg liter\(^{-1}\)) were selected and *E. coli* SM10 (pFLP2) was used to introduce the excision plasmid into the Tc transconjugants. Several colonies showing carbenicillin resistance (Cb\(^{R}\)) and TC susceptibility (Tc\(^{S}\)) were selected to cure the pFLP2 plasmid from putative reporter strains after FRT excision, cultures were grown overnight in the absence of antibiotics at 42°C and then plated on PIA containing 6% sucrose (pFLP2 carries the *Bacillus subtilis* sacB gene). Plasmid preparations were used to verify absence of the plasmid. A control strain, PAO1(lacI), which contained a promoterless lacZ gene inserted at the atb site, was also constructed by the same technique. PCR primers (designed from a template obtained from GenBank accession no. D13407) were designed to verify integration of pGJE02 and excision of the unwanted vector sequences. To verify integrate junctions, PCR products were sequenced with an ABI Prism BigDye kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) and an ABI model 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

**TABLE 2. Planktonic-specific catalase activity of strain PAO1 and katA\(^{-}\) and katB\(^{-}\) mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catalase activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
</tr>
<tr>
<td>PAO1</td>
<td>426 ± 6</td>
</tr>
<tr>
<td>PAO1(katA)</td>
<td>ND</td>
</tr>
<tr>
<td>PAO1(katB)</td>
<td>430 ± 10</td>
</tr>
</tbody>
</table>

\(^*\) Results are the means ± the standard errors of three replicate cultures for each strain. Results are typical for one of three independent experiments.

\(^\dagger\) Stationary-phase cultures were treated with 2 mM doses of H2O2 every 10 min for 1 h while shaking at 25°C.

\(^\ddagger\) ND, not detectable.
Although catalase expression was influenced by treatment with H₂O₂, the specific catalase activities of the PAO1 and the katA and katB mutant biofilms were determined before and after exposure to H₂O₂ (Fig. 3). Biofilms were collected at 20-min intervals during exposure to a constant flow of 50 mM H₂O₂. Initial specific catalase activity in PAO1 biofilms did not differ from that in stationary-phase planktonic cultures (Table 1). Like planktonic cells, biofilm catalase expression was influenced by treatment with H₂O₂. As soon as 20 min after exposure to H₂O₂, specific catalase activity increased in PAO1 biofilms and continued to increase throughout the exposure period, reaching approximately 700 U in 1 h. The approximate 50% increase over the initial activity was nearly the same as that observed for planktonic cells (Table 2). KatB⁻ biofilms yielded similar initial specific catalase activities but displayed no increase during treatment, suggest-
50 mM H$_2$O$_2$ for 1 h. Cell-free extracts were prepared and assayed for catalase activity as described in Materials and Methods. Data points are the means of three independent experiments (one biofilm per experiment for each strain) and each error bar represents one standard error of the mean. Duplicate assays were performed on each cell-free extract preparation.

FIG. 3. Total catalase activity of PAO1(katB::lacZ) biofilms exposed to 50 mM H$_2$O$_2$ for 1 h. Cell-free extracts were prepared and assayed for catalase activity as described in Materials and Methods. Data points are the means of three independent experiments (one biofilm per experiment for each strain) and each error bar represents one standard error of the mean. Duplicate assays were performed on each cell-free extract preparation.

The expression patterns for katA and katB are similar for both cell types (Fig. 5), although the conditions required for induction differed. Similar to the results of experiments depicted in Table 2 and Fig. 4, PAO1(katB::lacZ) biofilms exhibited a twofold induction of reporter gene activity over the 1-h H$_2$O$_2$ exposure period (Fig. 5A). Reporter enzyme levels did not increase in PAO1(katB::lacZ) biofilms that were not exposed to H$_2$O$_2$. When planktonic cells were exposed to 2 mM doses of H$_2$O$_2$ every 10 min for 1 h, a response similar to that of biofilm cells was observed (Fig. 5B). However, when stationary-phase planktonic cells of the reporter strain were exposed to a single 50 mM dose of H$_2$O$_2$, no katB::lacZ induction was observed (Fig. 5B); this was likely due to rapid killing of the planktonic cells (Fig. 1). Initial levels of reporter enzyme in biofilm and planktonic cells were nearly equal, averaging roughly 30 Miller units, and suggested that katB expression levels were very similar for both cell types. The promoterless lacZ control construct, PAO1(lacZ), consistently yielded approximately 30 Miller units and was unaffected by single 50 mM or multiple 2 mM H$_2$O$_2$ treatments (results not shown).

DISCUSSION

Due to its ubiquity in nature and tendency to form biofilms, P. aeruginosa has been used as a model organism for studying biofilm behavior and for the development of biofilm control strategies (10). Though recent progress has been significant, P. aeruginosa biofilm cell physiology is still only poorly understood. Gradients in metabolic activity have been shown to exist in P. aeruginosa biofilms, and some information regarding adaptive gene regulation has also been recently published (20, 38). In continuing attempts to expand our understanding of biofilm cell physiology, we are examining oxidative stress responses in P. aeruginosa biofilms, using H$_2$O$_2$ as a model antimicrobial agent for studying biofilm resistance mechanisms (17). Significant genetic and biochemical information regarding the oxidative stress response of this organism to H$_2$O$_2$ is already available (16, 17) and should facilitate efficient progress in determining the basis for the significant resistance of P. aeruginosa biofilms to oxidizing biocides.

FIG. 4. PAO1 biofilm catalase isozyme expression patterns during treatment with a continuous dose of 50 mM H$_2$O$_2$. Samples were harvested at 20-min intervals for a period of 1 h. Cell-free extracts were loaded on a 5% nondenaturing polyacrylamide gel, electrophoresed to allow separation of the KatA and KatB enzymes, and then stained for catalase activity. Each lane was loaded with 15 μg of total protein. Results are from a single experiment but are representative of three experiments conducted.
regulatory responses to oxidative stress differ between planktonic cells and biofilm cells. In planktonic culture, *P. aeruginosa* primarily expresses two catalases, KatA and KatB, in defense against H$_2$O$_2$ (7, 16). KatA is expressed throughout the growth cycle, with a marked increase of expression at the onset of stationary phase (7). KatB is not expressed during the aerobic growth cycle but is inducible upon exposure to H$_2$O$_2$, making KatB a marker enzyme for H$_2$O$_2$-mediated oxidative stress in *P. aeruginosa*. Expression patterns of both KatA and KatB were similar in planktonic cells (Table 2) and biofilms (Fig. 3 and 4). In the absence of H$_2$O$_2$, biofilms formed by PAO1 contained only KatA activity (Fig. 4), with levels being equivalent to those of planktonic cultures (Table 2; Fig. 3). Similar to that of planktonic cells treated with 2 mM pulses of H$_2$O$_2$, the specific catalase activity of biofilms treated with a constant exposure to 50 mM H$_2$O$_2$ increased; the increase observed in biofilm cells was of the same magnitude as that measured in planktonic cultures (Table 2; Fig. 3). The increase in total catalase activity in biofilm cells in response to oxidative stress (Fig. 3) appeared to be exclusively due to induction of katB. This was shown with both native gel analysis and reporter gene data (Fig. 4 and 5). In summary, biofilm cells appear to respond to oxidative stress in a manner that is similar, if not identical, to that observed with planktonic cells. We note, however, that this conclusion is based upon the assumption that KatA expression was uniform throughout the biofilm, and we also draw attention to the observation that induction of katB in biofilms apparently required substantially more H$_2$O$_2$.

Another specific interest in this study was to determine whether biofilms possess special mechanisms that aid in guarding the cell against killing by oxidative biocides such as H$_2$O$_2$. An obvious physiologic trait of importance to inactivating H$_2$O$_2$ would be the cellular level of catalase activity, and therefore we focused efforts on determining the role of this enzyme in protecting *P. aeruginosa* biofilms from H$_2$O$_2$. The extraordinary recalcitrance of *P. aeruginosa* biofilms to H$_2$O$_2$ treatment reported here is in agreement with previous studies with other oxidizing biocides (21, 30). Even after 1 h of exposure to a continuous flow of 50 mM H$_2$O$_2$, PAO1 biofilm integrity remained largely intact and nearly 80% of the cells survived (Fig. 2). To assess the relative contribution of the catalase isozymes to biofilm resistance to H$_2$O$_2$, we created defined *katA* and *katB* mutations in the wild-type strain PAO1. In planktonic cultures, the KatA$^\Delta$ mutant was extremely sensitive to H$_2$O$_2$, with cell viability decreasing by more than six orders of magnitude within only 20 min. The KatB$^\Delta$ mutant appeared somewhat more sensitive than the wild type, but cell survival was not statistically different. In biofilms, the effect of KatB also appeared to be marginal, as the biofilm structural integrity and rates of cell survival (Fig. 2) of the katB mutant were not different from those of PAO1.

In contrast to the role of KatB, lack of the constitutively expressed KatA isozyme resulted in hypersusceptibility to H$_2$O$_2$ (Fig. 1) and a complete loss of catalase-mediated H$_2$O$_2$ resistance activity (Table 2). This effectively resulted in these cells exhibiting a catalase-negative phenotype. KatB induction did not occur in the KatA$^\Delta$ strain after treatment with H$_2$O$_2$ in either planktonic cultures (Table 2) or biofilms (data not shown), an observation which is consistent with a recent report by Ma et al. (24). It is likely that this was due to the KatA$^\Delta$ cells being rapidly overwhelmed and killed before the cells had an opportunity to induce measurable katB. KatA$^\Delta$ biofilms also displayed a consistent pattern of sloughing when exposed to H$_2$O$_2$ (Fig. 2). Approximately 30 min after initial exposure to H$_2$O$_2$, much of the KatA$^\Delta$ biofilm sloughed from the substratum (results not shown), and after 1 h, roughly 90% of the KatA$^\Delta$ biofilm was removed from the substratum (Fig. 2). We note that this observation was highly reproducible, as it was measured in three independent experiments. In summary, the results of these experiments imply that even in the absence of catalase activity, *P. aeruginosa* biofilms remain relatively resistant to H$_2$O$_2$. However, biofilm structural integrity is highly dependent on the ability to neutralize peroxide with catalase.

In the present study, we have assessed the relative importance of the different catalase enzymes for protection against H$_2$O$_2$. Based on the similarity of KatA and KatB catalase expression and activity levels in planktonic and biofilm cells, we conclude that the remarkable resistance of PAO1 biofilms to H$_2$O$_2$ cannot be attributed to abnormally high initial or induced levels of catalase activity. Although catalase expression is critical to survival in this setting, other mechanisms of bio-

FIG. 5. PAO1 katB::lacZ reporter activity of biofilm (A) and planktonic cultures (B). Biofilms were treated with a continuous dose of 0 mM (□) or 50 mM (■) H$_2$O$_2$ for a period of 1 h. Stationary-phase planktonic cultures were exposed to a single 0 mM (□) or 50 mM (■) H$_2$O$_2$ dose or to 2 mM H$_2$O$_2$ pulses every 10 min for 1 h (▲). Data points are the means of three observations (one biofilm or planktonic culture per observation for each strain) and each error bar (where visible) represents one standard error of the mean.
film resistance appear to be involved. Indeed, it is important to note that under the conditions of the experiments used in this study, even the katA mutant still survived at relatively high levels. This is an important observation, since conclusions based solely on the planktonic viability data would predict that catalase was essential for \emph{P. aeruginosa} resistance to H$_2$O$_2$ and overestimate its actual importance in natural biofilm populations. Further investigation of these mechanisms, as well as of potential novel aspects of biofilm physiology, will eventually reveal assailable approaches to eradicating problematic biofilms in the future.

**ACKNOWLEDGMENTS**

This material is based on work supported by the National Science Foundation Center for Biofilm Engineering Cooperative Agreement EEC-9907039 and by National Institutes of Health grants AI-40541 (D.J.H.) and GM56685 (H.P.S.).

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