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

JAMES G. ELKINS,[1,2] DANIEL J. HASSETT,[3] PHILIP S. STEWART,[2,4]
HERBERT P. SCHWEIZER,[5] AND TIMOTHY R. MCDERMOTT[1,2]*

*Department of Land Resources and Environmental Sciences,[1] Center for Biofilm Engineering,[2] and
Department of Chemical Engineering,[4] Montana State University, Bozeman, Montana 59717;
Department of Molecular Genetics, Biochemistry, and Microbiology, University of
Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524[3]; and Department
of Microbiology, Colorado State University, Fort Collins, Colorado 80523[5]*

The role of the two known catalases in *Pseudomonas aeruginosa* in protecting planktonic and biofilm cells against hydrogen peroxide (H_2O_2) was investigated. Planktonic cultures and biofilms formed by the wild-type strain PAO1 and the *katA* and *katB* catalase mutants were compared for their susceptibility to H_2O_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_2O_2, whereas biofilm cell viability remained at approximately 90% when cells were exposed to a flowing stream of 50 mM H_2O_2. The *katB* mutant, lacking the H_2O_2-inducible catalase KatB, was similar to the wild-type strain with respect to H_2O_2 resistance. The *katA* mutant possessed undetectable catalase activity. Planktonic *katA* mutant cultures were hypersusceptible to a single dose of 50 mM H_2O_2, while biofilms displayed a 10-fold reduction in the number of culturable cells after a 1-h exposure to 50 mM H_2O_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 catalase induction occurred in biofilms after a 20-min exposure to H_2O_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_2O_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_2O_2, particularly at high H_2O_2 concentrations; KatB is induced in both planktonic and biofilm cells in response to H_2O_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_2O_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 impli-

cated 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_2O_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 biocide attack. Biofilms have been shown to become increasingly resistant to repeated doses of antibiotics (14) or nonspecific 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_2O_2. *P. aeruginosa* expresses several catalases that catalyze the disproportionation of H_2O_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 (7, 16). KatB expression is repressed during aerobic growth but is highly inducible upon exposure to H_2O_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_2O_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 Mineral (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 10 μM, which was found in preliminary studies to yield maximum catalase activity in PBM medium (results not shown). Strains were maintained on PBM medium solidified with 1.5% Noble agar (Difco) containing 300 mg of gentamicin per liter (Sigma) when appropriate. All planktonic cultures were incubated at 25°C in a rotary shaker water bath at 300 rpm. Culture volumes did not exceed 10% of the flask volume to ensure maximum aeration.

Biofilms were cultured using a drip-flow reactor system previously described by Huang et al. (20) and included 316L stainless steel slides (1.3 by 7.6 cm) as the substratum. Briefly, 10 ml of PBM medium was added to each chamber (four chambers per reactor), followed by inoculation with 1 ml of stationary-phase culture of the test strain grown in the same medium. The reactor was then incubated horizontally at room temperature for 24 h to allow bacterial attachment to the substratum. Following the attachment period, the reactor was inclined 10° and a constant drip of half-strength PBM (except 200 mg of glucose per liter) was allowed to flow over the slides at a rate of 50 ml h⁻¹ for 72 h. Biofilm thickness was determined by cryosectioning and microscopic analysis as previously described (20).

For construction and isolation of catalase *lacZ* reporter strains, *Pseudomonas* isolation agar (PIA) (Difco) was employed as the sole medium for *P. aeruginosa* strain selection and maintenance and was supplemented with carbenicillin (300 mg · liter⁻¹), tetracycline (TC) (100 mg · liter⁻¹), or sucrose (5%) as required. Plates were incubated at 37°C for 24 to 48 h. *Escherichia coli* strains were cultured in Luria-Bertani (LB) (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) broth or on LB agar plates solidified with 1.5% Bacto agar (Difco). For maintenance and selection of plasmids, liquid and solid LB media were supplemented with TC (15 mg · liter⁻¹), ampicillin (100 mg · liter⁻¹) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 mg · liter⁻¹). Liquid cultures were incubated overnight at 37°C in a rotary shaker water bath set at 300 rpm; agar plate cultures were also incubated overnight at 37°C.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic[a]	Source or reference
E. coli		
DH5α	F⁻ *lacZ*ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA argF*)	22
SM10	*thi thr leu tonA lacY supE recA* Muc⁺	34
HB101	*recA thi pro leu hsdRM*	4
P. aeruginosa		
PAO1	Wild type	19
PAO1 *katA*	PAO1 *katA*::Gm	24
PAO1 *katB*	PAO1 *katB*::Gm	This study
PAO1 *katB*::*lacZ*	PAO1 *katB* reporter	This study
PAO1 *lacZ*	PAO1 *lacZ* control	This study
Plasmids		
pBluescript (KS⁻)	Extended polylinker, pUC derivation	Stratagene
pRK2013	Km^r, *oriT* helper	13
pZ1918	Ap^r, pUC19/18 with 3.2-kb *lacZ* cassette	31
pMini-CTX	Tc^r, *attP* site-specific integration vector	18
pFLP2	Ap^r, Suc^r, broad-host-range Flp expression vector	18
pJFM12	pBluescript with ~3.6-kb *Eco*RI-*Eco*RV that contains *katA*	24
pJFM13	pEX100T with blunted ~3.6-kb *Eco*RI-*Eco*RV from pJFM12	24
pJFM14	pJFM13 with 850-bp Gm^r cassette in *Sma*I site of *katA*	24
pSMB2	pBluescript with 5.4-kb *Eco*RI fragment of *P. aeruginosa* containing *katB*	7
pEX100T	Gene replacement vector	32
pJE26	Ap^r, *Xba*I-*Hin*dIII *katB* fragment in pQF50 forming a *katB*::*lacZ* transcriptional fusion	This study
pJGE02	Tc^r, pMini-CTX containing *lacZ* cassette from pZ1918 on *Bam*HI fragment	This study
pJGE03	Tc^r, pJGE02 with *Xho*I-*Hin*dIII fragment from pJE26 containing *katB* promoter	This study
pUCGM	Ap^r, Gm^r, pUC19 with 850-bp Gm^r cassette	32

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Suc^r, sucrose resistance.

H_2O_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 cultures. Stationary-phase cells were harvested and diluted to a cell density of approximately 3.0×10^7 CFU · ml⁻¹ in PBM medium that did not contain glucose. To determine initial cell viability prior to H_2O_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 (wt/vol) (used in the H_2O_2 treatments to neutralize H_2O_2) and then plated on R2A agar (Difco). For H_2O_2 treatments, H_2O_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_2O_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_2O_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_2O_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_2O_2) and then for the remaining biofilms the culture medium inflow was switched to PBM medium that included 50 mM H_2O_2. The PBM-H_2O_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_2O_2. Biofilm biomass was then homogenized by using a PT 10/35 Brinkman homogenizer (Brinkman Instruments, Westbury, N.Y.) for 15 s at setting 4. Biofilm cell

viability was determined by diluting homogenized biofilm suspensions and enumerating CFUs on R2A agar as described for the planktonic culture H_2O_2 treatment experiments. In addition to the viable cell count, the total number of cells harvested from each 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 H_2O_2-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 polyacrylamide 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 H_2O_2 every 10 min for a period of 1 h, while biofilms were treated with a constant stream of 50 mM H_2O_2 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 H_2O_2 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 8 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 for sonication. 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 μmol of H_2O_2 degraded · min^{-1} · mg of total protein^{-1}. Catalase expression was also analyzed by nondenaturing 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 Sambrook et al. (29). Enzymes were purchased from either Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.). Oligonucleotides were designed with OLIGO software and purchased from Integrated DNA Technologies Inc. (Coralville, Iowa).

Insertional inactivation of the *katA* and *katB* genes was facilitated by using the gene replacement vector pEX100T (33) that selected for double-crossover events by using positive selection on agar media containing 6% sucrose. To construct a *katA* mutant, an ~3.6-kb *Eco*RI-*Eco*RV fragment from pJFM12 (24) was filled in with Klenow fragment and ligated into the unique *Sma*I site within pEX100T, forming pJFM13 (24). This plasmid was cut with *Sma*I, a unique site within the *katA* coding region, and ligated to an 850-bp *aaC1* (encoding gentamicin resistance [Gm^r]) cassette excised from pUCGM (32), forming pJFM14 (24). Plasmid pSMB5 (7), harboring the Gm^r cassette within the *Eco*RV site of *katB*, was used in a similar fashion for construction of an isogenic *katB* mutant. Plasmids were mobilized by conjugation by either triparental or biparental matings between *E. coli* and *P. aeruginosa* PAO1. Replacement of each wild-type allele with the mutated gene was verified by Southern blot analysis and resulted in the creation of isogenic strains that lacked either KatA or KatB catalase activity.

A novel system developed by Hoang et al. (18) was employed to introduce a site-directed, single copy *katB::lacZ* transcriptional fusion into the *P. aeruginosa* PAO1 genome. Briefly, the mini-CTX suicide vector (parent plasmid for pJGE03 [Table 1]) carrying the reporter gene construct integrates into the *P. aeruginosa* genome at the *attB* site via an integrase (*int*)-mediated recombination. TC-resistant (Tc) PAO1 colonies were selected, and a second plasmid, pFLP2, was introduced. The pFLP2 construct is a broad-host-range vector that expresses yeast Flp recombinase leading to excision of DNA sequences included within Flp recombinase target (FRT) sites. The integrated mini-CTX vector contains two FRT sites that flank the Tc^r, *int*, and *oriT* markers. Therefore, introduction of the pFLP2 construct eliminates these markers from the integrate. After the pFLP2 plasmid is cured from the reporter strain, only the inserted gene fusion, which is also flanked by T4-derived transcriptional terminators, is left remaining.

To construct the *katB::lacZ* reporter gene in mini-CTX, a promoterless *lacZ* cassette containing the native *lacZ* ribosome-binding site was excised from pZ1918 (31) on a 3.4-kb *Bam*HI fragment and then ligated into the unique

TABLE 2. Planktonic-specific catalase activity of strain PAO1 and KatA⁻ and KatB⁻ mutants[a]

Strain	Catalase activity (U)	
	Before treatment	After treatment[b]
PAO1	426 ± 6	609 ± 14
PAO1(*katA*)	ND[c]	ND
PAO1(*katB*)	430 ± 10	432 ± 9

[a] Results are the means ± the standard errors of three replicate cultures for each strain. Results are typical for one of three independent experiments.

[b] Stationary-phase cultures were treated with 2 mM doses of H_2O_2 every 10 min for 1 h while shaking at 25°C.

[c] ND, not detectable.

*Bam*HI site in the multicloning site of mini-CTX to create pJGE02. A 580-bp *Xho*I-*Hin*dIII fragment containing the *katB* promoter was then subcloned from pSMB2 (7) into the corresponding sites of the polylinker in pJGE02 to create pJGE03. This places the *katB* promoter upstream of *lacZ* and generates a *katB::lacZ* transcriptional fusion. The pJGE03 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(pRK2013) as the helper strain. Tc^r 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^r 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(*lacZ*), which contained a promoterless *lacZ* gene inserted at the *attB* 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 pJGE02 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).

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

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 batch conditions. Specific catalase activity levels in PAO1 were typically about 400 U (Table 2). After repeated doses of low levels of H_2O_2, total catalase levels increased by roughly 50%. No catalase activity was observed in untreated stationary-phase cultures of the *katA* mutant, nor was activity detectable in equivalent *katA* mutant cultures challenged with repeated doses of H_2O_2. 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 H_2O_2. 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.

H_2O_2 sensitivity of planktonic cells. In order to assess the importance of catalase in the protection of planktonic cells from H_2O_2, each strain was subjected to a single dose of 50 mM H_2O_2. Reductions in cell viability were determined at 20-min intervals for a period of 1 h. The strains were variably sensitive to the H_2O_2 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 H_2O_2, with a mean $\log_{10}$ reduction of 5.0 after 1 h, though this result was not statistically
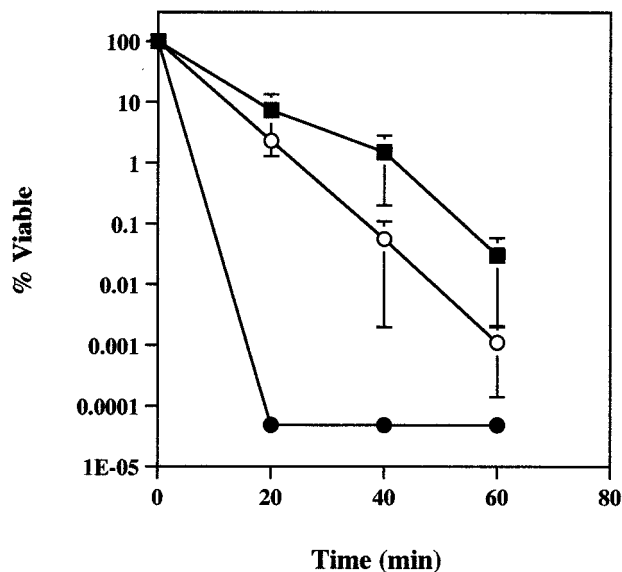
FIG. 1. Planktonic cell survival following treatment with a single dose of 50 mM H_2O_2. Samples from strain PAO1 (■) and KatA$^-$ (●) and KatB$^-$ (○) mutants were collected and neutralized at 20-min intervals during 1 h of exposure. Note that *y*-axis data is presented in $\log_{10}$ units. Data points are the means of three independent experiments for each strain (one culture per experiment) and each error bar represents one standard error of the mean. The responses of PAO1 and the *katB* mutant were not significantly different, but both were statistically significantly greater than that of the *katA* mutant ($P < 0.01$).

significantly different from that of PAO1. Cells lacking KatA, and therefore having no constitutive catalase activity (Table 2), were hypersensitive to H_2O_2. In all experiments, *katA* mutant CFUs could not be detected at the 20-min sampling time. Therefore, at least a 6.3 $\log_{10}$ reduction in viable cells (detection limit of viable counts) occurred as soon as 20 min after addition of H_2O_2. The lack of KatA clearly had a significant effect on the ability of planktonic cells to survive H_2O_2 treatment when compared to PAO1 ($P < 0.01$) or the *katB* mutant ($P < 0.01$).

H_2O_2 sensitivity of biofilm cells. Biofilms formed by PAO1 and the catalase mutant strains were also tested for their susceptibility to 50 mM H_2O_2. However, in these experiments H_2O_2 was delivered as a constant flow (50 ml · h^{-1}) rather than as a single pulse as with planktonic cultures. The catalase mutations had no apparent effect on the ability of these strains to form a biofilm, and for all three strains biofilm thickness was measured to be 114 ± 13 μm and from the total cell counts it was estimated that cell density averaged 5.4 × 10^9 total cells · cm^{-2}.

Biofilm cell survival was expressed as the viable fraction of the total number of cells present on the substratum after 1 h of exposure to 50 mM H_2O_2. The viable fraction was also determined for the control biofilms to serve as an internal check for verifying the accuracy and precision of the direct counts. Averaged over all PAO1, KatA$^-$, and KatB$^-$ control biofilms ($n = 9$), viable plate counts were 99.17% ± 0.03% of that estimated with total direct counts and did not vary between strains. Therefore, it was concluded that differences in percent viability between strains should reflect the role of catalase in protecting the biofilm cells and would not be attributable to random counting errors. When the levels of killing observed with biofilm cells were compared to those observed with planktonic cells, biofilm cells from all strains were shown to be much more resistant to H_2O_2 (Fig. 2). For the wild-type strain and the *katB* mutant, survival rates did not differ, as viability was

nearly constant at 80 and 77%, respectively, after 60 min. However, the lack of KatA significantly ($P < 0.05$) increased the susceptibility of biofilms to H_2O_2, resulting in nearly 90% killing.

Biofilm removal with H_2O_2 treatment. As biofilm formation was relatively uniform within and between strains, it was possible to use changes in direct count data to assess the extent to which the H_2O_2 treatment removed biofilm cells (Fig. 2). Even with profuse effervescence due to high catalase-specific activity (Fig. 3, and see below), PAO1 and *katB* mutant biofilms remained largely intact, with more than 70% of the initial total biomass remaining on the stainless steel surface after 1 h. By contrast, virtually no oxygen evolution was observed in the *katA* mutant biofilms during treatment with H_2O_2. Nevertheless, biofilm breakup occurred such that 93% of the initial biomass was removed, leaving a thin layer of cells remaining on the slide. Differences between *katA* mutant biofilm biomass remaining and the amounts measured for the PAO1 and *katB* strains were statistically significant ($P = 0.01$).

Catalase activity of *P. aeruginosa* biofilms. In addition to assessing susceptibility to H_2O_2, the specific catalase activities of the PAO1 and the *katA* and *katB* mutant biofilms were determined before and after exposure to H_2O_2 (Fig. 3). Biofilms were collected at 20-min intervals during exposure to a constant flow of 50 mM H_2O_2. 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 H_2O_2. As soon as 20 min after exposure to H_2O_2, 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-
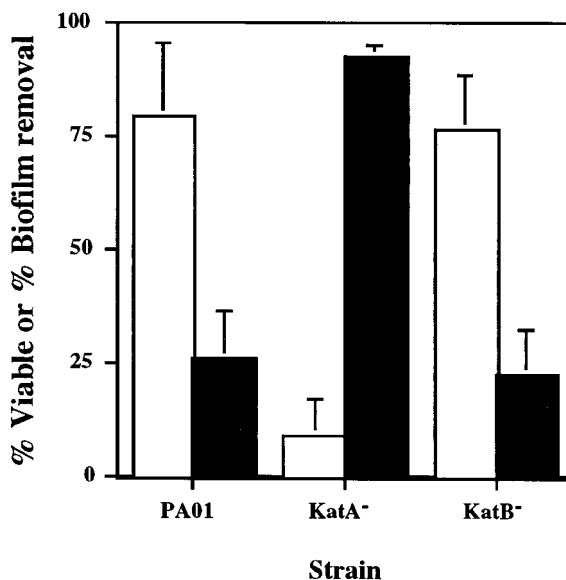

FIG. 2. Biofilm cell viability and removal after exposure to a continuous dose of 50 mM H_2O_2 for 1 h. Open bars, percent viability of remaining cells; filled bars, percent of biofilm removed. Percent viability is expressed as the ratio (×100) of CFUs obtained on agar plates to the total number of cells present on the substratum as determined with nonspecific staining and direct counts. Percent removal was based on the decrease in total direct counts. 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.
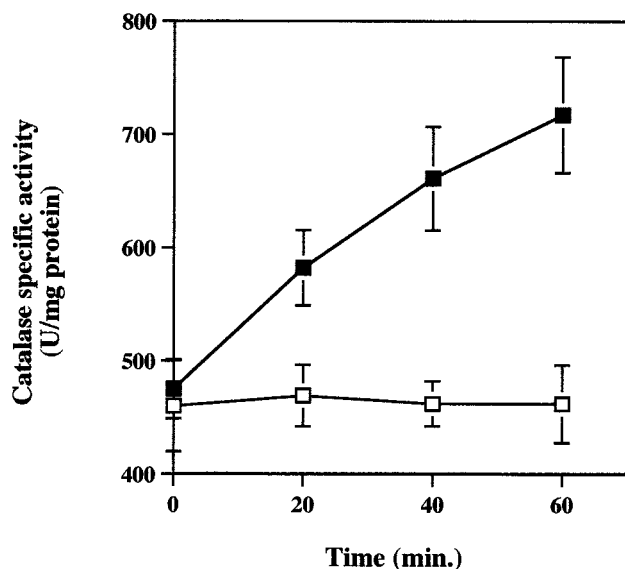
FIG. 3. Total catalase activity of PAO1 (■) and *katB* (□) biofilms exposed to 50 mM H₂O₂ for 1 h. Cell-free extracts were prepared and assayed for catalase activity as described in Materials and Methods. Data points are the means of three independent experiments (one biofilm per experiment for each strain) and each error bar represents one standard error of the mean. Duplicate assays were performed on each cell-free extract preparation.

ing that *katB* induction was primarily responsible for the significant ($P = 0.01$) increase in catalase activity observed in PAO1 biofilms. Total specific catalase activities for *katA* mutant biofilms could be measured only at 0 and 20 min since much of the biomass had already sloughed from the substratum (Fig. 2). No catalase activity was detected in cell-free extracts collected from KatA⁻ biofilms at either time point (data not shown). Native PAGE and catalase activity stains with biofilm cells extracts (Fig. 4) showed that KatA was the sole catalase being expressed before H₂O₂ exposure and that *katB* was induced in response to the 50 mM H₂O₂ treatment.

PAO1(*katB*::*lacZ*) reporter construction and analysis. The PAO1 *katB* *lacZ* reporter strain was used to assess whether gene regulation patterns routinely observed with planktonic cells would also hold for *P. aeruginosa* biofilms. The method of incorporating the reporter fusion into a specific location in the chromosome is advantageous for biofilm studies since chromosomal fusions are stable in the absence of antibiotic selection. To verify correct integration into the *attP* site, PCR primers specific for the flanking chromosomal DNA and integrated fusion were used to amplify the junction sequences. In each case, single amplification products of the predicted size were obtained from genomic templates (data not shown). These PCR products were also sequenced as an additional verification that the constructions were correct. Sequence data verified that both the pJGE03 and mini-CTX (*lacZ* control) vectors had integrated into the chromosome at the *attP* site and that pFLP2-mediated excision had occurred precisely. In preliminary measurements, it was empirically determined that culture CFU-per-milliliter optical density units for washed cell suspensions of both cell types were equivalent (results not shown), therefore allowing reporter enzyme units to be conveniently calculated in terms of cell suspension optical density.

The induction profile of *katB*::*lacZ* was 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 H₂O₂ exposure

period (Fig. 5A). Reporter enzyme levels did not increase in PAO1(*katB*::*lacZ*) biofilms that were not exposed to H₂O₂. When planktonic cells were exposed to 2 mM doses of H₂O₂ 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 H₂O₂, 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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.

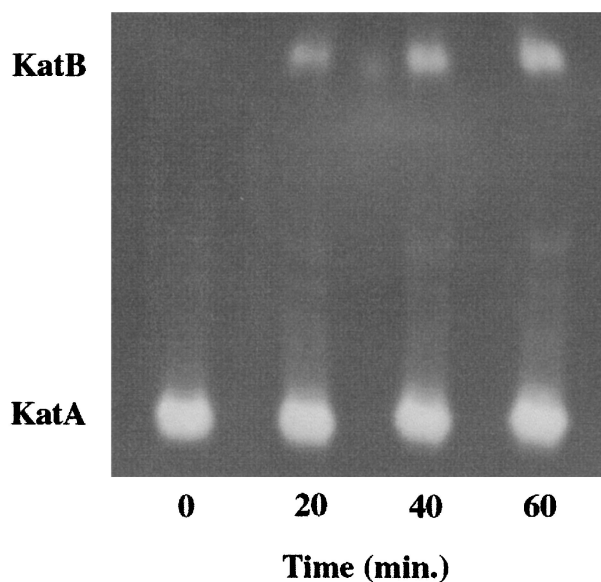One of the goals of this study was to determine whether cell

FIG. 4. PAO1 biofilm catalase isozyme expression patterns during treatment with a continuous dose of 50 mM H₂O₂. 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.
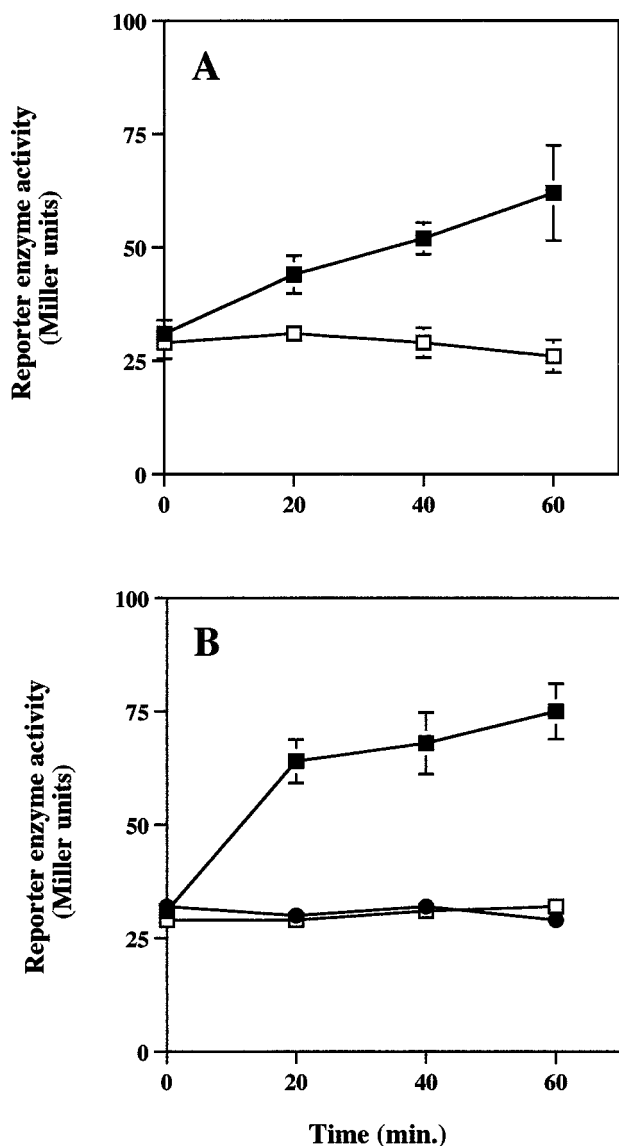
FIG. 5. PAO1 *katB::lacZ* reporter activity of biofilm (A) and planktonic cultures (B). Biofilms were treated with a continuous dose of 0 mM (□) or 50 mM (■) H_2O_2 for a period of 1 h. Stationary-phase planktonic cultures were exposed to a single 0 mM (□) or 50 mM (●) H_2O_2 dose or to 2 mM H_2O_2 pulses 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.

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_2O_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_2O_2, making KatB a marker enzyme for H_2O_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_2O_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_2O_2, the specific catalase activity of biofilms treated with a constant

exposure to 50 mM H_2O_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_2O_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_2O_2. An obvious physiologic trait of importance to inactivating H_2O_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_2O_2. The extraordinary recalcitrance of *P. aeruginosa* biofilms to H_2O_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_2O_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_2O_2, we created defined *katA* and *katB* mutations in the wild-type strain PAO1. In planktonic cultures, the KatA⁻ mutant was extremely sensitive to H_2O_2, with cell viability decreasing by more than six orders of magnitude within only 20 min. The KatB⁻ 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_2O_2 (Fig. 1) and a complete loss of catalase-mediated H_2O_2 resistance activity (Table 2). This effectively resulted in these cells exhibiting a catalase-negative phenotype. KatB induction did not occur in the KatA⁻ strain after treatment with H_2O_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⁻ cells being rapidly overwhelmed and killed before the cells had an opportunity to induce measurable *katB*. KatA⁻ biofilms also displayed a consistent pattern of sloughing when exposed to H_2O_2 (Fig. 2). Approximately 30 min after initial exposure to H_2O_2, much of the KatA⁻ biofilm sloughed from the substratum (results not shown), and after 1 h, roughly 90% of the KatA⁻ 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_2O_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_2O_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_2O_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-

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 *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.

REFERENCES

1. **Ashby, M. J., J. E. Neale, S. J. Knott, and I. A. Critchley.** 1994. Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. J. Antimicrob. Chemother. **33:**443–452.
2. **Atlas, R. M.** 1997. R2A agar, p. 1176. *In* L. C. Parks (ed.), Handbook of microbiological media. CRC Press, Ann Arbor, Mich.
3. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195:**133–140.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, M. R. W., and P. Gilbert.** 1993. Sensitivity of biofilm to antimicrobial agents. J. Appl. Bacteriol. **74**(Suppl.):87S–97S.
7. **Brown, S. M., M. L. Howell, M. L. Vasil, A. J. Anderson, and D. J. Hassett.** 1995. Cloning and characterization of the *katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. J. Bacteriol. **177:**6536–6544.
8. **Chen, X., and P. S. Stewart.** 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. Environ. Sci. Technol. **30:**2078–2083.
9. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35:**299–324.
10. **Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James.** 1994. Biofilms, the customized microniche. J. Bacteriol. **176:**2137–2142.
11. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. **25:**315–337.
12. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
13. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
14. **Giwercman, B., E. T. Jensen, N. Høiby, A. Kharazmi, and J. W. Costerton.** 1991. Induction of β-lactamase production in *Pseudomonas aeruginosa* biofilm. Antimicrob. Agents Chemother. **35:**1008–1010.
15. **González-Flecha, B., and B. Demple.** 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J. Biol. Chem. **270:**13681–13687.
16. **Hassett, D. J., L. Charniga, K. Bean, D. E. Ohman, and M. S. Cohen.** 1992. Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. Infect. Immun. **60:**328–336.
17. **Hassett, D. J., J. G. Elkins, D.-J. Ma, and T. R. McDermott.** *Pseudomonas aeruginosa* biofilm sensitivity to biocides: use of hydrogen peroxide as a model antimicrobial agent for examining resistance mechanisms. Methods Enzymol., in press.
18. **Hoang, T. T., A. J. Kutchma, A. Becher, and H. P. Schweizer.** A site-specific gene integration system for *Pseudomonas aeruginosa*. Submitted for publication.
19. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. 419–443.
20. **Huang, C.-T., K. D. Xu, G. A. McFeters, and P. A. Stewart.** 1998. Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilm in response to phosphate starvation. Appl. Environ. Microbiol. **64:**1526–1531.
21. **LeChevallier, M. W., C. D. Cawthon, and R. G. Lee.** 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. **54:**2492–2499.
22. **Liss, L.** 1987. New M13 host: DH5αF′ competent cells. Bethesda Res. Lab Focus **9:**13.
23. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^s (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
24. **Ma, J.-F., U. A. Ochsner, M. G. Klotz, V. K. Nanayakkara, M. L. Howell, Z. Johnson, J. E. Posey, M. L. Vasil, J. J. Monaco, and D. J. Hassett.** 1999. Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**3730–3742.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
26. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1–23. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York, N.Y.
27. **Nichols, W. W., M. J. Evans, M. P. E. Slack, and H. L. Walmsley.** 1989. The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. J. Gen. Microbiol. **135:**1291–1303.
28. **Östling, J., L. Holmquist, K. Flärdh, B. Svenblad, Å. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103–127. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York, N.Y.
29. **Sambrook, J., E. F. Frisch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Sanderson, S. S., and P. S. Stewart.** 1997. Evidence of bacterial adaptation to monochloramine in *Pseudomonas aeruginosa* biofilms and evaluation of biocide action model. Biotechnol. Bioeng. **56:**201–209.
31. **Schweizer, H. P.** 1993. Two plasmids, X1918 and Z1918, for easy recovery of *xylE* and *lacZ* reporter genes. Gene **134:**89–91.
32. **Schweizer, H. P.** 1993. Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
33. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15–22.
34. **Simon, R., U. Priefer, and A. Puehller.** 1983. A broad host range mobilization system for *in-vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
35. **Stewart, P. S.** 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother. **40:**2517–2522.
36. **Tresse, O., T. Jouenne, and G.-A. Junter.** 1995. The role of oxygen limitation in the resistance of agar-entrapped, sessile-like *Escherichia coli* to aminoglycoside and β-lactam antibiotics. J. Antimicrob. Chemother. **36:**521–526.
37. **Wayne, L. G., and G. A. Diaz.** 1986. A double staining method for differentiating between two classes of mycobacterial catalases in polyacrylamide gels. Anal. Biochem. **157:**89–92.
38. **Xu, K. D., P. S. Stewart, F. Xia, C.-T. Huang, and G. A. McFeters.** 1998. Physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. **64:**4035–4039.