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A Protease-Resistant Catalase, KatA, Released upon Cell Lysis during Stationary Phase Is Essential for Aerobic Survival of a *Pseudomonas aeruginosa oxyR* Mutant at Low Cell Densities

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A *Pseudomonas aeruginosa oxyR* mutant was dramatically sensitive to H_2O_2, despite possessing wild-type catalase activity. Oxygen-dependent *oxyR* phenotypes also included an inability to survive aerobic serial dilution in Luria broth and to resist aminoglycosides. Plating the *oxyR* mutant after serial dilution in its own spent culture supernatant, which contained the major catalase KatA, or under anaerobic conditions allowed for survival. KatA was resistant to sodium dodecyl sulfate, proteinase K, pepsin, trypsin, chymotrypsin and the neutrophil protease cathepsin G. When provided in *trans* and expressed constitutively, the OxyR-regulated genes *katB*, *ahpB*, and *ahpCF* could not restore both the serial dilution defect and H_2O_2 resistance; only *oxyR* itself could do so. The aerobic dilution defect could be complemented, in part, by only *ahpB* and *ahpCF*, suggesting that the latter gene products could possess a catalase-like activity. Aerobic Luria broth was found to generate ~1.2 μM H_2O_2 min^{-1} via autoxidation, a level sufficient to kill serially diluted *oxyR* and *oxyR katA* bacteria and explain the molecular mechanism behind the aerobic serial dilution defect. Taken together, our results indicate that inactivation of OxyR renders *P. aeruginosa* exquisitely sensitive to both H_2O_2 and aminoglycosides, which are clinically and environmentally important antimicrobials.

The major response of *Escherichia coli* to hydrogen peroxide (H_2O_2) is governed by a 34-kDa transactivator, OxyR (5, 6, 30, 31). OxyR positively regulates *katG* (encoding hydroperoxidase I), *gorA* (encoding glutathione reductase), *ahpCF* (encoding alkyl hydroperoxide reductase), *dps* (encoding a nonspecific DNA-binding protein) (1, 5, 6), and *fur* (encoding ferric uptake regulatory protein) (31). Each of these genes is important in combating H_2O_2-mediated stress. OxyR acts as a transcriptional autorepressor under noninducing conditions. However, in the presence of its inducer, H_2O_2, an intramolecular disulfide bond that activates OxyR is formed, allowing it to then govern transcription of OxyR-dependent promoters (24, 26).

In contrast to the case for *E. coli*, genes under OxyR control in *Pseudomonas aeruginosa* include the *katB-ankB* operon, *ahpB*, and *ahpCF* (17). These genes encode catalase B (KatB), an ankyrin-like protein that is necessary for optimal KatB activity (AnkB), and two alkyl hydroperoxide reductases, AhpB and AhpCF, respectively (17). In this work, we report dramatic sensitivity of a *P. aeruginosa oxyR* mutant to H_2O_2 and aminoglycosides, despite possessing wild-type catalase activity. Growth of serially diluted *oxyR* organisms under aerobic conditions was found to require the major housekeeping catalase, KatA, which was released into the extracellular milieu upon cell lysis. KatA was found to be resistant to a number of proteases, including the human neutrophil serine protease cathepsin G. Our data suggest that released KatA could remain for extended periods, especially in *P. aeruginosa* biofilms of environmental, industrial, and clinical importance. Such resilience would afford viable organisms, especially those in a biofilm (10), additional protection against H_2O_2.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All *P. aeruginosa* and *E. coli* strains used in this study are listed in Table 1 and were maintained on Luria (L) agar (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl) or M9 minimal medium agar (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 1 mM $MgSO_4 \cdot 7H_2O$, and 0.2% glucose [per liter]) plates, with each medium solidified with 15 g of Bacto agar per liter. All strains were stored indefinitely at −80°C in either 12.5% glycerol or 10% skim milk.

Chemicals. Bovine serum albumin, carbenicillin, chloramphenicol, horseradish peroxidase, H_2O_2, cumeme hydroperoxide (CHP), potassium ferricyanide, ferric chloride, *o*-dianisidine dihydrochloride, pepsin, trypsin, and chymotrypsin were from Sigma Chemical Company (St. Louis, Mo.). Gentamicin, tetracycline and Triton X-100 were from Fisher Scientific. Human neutrophil cathepsin G was from Calbiochem (La Jolla, Calif.). Tobramycin was from Eli Lilly (Indianapolis, Ind.), and amikacin was from Bristol-Myers Squibb (Princeton, N.J.).

Growth conditions. All bacteria were propagated from single colonies or stationary-phase suspensions that had been grown in either L broth or M9 minimal medium. Liquid cultures were grown at 37°C with shaking at 300 rpm or on a roller wheel at 70 rpm unless otherwise indicated. Culture volumes were 1/10 of the total Erlenmeyer flask volume to ensure proper aeration. Bacteria were also grown under anaerobic conditions in an anaerobic chamber (model 1024; Forma Scientific, Marietta, Ohio).

Serial dilution experiments. Bacteria were grown aerobically in L broth or anaerobically in L broth containing 1% KNO_3 for 17 h at 37°C. Suspensions were serially diluted in sterile aerobic or anaerobic L broth or filter-sterilized (0.2-μm-pore-size filter) spent culture supernatants from various bacteria in microtiter dishes (Becton Dickinson, Franklin Lakes, N.J.). Five or 20 microliters of each dilution was spotted onto L-agar or L-agar–1% KNO_3 plates and incubated aerobically or anaerobically, respectively.

Identification of OxyR-regulated genes that rescue the aerobic dilution defect. Two phenotypes of the *oxyR* mutant constructed in this study were a dramatic sensitivity to H_2O_2 and an inability to survive aerobic serial dilution in L broth. To identify OxyR-regulated or other genes that could rescue the dilution defect, the *oxyR*, *ahpB-tdr*, *ahpCF*, *katB*, *dps*, and *recG* genes were expressed constitutively in the *oxyR* mutant via the *lac* promoter of pUCP19 or pUCP22 (29). The

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Source or reference
E. coli strains		
DH5α	*F− lacZΔM15 recA1 hsdR17 supE44* Δ(*lacZYA argF*)	Bethesda Research Laboratories
SM10	Km^r; mobilizer strain	21
P. aeruginosa strains		
PAO1	Prototrophic, wound isolate	11
ahpB	Gm^r; Δ*ahpB*::Gm mutant of PAO1	17
ahpBkatA	Gm^r Tc^r; Δ*ahpB*::Gm *katA*::Tc mutant of PAO1	17
ahpCF	Gm^r; Δ*ahpCF*::Gm mutant of PAO1	17
ahpBahpCF	Gm^r Tc^r; Δ*ahpB*::Gm *ahpCF*::Tc mutant of PAO1	17
katA	Gm^r Δ*katA*::Gm mutant of PAO1	15
katAoxyR	Gm^r Tc^r; Δ*katA*::Gm *katB*::Tc mutant of PAO1	This study
katB	Gm^r; Δ*katB*::Gm mutant of PAO1	10
katAkatB	Gm^r Tc^r; Δ*katA*::Gm *katB*::Tc mutant of PAO1	10
lasIrlhI	*lasI*::Tn*10 rhlI*::Tn*501-2*	18
oxyR-um	Unmarked Δ*oxyR* mutant of PAO1	17
oxyR	Gm^r; Δ*oxyR*::Gm mutant of PAO1	17
recG	Gm^r; Δ*recG*::Gm mutant of PAO1	17
Plasmids		
pCR-Blunt	Km^r; PCR cloning vector	Invitrogen
pEX100T	*sacB* Ap^r *oriT mob*	19
pUCP19	Ap^r; broad-host-range expression vector	29
pUCP22	Ap^r; broad-host-range expression vector	29
pUCP-*oxyR*	Ap^r; pUCP19 containing *oxyR* under P_{lac} control	17
pUCP-*katB*	Ap^r; pUCP19 containing *katB* under P_{lac} control	17
pUCP-*ahpB-tdr*	Ap^r; pUCP19 containing *ahpB* under P_{lac} control plus the *tdr* gene	This study
pUCP-*ahpCF*	Ap^r; pUCP19 containing *ahpCF* under P_{lac} control	17
pUCP-*recG*	Ap^r; pUCP19 containing *recG* under P_{lac} control	17
pUCP-*dps*	Ap^r; pUCP19 containing *dps* under P_{lac} control	This study

[a] Abbreviations used for genetic markers were as described by Holloway et al. (12). *mob*, mobilization site (ColE1); Tra$^+$, conjugative phenotype; *oriT*, origin of transfer (RK2); Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Tc^r, tetracycline resistance.

construction of pUCP-*oxyR*, pUCP-*katB*, pUCP-*ahpCF*, and pUCP-*recG* is described in an accompanying paper by Ochsner et al. (17). Plasmid pUCP-*ahpB-tdr* was isolated from a pUCP19-based plasmid library of *P. aeruginosa* PAO1 and contained a 6-kb partial *Sau*3A fragment that carried the *ahpB* gene under the control of the plasmid-borne P_{lac} promoter and also harbored the *tdr* gene. The *dps* gene was PCR amplified using primers *dps*-845 (5′-*Xba*I-tctaGAGTGCCC CATTCACAAGG and *dps*-1377 (5′-*Hin*dIII-aaGCTTTCTATCGAGCGAT CGA (bases in the restriction enzyme cleavage site are underlined, and designed and mismatched bases are in lowercase type). The 539-bp PCR product was then cloned into pCR-Blunt and ligated directionally as an *Xba*I-*Hin*dIII fragment into pUCP22, yielding pUCP-*dps*, where the *dps* gene is expressed from the plasmid-borne P_{lac} promoter. The strains containing these recombinant plasmids were grown aerobically for 17 h in L broth and serially diluted in sterile, aerobic L broth in microtiter dishes as described above. Suspensions (20 μl) of each dilution were spotted on L-agar plates containing 400 μg of carbenicillin per ml.

Manipulation of recombinant DNA. Plasmid DNA was transformed into *E. coli* DH5α-MCR (Gibco-BRL, Gaithersburg, Md.) or SM10 (21). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) was routinely added to agar medium to detect the presence of insert DNA. Restriction endonuclease, alkaline phosphatase, Klenow fragment, T4 DNA polymerase, and T4 DNA ligase were used as specified by the vendor (Gibco-BRL). Plasmid DNA was isolated using miniprep kits from Qiagen. DNA fragments used for cloning or in the construction of radiolabeled probes were recovered from agarose gels using SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, Maine). Gene replacements were done using the pEX100T vector (19) as described in detail in the accompanying paper by Ochsner et al. (17).

H_2O_2, CHP, and antibiotic sensitivity assays. *P. aeruginosa* strains were grown aerobically in M9 minimal medium for 17 h at 37°C. Each culture was diluted in 3 ml of 0.8% low-melting-point M9 agarose (SeaPlaque) to a final optical density at 600 nm of 0.2 and maintained at 37°C. These suspensions were then distributed evenly on M9 agar plates, and the top agarose was allowed to solidify. Filter paper disks containing 10 μl of either 0.2% H_2O_2, 20% CHP, gentamicin (40 mg/ml), amikacin (40 mg/ml), tobramycin (25 mg/ml), carbenicillin (50 mg/ml), chloramphenicol (34 mg/ml in 95% ethanol), or tetracycline (15 mg/ml in 50% ethanol) solution were placed on the top agar surface, and the plates were incubated aerobically at 37°C for 17 to 24 h. The zones of growth inhibition were then recorded in triplicate.

Cell extract preparation and biochemical assays. Cell extracts were prepared from bacteria harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Bacteria were washed twice in ice-cold 50 mM sodium phosphate buffer (pH 7.0) and sonicated in an ice-water bath for 10 s with a Heat Systems-Ultrasonics (Farmingdale, N.Y.) model W-225 sonicator equipped with microtip at output setting 5. The sonicate was clarified by centrifugation at 13,000 × *g* for 10 min at 4°C. Cell extract preparation for native gel electrophoresis was performed as described above except that 50 mM Tris-HCl (pH 7.4) was used as the diluent. Catalase activity was assayed spectrophotometrically at 240 nm by monitoring the decomposition of 18 mM H_2O_2 using a Spectronic Genesys 5 spectrophotometer (Spectronic Unicam, Rochester, N.Y.) (2). Catalase activity gels (5%) were stained according to the method of Wayne and Diaz (28). One unit of catalase activity was that which degraded 1 μmol of H_2O_2 per min at 23°C. The production of H_2O_2 in aerobic L broth was monitored as follows. For determination of H_2O_2 formation by autoxidation of aerobic L broth, L broth was diluted 1:1 with a solution containing 0.01% horseradish peroxidase, 0.2% Triton X-100, 0.63 mM *o*-dianisidine dihydrochloride, and 1 mM EDTA in 50 mM acetate buffer, pH 5.0. The production of H_2O_2 was monitored spectrophotometrically at 460 nm by the oxidation of *o*-dianisidine dihydrochloride. Bovine liver catalase (100 U/ml) (Boehringer-Mannheim) was used to inhibit H_2O_2 production. Susceptibility of *P. aeruginosa* KatA to proteolysis was assayed by incubating sterile spent culture supernatant containing KatA activity with an equal volume of various proteases (1 mg/ml) or 1 mM dithiothreitol for 1 h at 37°C, after which catalase activity was recorded. Neutrophil cathepsin G was used at 100 mU/ml. Protein concentrations in cell extracts were estimated by the method of Bradford (3), using bovine serum albumin (fraction V; Sigma) as a standard.

RESULTS

Phenotypes of a *P. aeruginosa oxyR* mutant. (i) H_2O_2 sensitivity despite possession of normal catalase activity. In our accompanying work (17), we describe the cloning and characterization of genes under the control of the global regulator OxyR in *P. aeruginosa*. These were found to include the *katB-ankB* operon, encoding a 228-kDa catalase and an ankyrin-like protein, AnkB, that is essential for optimal KatB activity, and *ahpB* and *ahpCF*, encoding two alkyl hydroperoxide reductases, AhpB and AhpCF, respectively. When we initially con-

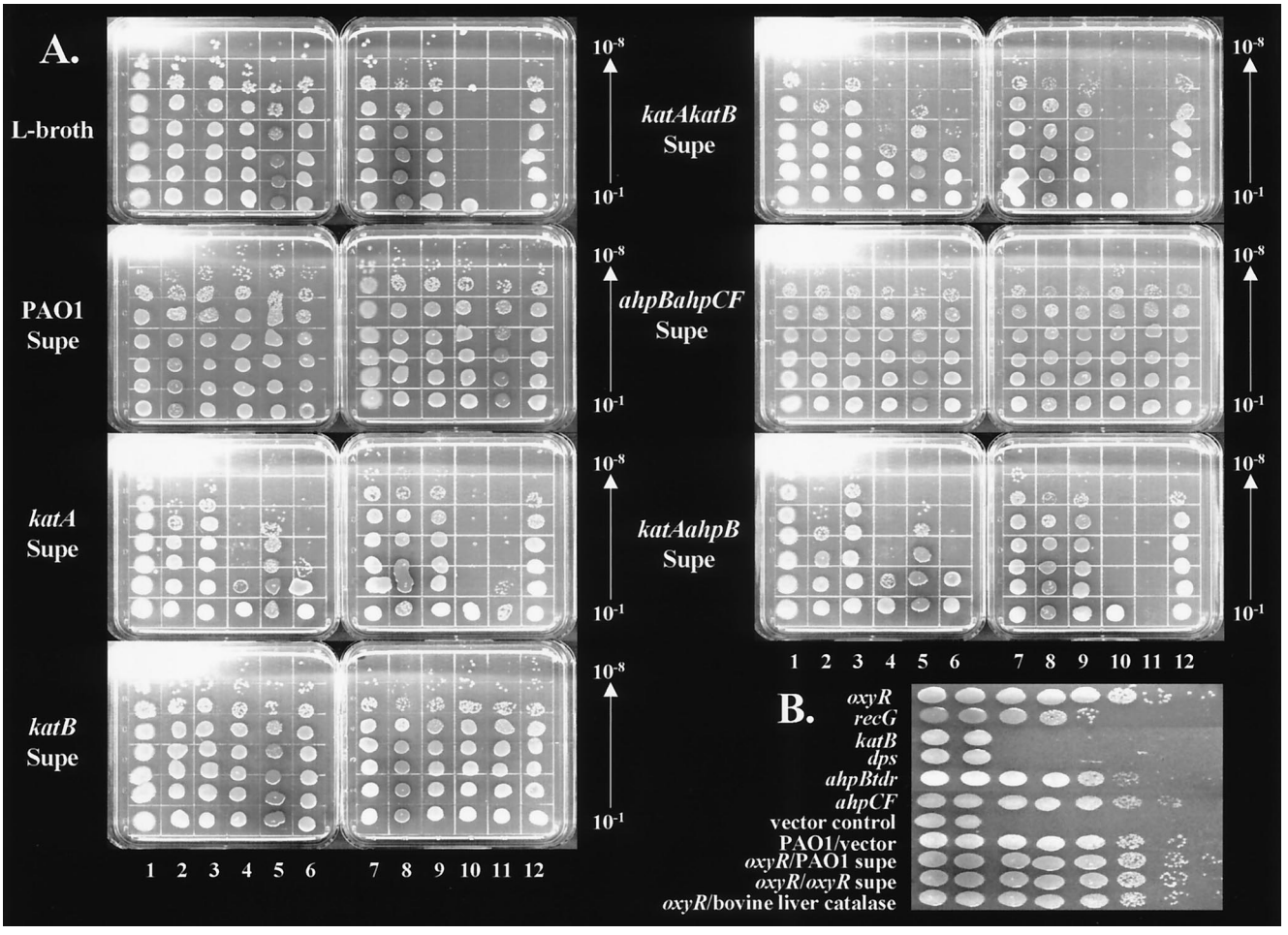

FIG. 1. Aerobic serial dilution defect of *P. aeruginosa oxyR*, *oxyR katA*, and *katA katB* strains. (A) Bacteria were grown aerobically in L broth until stationary phase. Organisms were serially diluted in either L broth or filter-sterilized spent culture supernatants (Supe) from different organisms to determine the effect of various gene products on rescue of the serial dilution defect of the *oxyR* mutant. Five-microliter suspensions of 10^{-1} through 10^{-8} serial dilutions were spotted on square L-agar plates and incubated at 37°C under aerobic conditions for 17 h. The top of each plate was removed, the plate was placed top-side-down on a scanner, and a black sheet of paper was laid carefully on top of each plate to enhance contrast. The images were scanned using ScanGal software and finally stored as a PowerPoint 98 file. Lanes: 1, PAO1; 2, *katA*; 3, *katB*; 4, *katA katB*; 5, *ahpB*; 6, *ahpB katA*; 7, *ahpCF*; 8, *ahpB ahpCF*; 9, *recG*; 10, *oxyR*; 11, *oxyR katA*; 12, *lasI rhlI*. (B) Bacteria harboring various pUCP-P_{lac} based plasmids containing the genes listed to the left were grown aerobically in L broth containing 400 μg of carbenicillin for 17 h per ml at 37°C. Serial dilutions were made in either fresh L broth, filter-sterilized (0.2-μm-pore-size filter) spent culture supernatants of wild-type PAO1 or the *oxyR* mutant, or L broth containing 100 U of bovine liver catalase per ml. Twenty microliters of each dilution was spotted onto square L-agar–carbenicillin plates and incubated aerobically for 17 h at 37°C.

structed an isogenic *P. aeruginosa oxyR* mutant (17), we postulated that it would be very sensitive to H_2O_2. Although the *oxyR* mutant possessed wild-type catalase activity (Table 2), we found the mutant to be to H_2O_2 sensitive but intermediate in sensitivity with respect to a catalase-deficient *katA katB* mutant when grown in L broth (Table 2) (10). Interestingly, the *oxyR* mutant was more sensitive to H_2O_2 than the *katA katB* mutant when grown in M9 minimal medium (Table 2).

(ii) Aerobic serial dilution defect of the *oxyR* mutant and protection by *P. aeruginosa* catalase KatA. During our selection for a double-crossover event while constructing our *oxyR* mutant, there were virtually no isolated colonies on the final selection medium, only patches of growth where high cell densities were distributed on the agar surface (data not shown). Hence, the *oxyR* mutant could not survive aerobic serial dilution, but it could in the presence of bovine liver catalase (Fig. 1B), in the presence of sterile spent culture supernatants of wild-type or *oxyR* mutant bacteria (Fig. 1), or when grown anaerobically. To determine the OxyR-dependent or -indepen-

TABLE 2. Catalase activity and H_2O_2 sensitivity of *P. aeruginosa* strains

Strain	Catalase activity (U/mg)[a] in:		H_2O_2 sensitivity[b] on:	
	Whole cells	Supernatant	L agar	M9 agar
PAO1	1,485 ± 76	1,391 ± 216	34.0 ± 0.8	20 ± −1
oxyR	1,406 ± 107	1,858 ± 230	41.8 ± 0.3	50 ± 0
katA katB	ND[c]	ND	51.8 ± 1.0	44 ± 2

[a] Bacteria were grown aerobically in L broth with vigorous shaking for 17 h at 37°C. Cell extracts were assayed for catalase activity as previously described (2). One unit was that activity which degraded 1 μmol of H_2O_2 min^{-1} mg^{-1} at room temperature. Results are means and standard errors.

[b] Stationary-phase bacteria grown in either L broth or M9 minimal medium were diluted 1:100 in 5 ml of 0.8% top agarose (low melting point) and poured evenly on the surfaces of 150-mm-diameter L-agar plates. Filter paper disks (7 mm) saturated with 10 μl of 8.8 M (L agar) or 59 mM (M9 medium) H_2O_2 were placed on the bacterial lawn, and the plates were incubated aerobically at 37°C for 17 h. The zones of growth inhibition (millimeters) were recorded in triplicate and are given as means and standard errors.

[c] ND, none detected.

TABLE 3. Effect of various gene products on sensitivity to H_2O_2, CHP, and antibiotics[a]

Strain	Sensitivity[b] to:							
	H_2O_2	CHP	GM	TB	AK	CB	CM	TC
PAO1	—[c]	—	31	25	27	15	22	19
oxyR	—	—	42	30	36	15	22	19
oxyR + pUCP-*oxyR*	15	18	ND[d]	ND	ND	ND	ND	ND
oxyR + pUCP-*recG*	37	23	ND	ND	ND	ND	ND	ND
oxyR + pUCP-*katB*	36	30	ND	ND	ND	ND	ND	ND
oxyR + pUCP-*dps*	25	31	ND	ND	ND	ND	ND	ND
oxyR + pUCP-*ahpB-tdr*	27	23	ND	ND	ND	ND	ND	ND
oxyR + pUCP-*ahpC-ahpF*	26	22	ND	ND	ND	ND	ND	ND
oxyR + vector	33	24	ND	ND	ND	ND	ND	ND
PAO1 + vector	12	19	ND	ND	ND	ND	ND	ND

[a] Bacteria were grown aerobically in M9 minimal medium containing 400 μg of carbenicillin per ml for 17 h at 37°C. The suspensions were diluted a final optical density at 600 nm of 0.2 in 3 ml of 0.8% M9 top agarose (low-melting-point; 37°C) and layered onto M9 plates. Filter paper disks were impregnated with 10 μl of either 0.2% H_2O_2 or 20% CHP. Filter paper disks were also impregnated with 10 μl of various antibiotics, including gentamicin (GM) (40 mg/ml), tobramycin (TB) (25 mg/ml), amikacin (AK) (40 mg/ml), carbenicillin (CB) (50 mg/ml), chloramphenicol (CM) (34 mg/ml), and tetracycline (TC) (15 mg/ml).
[b] The zones of growth inhibition, in millimeters ($n = 3$), were recorded after 17 h of growth at 37°C. The standard errors of the mean zones of inhibition were <5%.
[c] —, see below with appropriate vector controls.
[d] ND, not determined.

dent gene product(s) that could rescue this defect, a panel of mutants that had been compromised in some facet of oxidative stress machinery was screened for a potential aerobic serial dilution defect. These organisms were also serially diluted in sterile spent culture supernatants from seven different bacteria that lacked one or both catalases, alkyl hydroperoxide reductases, or various combinations of both. As shown in Fig. 1A, when diluted in L broth, the *oxyR* (lane 10) and *oxyR katA* (lane 11) mutants could survive at only a 10-fold or less serial dilution. Interestingly, serial dilution in sterile supernatants of wild-type, *katB*, and *ahpB ahpCF* bacteria rescued both *oxyR* and *oxyR katA* bacteria. Sterile spent supernatant from *katA*, *ahpB katA*, or *katA katB* mutants could not completely rescue diluted *oxyR*, *katA oxyR*, *katA ahpB*, or *katA katB* mutants. In fact, even the *ahpB* mutant alone could not be fully serially diluted in the latter supernatants, yet the strain was fully viable when diluted in L broth. Thus, an autoxidizable factor present in the spent culture supernatant of *katA*, *katA katB*, and *katA ahpB* strains sensitized *ahpB*, *ahpB katA*, and *katA katB* bacteria. Although this factor is unknown at present, we believe it to be the redox-cycling antibiotic pyocyanin.

(iii) Constitutive expression of OxyR-controlled AhpB and AhpCF partially rescues the aerobic dilution defect but not H_2O_2 or CHP resistance. One simple means to identify OxyR-regulated genes that restore the aerobic dilution defect and/or wild-type H_2O_2 resistance was to express *ahpB*, *ahpCF*, and *katB* in the *oxyR* mutant via the *lac* promoter of pUCP19 (29). This allows for constitutive expression of what are otherwise stringently regulated loci. Predictably, the dilution defect (Fig. 1B) and H_2O_2 and CHP resistance (Table 2) were rescued in *trans* by *oxyR* itself. The *recG*, *ahpB*, and *ahpCF* genes provided in *trans* could partially restore the dilution defect (Fig. 1B). Another OxyR-controlled gene, *katB* (4, 17), encoding an H_2O_2-inducible catalase, KatB, could not rescue the dilution defect, nor could the Dps protein. The *dps* gene is regulated by OxyR in *E. coli* (1) but not in *P. aeruginosa* (data not shown). Interestingly, Dps, AhpB-Tdr, and AhpCF could provide some protection against H_2O_2, with only OxyR providing full protection. CHP resistance was more than fully restored by provision of *oxyR* in *trans*. Interestingly, AhpB and AhpCF overexpression in the *oxyR* mutant did not significantly alter sensitivity to CHP, and overexpression of KatB and Dps actually increased sensitivity to CHP (Table 3).

(iv) Hypersusceptibility to aminoglycoside but not to other antibiotics that do not cause an oxidative stress. Aminoglycosides are a class of antibiotics that require oxidative phosphorylation for antimicrobial activity. In fact, it was recently shown that gentamicin exposure evokes production of reactive oxygen species (20, 27). Thus, if oxidative stress defense systems are compromised, as in the *oxyR* mutant, we predicted that it would be more sensitive to aminoglycosides. To test this hypothesis, an unmarked *oxyR* deletion strain, *oxyR*-um, was constructed. As shown in Table 3, the *oxyR*-um mutant was far more sensitive than wild-type bacteria to several aminoglycosides, including gentamicin, tobramycin, and amikacin. In contrast, there was no difference in sensitivity to antibiotics that do not evoke an oxidative stress, including carbenicillin, tetracycline, and chloramphenicol.

Catalase (KatA) activity is present in spent culture supernatants of the *oxyR* mutant. To identify the catalase released upon cell lysis of stationary-phase organisms, culture supernatants from several strains were assayed for catalase activity.

FIG. 2. Catalase activity and gel staining of spent culture supernatants of *P. aeruginosa*. *P. aeruginosa* strains were grown aerobically in L broth for 17 h at 37°C. The bacteria were harvested by centrifugation at 10,000 × *g* for 10 min, and the supernatants were filtered through 0.2-μm-pore-size filters. Culture supernatants were subjected to electrophoresis using 6% nondenaturing acrylamide gels and stained for catalase activity. The number with each lane is the percent catalase activity with respect to that of wild-type bacteria.

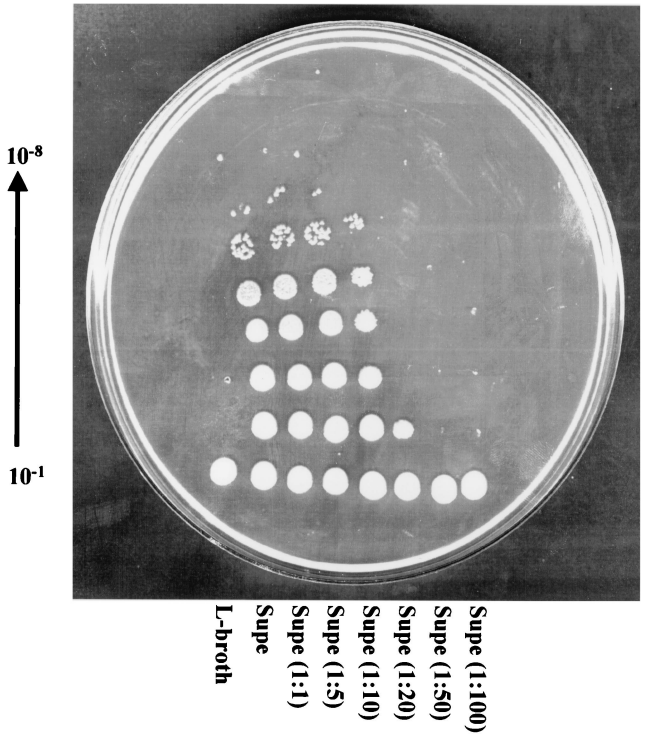

FIG. 3. Titration of KatA activity required for aerobic rescue of serially diluted *oxyR* bacteria. *P. aeruginosa oxyR* was grown aerobically to stationary phase in L broth. The bacteria were harvested by centrifugation at 10,000 × *g* for 10 min, and the supernatants were filtered through 0.2-μm-pore-size filters. The bacterial suspension was serially diluted in either L broth or various dilutions of wild-type spent culture supernatant (Supe) in microtiter dishes with a well volume of 200 μl. In this particular experiment, the catalase specific activity of the culture supernatant was 688 U/mg.

Table 2 demonstrates that wild-type and *oxyR* mutant bacteria possess 1,391 ± 216 and 1,858 ± 230 U of catalase activity per mg in their spent culture supernatants, respectively, while the *katA katB* mutant possesses none. In either case, supernatant catalase activity was attributable to KatA, since KatB was predictably absent because bacteria were not treated with H_2O_2 (4). As expected, no catalase activity was present in the *katA* (Fig. 2, lane 2), *katA katB* (lane 4), and *ahpB katA* (lane 5) strains relative to the wild-type (lane 1), *katB* (lane 3), or *ahpB ahpCF* (lane 6) strains. Thus, KatA is likely released into the culture supernatant upon cell lysis, a result confirmed by detection of fumarase (a cytoplasmic enzyme) activity, in the supernatants (data not shown). Additional evidence that KatA was not secreted came from construction of a KatA-PhoA translational fusion plasmid that yielded no alkaline phosphatase activity in the periplasmic space (data not shown). Interestingly, KatA was found to be a very stable enzyme, since it was highly resistant to sodium dodecyl sulfate, proteinase K, sodium dodecyl sulfate-proteinase K, pepsin, chymotrypsin, and trypsin but was inactivated by dithiothreitol and boiling. KatA activity was also retained after exposure to the human neutrophil protease cathepsin G (data not shown).

How much KatA is needed for rescue of the *oxyR* mutant in L broth? To determine the precise amount of KatA required for aerobic rescue of the *oxyR* mutant, wild-type spent culture supernatants were serially diluted in L broth and plated on L-agar plates as described above. As shown in Fig. 3, a 10-fold dilution of the *oxyR* mutant in L broth alone allowed for survival. A weakening of the rescue effect could be seen at the 10^{-6} to 10^{-8} dilutions when the supernatant was diluted 10-fold. Still, the most dramatic effect was observed when the supernatant was diluted 20-fold or greater. In this particular experiment, the stationary-phase suspension contained 2×10^{10} CFU/ml and the supernatant (~120 μg of protein per ml) contained ~688 U of KatA activity per mg. Thus, the total number of organisms present in the first 5-μl spot of the 10^{-1} dilutions is $\sim 1 \times 10^7$ CFU/ml. On a per-organism basis in a microtiter well volume of 200 μl, and based upon the near-complete inhibition by 20-fold-diluted supernatant of the growth of the *oxyR* mutant, we calculate that at least 16.5 U of KatA activity per well is required for dilution beyond 10^7 CFU/ml. This corresponds to a spent culture supernatant protein requirement, based upon this experiment, of at least 2.4 μg/200-μl well volume.

H_2O_2 is produced as an autoxidation product of aerobic L broth. Because of the dramatic sensitivity of *oxyR* bacteria to H_2O_2, coupled with its inability to be serially diluted in aerobic L broth, we predicted that H_2O_2 was generated in L broth via autoxidation. To test this hypothesis, H_2O_2 production in aerobic L broth was monitored by the horseradish peroxide-catalyzed oxidation of *o*-dianisidine (16). As shown in Fig. 4, a linear rate of 1.2 nmol of H_2O_2 s/min/ml (or 1.2 μM H_2O_2/min) was produced by aerobic L broth, an amount which gradually reached a plateau as oxygen became depleted in the cuvette (data not shown). Not surprisingly, H_2O_2 production was inhibited by the addition of 100 U of bovine liver catalase.

DISCUSSION

H_2O_2 is used to treat problematic bacterial biofilms in environmental and industrial settings (7, 23). It is also produced by phagocytic cells to kill invading microbes during the infection process (8–10, 14). In its presence, however, survival of *P.*

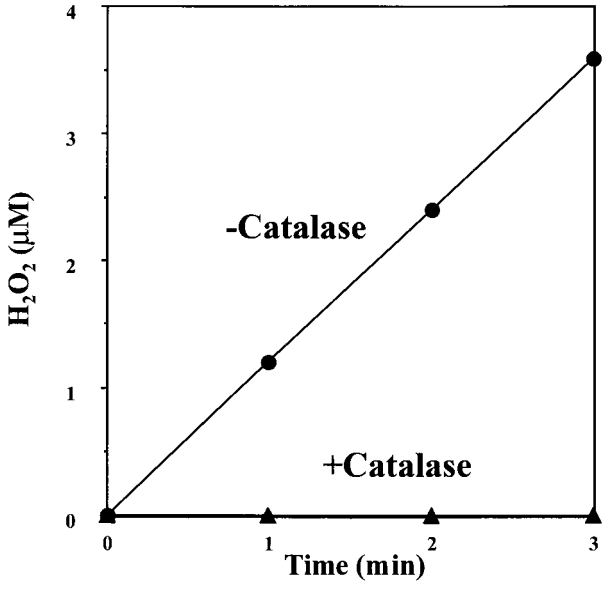

FIG. 4. H_2O_2 is produced as an autoxidation product of aerobic L broth. Ten milliliters of L broth was shaken vigorously at room temperature for 1 h. Samples were added to a solution containing 0.01% horseradish peroxidase, 0.2% Triton X-100, 0.63 mM *o*-dianisidine dihydrochloride, and 1 mM EDTA in 50 mM acetate buffer, pH 5.0. The production of H_2O_2 by autoxidation was monitored spectrophotometrically at 460 nm by the oxidation of *o*-dianisidine dihydrochloride. The addition of 100 U of bovine liver catalase per mg inhibited H_2O_2 production.

aeruginosa would depend upon an ability to rapidly mount a strategic defense against it. Such a response is governed, in part, by OxyR, which controls the *katB-ankB* operon, *ahpB*, and *ahpCF* (17). One likely reason for the dramatic H_2O_2 sensitivity of the *oxyR* mutant is that each OxyR-regulated gene product contributes some measure of protection against it (17). We have recently shown that KatB is required for optimal H_2O_2 resistance and that KatB activity is increased ~50-fold in its presence (4, 13). Because KatB can be detected in the cytoplasmic membrane and periplasmic fractions (13), it could act to protect H_2O_2-sensitive respiratory chain components. Optimal KatB-mediated protection also requires AnkB, a cytoplasmic membrane-periplasmic ankyrin-like protein that we believe anchors KatB to the inner membrane and/or stabilizes it is the periplasmic space (13). Although AhpB and AhpCF are predicted to encode proteins that are important in the detoxification of organic hydroperoxides, both AhpB and AhpCF appear to be important for some resistance to H_2O_2 when they are overexpressed in an *oxyR* mutant (Table 3). Interestingly, both *ahpCF* and *ahpB* are activated by H_2O_2 and CHP, and an *ahpB* mutant is very sensitive to H_2O_2 (17). AhpB could be the first line of defense in H_2O_2 detoxification, because AhpB-PhoA analyses indicate that it is a periplasmic protein (data not shown). Thus, AhpB, like KatB-AnkB (13), could also serve to protect H_2O_2-sensitive membrane components from oxidation by H_2O_2 or more hazardous downstream radicals (e.g., $HO^{\cdot}$).

Haemophilus influenzae, *Xanthomonas campestris* (15a), and *E. coli oxyR* mutants require catalase for normal aerobic growth. In this study, we found that the aerobic dilution defect was due to the production of ~1.2 μM H_2O_2 min^{-1} via low-level autoxidation of components present in L broth. The H_2O_2 produced simply by aerobic L broth was sufficient to kill serially diluted *oxyR* and *oxyR katA* bacteria, and KatA, when present in the spent culture supernatant, and to a lesser extent periplasmic AhpB protected the diluted organisms. This suggests that *P. aeruginosa* possesses an exquisitely H_2O_2-sensitive protein that is likely in the cytoplasmic membrane. Interestingly, the β subunit of F_1F_o-ATPase of *E. coli* is oxidized by H_2O_2 and could represent such a target in *P. aeruginosa* (25). Because we also found that KatA is very resistant to multiple proteases, including the relatively nonspecific proteinase K and the clinically relevant neutrophil cathepsin G, it could persist for extended periods when release from lysed cells. Thus, KatA could prevent H_2O_2 penetration and killing of bacteria, especially those growing as a biofilm, because it would essentially be an antioxidant "land mine" (7, 23). KatA-mediated protection against H_2O_2 is also clinically relevant because human phagocytic cells produce copious quantities of H_2O_2 as part of an oxygen-dependent antimicrobial regimen against bacterial infections. In seven different sputum samples from cystic fibrosis patients, we were unable to detect *P. aeruginosa* KatA activity, but we did find high levels of neutrophil catalase (data not shown). The likely reason that we could not detect KatA activity in the sputum samples is that *P. aeruginosa* titers are being controlled to ~10^4 organisms/ml of sputum with very aggressive antibiotic regimens.

Another phenotype of the *oxyR* mutant was a dramatic sensitivity to aminoglycosides, compounds whose antibacterial activity is dependent upon oxidative phosphorylation. Interestingly, aminoglycosides are used as part of aggressive antibiotic regimens (typically with β-lactams and/or quinolones) in the treatment of *P. aeruginosa* infections, especially in patients with cystic fibrosis (22). Thus, our results suggest that OxyR is essential for resistance to not only H_2O_2 but also aminoglycosides. Finally, this study implies that OxyR, which is present in a myriad of different bacteria, could be a target for novel pharmaceuticals. Successful compounds would essentially render bacteria *oxyR* mutants that would be unable to successfully colonize new niches in the presence of micromolar levels of H_2O_2, aminoglycosides, or a combination of both.

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