The Peptidoglycan-Associated Lipoprotein OprL Helps Protect a *Pseudomonas aeruginosa* Mutant Devoid of the Transactivator OxyR from Hydrogen Peroxide-Mediated Killing during Planktonic and Biofilm Culture^{∇†}

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OxyR controls H₂O₂-dependent gene expression in *Pseudomonas aeruginosa*. Without OxyR, diluted (<10⁷/ml) organisms are easily killed by micromolar H₂O₂. The goal of this study was to define proteins that contribute to *oxyR* mutant survival in the presence of H₂O₂. We identified proteins in an *oxyR* mutant that were oxidized by using 2,4-dinitrophenylhydrazine for protein carbonyl detection, followed by identification using a two-dimensional gel/matrix-assisted laser desorption ionization–time of flight approach. Among these was the peptidoglycan-associated lipoprotein, OprL. A double *oxyR oprL* mutant was constructed and was found to be more sensitive to H₂O₂ than the *oxyR* mutant. Provision of the OxyR-regulated alkyl hydroperoxide reductase, AhpCF, but not AhpB or the catalase, KatB, helped protect this strain against H₂O₂. Given the sensitivity of *oxyR oprL* bacteria to planktonic H₂O₂, we next tested the hypothesis that the biofilm mode of growth might protect such organisms from H₂O₂-mediated killing. Surprisingly, biofilm-grown *oxyR oprL* mutants, which (in contrast to planktonic cells) possessed no differences in catalase activity compared to the *oxyR* mutant, were sensitive to killing by as little as 0.5 mM H₂O₂. Transmission electron microscopy studies revealed that the integrity of both cytoplasmic and outer membranes of *oxyR* and *oxyR oprL* mutants were compromised. These studies suggest that sensitivity to the important physiological oxidant H₂O₂ in the exquisitely sensitive *oxyR* mutant bacteria is based not only upon the presence and location of OxyR-controlled antioxidant enzymes such as AhpCF but also on structural reinforcement by the peptidoglycan-associated lipoprotein OprL, especially during growth in biofilms.

Pseudomonas aeruginosa is an important opportunistic pathogen and a leading cause of global nosocomial infections (46). In human disease, the organism is most frequently encountered in patients whose immune system has been compromised, including individuals suffering from burns (30), cancer chemotherapy (55), organ transplantation (16), and various pneumonias (34). However, the organism receives its greatest notoriety because it is the predominant pathogen during airway infection of patients afflicted with the inherited disease, cystic fibrosis (CF) (3, 23). Within the thick CF airway mucus, *P. aeruginosa* forms highly refractory communities known as biofilms. In this remarkably dynamic niche, the bacteria are tightly packed against one another and encased in oblong spheroid-shaped biofilms composed of both human and bacterial products (63). Arguably, the biofilm mode of growth is one that inherently resists killing by conventional antibiotics

regimens and professional phagocytes that include alveolar macrophages. However, during CF airway disease, it is the neutrophil whose titers rise nearly 1,500-fold in the chronically infected airways (5).

During infection, *P. aeruginosa* faces potential death when the organism encounters stimulated neutrophils that have undergone what is commonly referred to as the oxidative (or respiratory) burst. One toxic product of the respiratory burst is hydrogen peroxide (H₂O₂) (27), which is generated through protonation of O₂[−] in the acidic milieu of the phagolysosomal vacuole. Within this vacuole, H₂O₂ concentrations have been estimated to be as high as 100 mM (32), a level that easily causes the death of both planktonic and biofilm *P. aeruginosa* (7, 26, 28, 38). In fact, stimulated neutrophils release even significant H₂O₂ (~12 μM) in the extracellular milieu (59). Despite these oxidative perils, *P. aeruginosa* is remarkably equipped with a powerful battery of either constitutive or inducible enzymatic defenses to help collaboratively detoxify H₂O₂ and/or organic peroxides. These include three catalases (KatA, KatB, and KatC) (7, 38, 42), and at least four alkyl hydroperoxide reductases, AhpA, AhpB, and AhpCF (45), and Ohr (organic hydroperoxide reductase, (43)). Thus, given these advantageous defenses, the organism is inherently able to respond quickly to sudden changes in H₂O₂ levels, especially in

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the context of extracellular versus intraphagosomal concentrations, to avoid death. However, the most robust genetic response to H₂O₂ in *P. aeruginosa* is governed by the global transactivator OxyR (25, 45).

Activation of OxyR in *Escherichia coli* is initiated upon exposure to low levels of endogenous or exogenous H₂O₂. The transcriptionally dormant OxyR becomes an active transactivator when two sulfhydryl groups on cysteines 199 and 208 are oxidized in an H₂O₂-dependent fashion. This event allows OxyR and RNA polymerase (61) to transcribe genes whose products are specifically designed to cope with H₂O₂-mediated stress (see the recent review by Hassett and Imlay [27]). The three major OxyR-regulated antioxidant enzymes include the tetrameric catalase KatB, the alkylhydroperoxide reductase AhpCF, and periplasmic AhpB (25, 45). However, bacteria lacking OxyR exhibit truly remarkable aerobic phenotypes. First, *oxyR* mutant bacteria are unable to form isolated colonies on rich media (e.g., Luria-Bertani agar [L-agar] plates) (25). Second, when 5 μ l of overnight aerobic cultures was serially diluted and spotted onto L-agar plates, survivors grow from only undiluted cultures (25). An even more striking defect is highlighted when a *katA* mutation is introduced into an *oxyR* mutant background. This strain was unable to grow even from undiluted cultures on aerobic L agar (25). The mechanistic basis for this exquisite sensitivity is that L broth is known to contain autooxidizable components that are capable of generating $\sim 1.2 \mu\text{M}$ H₂O₂ per min (25), an amount that is sufficient to kill $\leq 10^7$ *oxyR* mutant bacteria per ml. Finally, we also showed that *oxyR* mutant bacteria have impaired virulence properties in mouse and fruit fly (*Drosophila melanogaster*) infection models and show increased susceptibility to killing by human neutrophils (33).

In the present study, we examined the mechanistic basis underlying the limited resistance of a *P. aeruginosa oxyR* mutant to H₂O₂ under aerobic conditions. Using both proteomic and genetic approaches, we discovered that the peptidoglycan-associated lipoprotein OprL is involved in what modest protection is afforded an *oxyR* mutant when exposed to exogenous H₂O₂. Transmission electron microscopy (TEM) studies suggest that it is likely that the altered membrane integrity of the *oxyR oprL* double mutant compromises H₂O₂-sensitive respiratory membrane components and, surprisingly, the cytoplasmic AhpCF provides some protection when provided in *trans*.

MATERIALS AND METHODS

Bacterial strains, plasmids and planktonic growth conditions. All bacteria and plasmids used in the present study are listed in Table 1. Organisms were grown in either Luria-Bertani broth (L broth), L broth containing 100 mM KNO₃, or 1% Trypticase soy broth (TSB). Cultures were grown at 37°C with shaking at 300 rpm. Culture volumes were 1/10 of the total Erlenmeyer flask volume to ensure maximum aeration. Media were solidified with 1.5% Bacto agar. Frozen bacterial stocks were stored at -80°C in a 1:1 mixture of 30% glycerol and stationary-phase bacterial suspension.

Manipulation of recombinant DNA and genetic techniques. All plasmid and chromosomal nucleic acid manipulations were by standard techniques (50). Plasmid DNA was transformed into *E. coli* strain DH5 α -MCR (Protein Express, Cincinnati, OH). To detect the presence of insert DNA, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 40 $\mu\text{g}/\text{ml}$) was added to agar media. Restriction endonucleases, the Klenow fragment of DNA polymerase I, T4 DNA polymerase, and T4 DNA ligase were used as specified by the vendor (Invitrogen/Gibco-BRL Corp., Gaithersburg, MD). Plasmid DNA was isolated by using plasmid miniprep isolation kits (Qiagen), and restriction fragments were recov-

ered from agarose gels by using SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, ME). PCRs were performed by using *Pfu* DNA polymerase (BRL) and appropriate primers in an MJ Research thermal cycler, with 30 cycles of denaturation (2 min, 94°C), annealing (1 min, 54°C), and extension (1 min 30 s, 72°C). Amplified DNA fragments were gel purified, cloned into pCR2.1 (Invitrogen), and sequenced.

Construction of *P. aeruginosa* mutants. The strategy for insertional inactivation of some of the genes listed in Table 1 was facilitated by gene disruption with either an 850-bp gentamicin resistance (Gm^r) cassette from pUCGM (52), and the gene replacement vector pEX100T (29), the latter of which allowed for selection of double-crossover events within putative recombinants cultured on agar containing 5% sucrose. All mutants were confirmed by PCR.

Semiquantitative expression of *oprL* by RT-PCR. Total RNA from stationary- and exponential-phase PAO1 and *oxyR* mutant bacteria was purified by using a RiboPure-Bacteria kit as specified by the manufacturer (Ambion). After treatment with DNase, 250 ng of total RNA was used to amplify the *oprL* and *omlA*, the latter of which is a constitutively expressed gene and internal control (44) by using one-step reverse transcription-PCR (RT-PCR) kit (Qiagen). Both RT-PCR analyses of *oprL* and *omlA* were performed using 25 PCR cycles. PCR products were separated by electrophoresis on 1.2% agarose gels. Band intensities were determined by using AlphaEase FC StandAlone software (Alpha Innotech).

H₂O₂ sensitivity measurements. Bacteria were grown aerobically in L broth for 17 h at 37°C. Cultures were diluted in 5 ml of 0.8% low-melting-point agarose (SeaPlaque) in an L-broth base to a final optical density at 600 nm of 0.01. Suspensions were distributed evenly on L agar plates, and the top agarose was allowed to solidify. Filter paper disks (7 mm) containing 10 μ l of either 4.4 M H₂O₂ or 1 M *t*-butyl hydroperoxide (*t*-BOOH) were placed on the top agar surface, and the plates were incubated at 37°C for 24 h. The zones of growth inhibition were measured from at least three experiments in triplicate. Using yet another measure of quantitative H₂O₂ susceptibility, the same titers of 17-h-old suspensions were treated with 5 mM H₂O₂ for 0, 20, 30, and 40 min, respectively. Serial dilutions were spotted on L agar plates containing filter-sterilized bovine liver catalase (Boehringer Mannheim), which allows the *oxyR* mutant to survive the $\sim 1.2 \mu\text{M}$ H₂O₂ produced in aerobic L agar plates by autooxidation (25). Surviving bacteria were enumerated after a 24-h incubation at 37°C.

Catalase assays. Cell extracts prepared from sonicated stationary-phase bacteria were prepared in 50 mM potassium phosphate buffer (pH 7.0; KP_i). Catalase activity was measured by the decomposition of 19.5 mM H₂O₂ in KP_i at 240 nm (7, 38). One unit of activity is defined as that which decomposes 1 μmol of H₂O₂ min⁻¹ mg of protein⁻¹. Protein concentrations were estimated by the method of Bradford (4) using bovine serum albumin fraction V (Sigma) as a standard. Where applicable, statistics were performed by using a Student *t* test, with all assays being performed in triplicate.

Detection of protein carbonyl formation using DNPH derivatization and two-dimensional (2-D) gel electrophoresis after H₂O₂ exposure. The oxidation of specific amino acids is considered an appropriate indicator of the level of oxidative stress mediated by H₂O₂ and other oxidants in aerobic cells. Upon exposure to such oxidants, carbonyl groups in specific proteins are formed which can react with 2,4-dinitrophenylhydrazine (DNPH). An antibody specific for DNP can then bind to the adduct, and protein carbonyl formation can be quantified. First, the *P. aeruginosa oxyR* mutant was grown aerobically overnight in L broth and then diluted 1:100 in fresh medium. Bacteria were then grown to mid-logarithmic phase and treated with 10 mM H₂O₂ for 30 min. The organisms were harvested by centrifugation at $13,000 \times g$ for 10 min at 4°C and subsequently washed twice in 10 mM Tris-HCl (pH 7.8). Cells were then disrupted with lysis buffer (9.5 M urea, 2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.8% ampholytes [Amersham], 1 mM EDTA, 50 mM dithiothreitol [DTT]) at room temperature for 30 min. Immobiline Drystrips (Amersham) were used for isoelectric focusing of 80 μg of cell extract in the first dimension using an IPGphor isoelectric focusing system (Pharmacia Biotech). The strips were then immediately subjected to "in-strip" DNPH derivatization as described previously (10, 13, 47). Briefly, the strips were incubated for 20 min in 2 N HCl–10 mM DNPH at room temperature, followed by equilibration in 50 mM Tris-HCl containing 6 M urea, 2% (wt/vol) sodium dodecyl sulfate (SDS), 30% (vol/vol) glycerol, and 1% DTT (pH 6.8) for 15 min. The strips were then reequilibrated in the same buffer containing 2.5% iodoacetamide, thereby replacing the DTT. Strip proteins were then separated in the second dimension by SDS–12% polyacrylamide gel electrophoresis using a vertical gel electrophoresis unit (SE 400; Hoefer). After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride membranes (Hybond-C; Amersham) and the carbonylated proteins were detected by using an OxyBlot kit (Chemicon International, Germany). Briefly, polyvinylidene difluoride membranes were incubated with (i)

TABLE 1. Strains and plasmids in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
<i>P. aeruginosa</i> PAO1	Wild type	31
<i>oxyR</i> UM	<i>oxyR</i> unmarked deletion mutant	25
<i>oxyR</i> UM <i>attB</i> - <i>oxyR</i>	<i>oxyR</i> unmarked deletion mutant containing <i>oxyR</i> at <i>attB</i> site	This study
<i>oxyR</i> UM PA3716::Gm	Gm ^r ; <i>oxyR</i> UM, PA3716::Gm	This study
<i>oxyR</i> UM PA1011::Gm	Gm ^r ; <i>oxyR</i> UM, PA1011::Gm	This study
<i>oxyR</i> UM <i>oprF</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>oprF</i> ::Gm	This study
<i>oxyR</i> UM <i>fkpP</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>fkpP</i> ::Gm	This study
<i>oxyR</i> UM <i>oprL</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>oprL</i> ::Gm	This study
<i>oxyR</i> UM PA0700::Gm	Gm ^r ; <i>oxyR</i> UM, PA0700::Gm	This study
<i>oxyR</i> UM <i>oprD</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>oprD</i> ::Gm	This study
<i>oxyR</i> UM <i>tpsA</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>tpsA</i> ::Gm	This study
<i>oxyR</i> UM <i>aguA</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>aguA</i> ::Gm	This study
<i>oxyR</i> UM PA4632::Gm	Gm ^r ; <i>oxyR</i> UM, PA4632::Gm	This study
<i>oxyR</i> UM <i>sdhB</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>sdhB</i> ::Gm	This study
<i>oxyR</i> UM <i>tpx</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>tpx</i> ::Gm	This study
<i>oxyR</i> UM <i>dnaK</i> ::Gm	Gm ^r ; <i>oxyR</i> UM, <i>dnaK</i> ::Gm	This study
<i>E. coli</i> DH5α-MCR	<i>mcrA</i> (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80Δ <i>lacZ</i> Δ <i>M15</i> (<i>lacZYA</i> - <i>argF</i>) <i>U169 deoR</i> <i>recA1 endA1 phoA supE44 thi-1 gyrA96 relA1</i>	Protein Express, Inc.
Plasmids		
pEX100T	Ap ^r ; <i>oriT</i> <i>mob</i> <i>sacB</i> gene replacement vector	53
pEX100T PA3716::Gm	pEX100T carrying a PA3716::Gm gene replacement construct	This study
pEX100T PA1011::Gm	pEX100T carrying a PA1011::Gm gene replacement construct	This study
pEX100T <i>oprF</i> ::Gm	pEX100T carrying a <i>oprF</i> ::Gm gene replacement construct	This study
pEX100T <i>fkpP</i> ::Gm	pEX100T carrying a <i>fkpP</i> ::Gm gene replacement construct	This study
pEX100T <i>oprL</i> ::Gm	pEX100T carrying a <i>oprL</i> ::Gm gene replacement construct	This study
pEX100T PA0700::Gm	pEX100T carrying a PA0700::Gm gene replacement construct	This study
pEX100T <i>oprD</i> ::Gm	pEX100T carrying a <i>oprD</i> ::Gm gene replacement construct	This study
pEX100T <i>rspA</i> ::Gm	pEX100T carrying a <i>rspA</i> ::Gm gene replacement construct	This study
pEX100T <i>aguA</i> ::Gm	pEX100T carrying a <i>aguA</i> ::Gm gene replacement construct	This study
pEX100T PA4632::Gm	pEX100T carrying a PA4632::Gm gene replacement construct	This study
pEX100T <i>sdhB</i> ::Gm	pEX100T carrying a <i>sdhB</i> ::Gm gene replacement construct	This study
pEX100T <i>tpx</i> ::Gm	pEX100T carrying a <i>tpx</i> ::Gm gene replacement construct	This study
pEX100T <i>dnaK</i> ::Gm	pEX100T carrying a <i>dnaK</i> ::Gm gene replacement construct	This study
pUCP22- <i>ahpB</i>	pUCP22 with <i>ahpB</i>	45
pCUP22- <i>ahpCF</i>	pUCP22 with <i>ahpCF</i>	45
pUCP22- <i>katB</i>	pUCP22 with <i>katB</i>	45

primary antibody (rabbit immunoglobulin G, 1:150 dilution) specific to the DNP moiety on the proteins, (ii) secondary antibody, and finally (iii) goat anti-rabbit immunoglobulin G (horseradish peroxidase conjugated, 1:300 dilution). Detection of oxidized proteins was assessed by chemiluminescence (RPN2109; Amersham). The membranes were exposed to Hybond-ECL film (Amersham) for either 3 s, 10 min, or 24 h, and the film was developed. Quantification of protein spots was performed by using ImageJ for the Macintosh (<http://www.versiontracker.com/dyn/moreinfo/macosx/19224>).

Protein identification by MALDI-TOF mass spectrometric protein analyses. Protein spots were excised from AgNO₃-stained polyacrylamide gels (54). Prior to the “in-gel” trypsin digestion step, the AgNO₃ was removed from protein spots by using H₂O₂ and ammonium bicarbonate as previously described (57). Next, selected proteins were digested with 125 ng of trypsin (Promega)/μl for 16 to 20 h, and the peptides obtained were analyzed by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry. The proteins were identified by searching a *P. aeruginosa* mass spectrometric database (Swiss-Prot). Briefly, the generated mass lists composed of monoisotopic [M+H]⁺ masses were first filtered for common contaminants (e.g., keratin) and subsequently used for database searches by using the ProFound search algorithm (64).

Flow-cell biofilm experiments. Biofilms were grown in confocal “friendly” flow chambers as previously described (36). Briefly, bacteria were grown aerobically in L broth at 37°C until the stationary growth phase, diluted 1:50 into 1% TSB, and a 0.2-ml suspension was used to inoculate flow cells (Stovall Life Sciences, Inc., Greensboro, NC). Flow was initiated at a rate of 0.17 ml min⁻¹ after the bacteria were allowed to attach for 1 h. After a 3-day incubation at room temperature (22 to 23°C), biofilms were treated with various concentrations of H₂O₂ for 30 min.

The biofilms were then stained with a live/dead viability stain composed of SYTO 9 and propidium iodine (Molecular Probes, Inc., Eugene, OR). Biofilm images were obtained by using an LSM 510 confocal microscope (Carl Zeiss, Inc., Germany). The excitation and emission wavelengths for green fluorescence were 488 and 500 nm, respectively, while those for red fluorescence were 490 and 635 nm, respectively. All biofilm experiments were repeated at least three times. The live/dead ratios of the biofilms were calculated by using 3D for LSM (v.1.4.2) software (Carl Zeiss).

TEM. All bacteria were grown in L-broth until the stationary growth phase, clarified by centrifugation at 13,000 × *g* for 10 min, and 0.5 ml suspensions treated with 50 mM H₂O₂ in fresh L-broth for 30 min. After a second centrifugation step, cell pellets were fixed with 2.5% glutaraldehyde and 4% formaldehyde (vol/vol) in 0.1 M cacodylate buffer, postfixed with OsO₄ in the presence of 2% (wt/vol) potassium ferrocyanide, and embedded in Eponate 12. Thin sections were poststained with (2%) uranyl acetate and lead citrate and examined with a Zeiss transmission electron microscope at an accelerating voltage of 80 kV. The TEM micrographs were taken at two different magnifications, ×40,000 and ×100,000, to indicate the overall cellular size and possible membrane integrity alterations.

RESULTS

Remarkable sensitivity of *oxyR* mutant bacteria to merely aerobic growth on rich media and protection by anaerobic growth. We have previously shown that *oxyR* mutant bacteria

FIG. 1. Sensitivity of *oxyR* mutant bacteria to serial dilution on aerobic but not anaerobic media. Bacteria were grown aerobically in L-broth until stationary phase. Organisms were serially diluted in either L broth or sterile-filtered spent culture supernatant, and 10- μ l aliquots were spotted onto L agar (+O₂ [A]) or L agar containing 100 mM KNO₃ (-O₂ [B]), the latter of which was incubated at 37° in a Coy anaerobic chamber for 48 h. Lane 1, PAO1; lane 2, *oxyR*-UM; lane 3, *oxyR* complemented strain; lane 4, *oxyR* diluted in its own sterile-filtered supernatant; lane 5, *oxyR katA* double mutant. UN, undiluted bacterial suspension.

are exquisitely susceptible to micromolar levels of H₂O₂ based upon the fascinating observation that L broth generates ~1.2 μ M H₂O₂/min through autoxidation of medium components (25). For example, note that when serial dilutions of stationary-phase *oxyR* mutant organisms are spotted onto L agar plates under aerobic conditions (+O₂), only the undiluted (UN) sample survives (Fig. 1A, lane 2), while wild-type organisms show the characteristic serial dilution pattern of cells that harbor the full OxyR-controlled antioxidant enzymatic repertoire, including KatB-AnkB (PA4613-PA4612), AhpB (PA0848), and AhpCF (PA0139-PA0140) (Fig. 1A, lane 1). Complementation in *cis* in the unique *attB* site on the *P. aeruginosa* chromosome with the wild-type *oxyR* gene restored the serial dilution capacity and growth on aerobic L agar of wild-type bacteria (Fig.

1A, lane 3). Similarly, serial dilution of the *oxyR* mutant in the filter-sterilized supernatant from stationary-phase wild-type bacteria, which contains the major, protease-resistant catalase KatA from lysed cells (25), also showed a protective pattern (Fig. 1A, lane 4). By far the most H₂O₂-sensitive strain was an *oxyR katA* double mutant, where organisms could not even survive spotting of undiluted cells onto aerobic L agar (Fig. 1A, lane 5). Interestingly, when the same serial dilutions were plated under anaerobic conditions where there is no possibility of medium H₂O₂ generation, the bacteria grew as wild-type organisms with negligible differences (Fig. 1B, -O₂).

Because of the remarkable aerobic sensitivity of the *P. aeruginosa oxyR* mutant to H₂O₂, our next goal was to identify proteins that protect the aerobic *oxyR* mutant from H₂O₂. We were particularly interested in proteins that are not involved in what might be predicted to be a classical antioxidant enzymatic response to oxidative stress (e.g., OxyR regulon) that has been demonstrated in the most heavily researched bacteria that include *E. coli* (65), *Salmonella enterica* serovar Typhimurium (19), and *Xanthomonas campestris* (9).

Determination of protein carbonylation by DNP derivatization of H₂O₂-treated *oxyR* mutant bacteria. Our next goal was to evaluate the role of proteins in the *oxyR* mutant that serve to provide limited protection to this extremely sensitive organism when faced with H₂O₂. Realizing that the oxidation of specific amino acids is considered an appropriate indicator of the level of oxidative stress mediated by H₂O₂ and other oxidants in aerobic cells, we embarked on the determination of carbonyl groups in proteins that can be formed upon oxidation by H₂O₂ that can react with DNPH. By using an antibody specific for DNP, protein carbonyl formation can be quantified. For example, using this technique, glyceraldehyde-3-phosphate dehydrogenases (both Tdh2p and Tdh3p) were found to play a role in defense against H₂O₂ in *Saccharomyces cerevisiae* (14).

Because wild-type bacteria have their full antioxidant enzymatic gamut (e.g., isozymes of catalase, peroxidase, Ahp, and superoxide dismutase [SOD]), we first discovered that the difference in carbonylated proteins between H₂O₂-treated and control organisms were only a few spots. Of these, we found that three hypothetical proteins (spots 1, 2, and 6) and also *oprD* and *aguA* were more susceptible to protein carbonylation in wild-type bacteria treated with H₂O₂ than in untreated bacteria (data not shown). In contrast, the *oxyR* mutant lacks (i) the H₂O₂-inducible catalase KatB, (ii) the cytoplasmic AhpCF, and (iii) the periplasmic AhpB. Thus, it is not surprising that this organism is severely impaired in its ability to cope with even micromolar levels of H₂O₂ (25, 45). Therefore, the *oxyR* mutant strain was used in the following experiments. First, the organism was exposed to H₂O₂ at a concentration that would minimally affect wild-type bacteria (10 mM H₂O₂, 30 min) to determine carbonylated proteins in H₂O₂-treated versus untreated bacteria using a coupled 2-D gel/MALDI-TOF mass spectrometric approach. Figure 2A depicts a silver-stained 2-D gel of untreated *oxyR* mutant bacteria, while Fig. 2C shows a similar gel of protein lysates derived from organisms treated with 10 mM H₂O₂. Figure 2B and D are representative Western blots of Fig. 2A and C obtained by using the Oxyblot technique (see Materials and Methods). Note the differences in the overall amount and intensity of spots 8 and 9, respec-

FIG. 2. Protein oxidation profile of *P. aeruginosa oxyR* mutant bacteria exposed to H_2O_2 . (A to D) $AgNO_3$ -stained 2-D gels of control (A) and H_2O_2 -treated (10 mM for 30 min) (C) bacteria; Western blot and DNP staining of control (B) and H_2O_2 -treated (10 mM for 30 min) (D) bacteria. The numbers represent the spots that have a changing signal after immunodetection of DNP-derivatized carbonylated proteins. The bracketed asterisk shown in Fig. 3D indicates a protein spot that cannot be identified by MALDI-TOF mass spectrometry. (E and F) Expanded sections and longer exposure times of immunodetection of DNP-derivatized carbonylated proteins which show clear overexpression of protein spots 8 and 9, respectively.

tively (also refer to Fig. 2E and F). Upon observation of distinct differences in oxidized protein profiles between control and H_2O_2 -treated *oxyR* bacteria (Fig. 2B and D), we next used MALDI-TOF mass spectrometry to identify proteins with increased oxidation patterns. Table 2 lists the 13 proteins that were identified in the present study, including the PA number, function (if known), the molecular mass and *pI* of each protein, and several mass spectrometric parameters. Several of these were the outer membrane porins OprF and OprD and the peptidoglycan-associated lipoprotein OprL. Others included four hypothetical proteins, a probable peptidyl-prolyl *cis-trans* isomerase (FkbP-type), 30S ribosomal protein S1, arginine deiminase, succinate dehydrogenase (SDH; β -subunit), a thiol

peroxidase, and DnaK. Unfortunately, the identification of many of the proteins that were clearly different in DNPH reactivity between *oxyR* control and H_2O_2 -treated organisms were not represented in sufficient quantity or quality for identification, even after overloading the gels.

The peptidoglycan-associated lipoprotein OprL is essential for the limited H_2O_2 resistance of an *oxyR* mutant. First, isogenic mutants were constructed in each of the 13 candidate genes encoding the oxidized proteins detected in the H_2O_2 -treated *oxyR* mutant (Fig. 2B and D) to assess their possible contribution to H_2O_2 resistance or susceptibility. There were no differences in the H_2O_2 sensitivity of each of these mutants compared to wild-type bacteria (data not shown). Therefore,

TABLE 2. Proteins in H₂O₂-treated *P. aeruginosa oxyR* mutant that were reproducibly oxidized after DNP derivatization and identified by MALDI-TOF mass spectrometric analysis^a

Spot no.	Gene	Protein	PA no.	Molecular mass (kDa)/pI	MOWSE score	Masses matched
1		Hypothetical protein	PA3716	60.504/4.4	1.15E + 08	18 (26)
2		Hypothetical protein	PA1011	43.056/4.9	1.72E + 10	19 (50)
3	<i>oprF</i>	Outer membrane protein OprF precursor	PA1777	37.640/5.0	3.67E + 05	10 (32)
4	<i>fkpP</i>	Probable peptidyl-prolyl <i>cis-trans</i> isomerase, FkpP-type	PA3262	26.846/5.1	14882	9 (47)
5	<i>oprL</i>	Outer membrane protein OprL precursor	PA0973	17.925/5.9	1.84E + 04	6 (8)
6		Hypothetical protein	PA0700	31.717/8.9	474	4 (10)
7	<i>oprD</i>	Outer membrane porin protein OprD precursor	PA0958	48.360/5.0	4.26E + 04	11 (23)
8	<i>rpsA</i>	30S ribosomal protein S1	PA3162	61.870/4.8	1.25E + 08	17 (26)
9	<i>aguA</i>	Agmatine deiminase	PA0292	41.191/4.8	251	5 (22)
10		Hypothetical protein	PA4632	29.159/7.0	22385	7 (36)
11	<i>sdhB</i>	Succinate dehydrogenase (β-subunit)	PA1584	25.155/6.6	2.81E + 04	9 (12)
12	<i>tpx</i>	Thiol peroxidase	PA2532	17.234/5.2	2117	4 (7)
13	<i>dnaK</i>	DnaK protein	PA4716	68.404/4.8	3.20E + 06	15 (53)

^a The identified proteins are listed with their PA numbers, gene name (if known), pI value, MOWSE (molecular weight search) score, and molecular mass.

we next constructed 13 isogenic double mutants in an unmarked *oxyR* mutant background. Surprisingly, we found that only the *oxyR oprL* double mutant was more sensitive to H₂O₂ than the *oxyR* mutant (Fig. 3A, lane 5 versus lane 2). Representative L agar plates are included on the top of Fig. 3A and B to reveal a visual representation of these data. In contrast, the *oxyR oprL* mutant with AhpB (lane 7 versus plasmid control [lane 6]) or KatB (lane 9 versus plasmid control [lane 6]) provided in *trans* could not contribute to protection of the *oxyR oprL* mutant in this assay system. Only provision of AhpCF (lane 8) afforded some protection of the *oxyR oprL* mutant against H₂O₂-mediated killing. In contrast, the *oxyR oprL* mutant was no more sensitive to *t*-butyl hydroperoxide (Fig. 3B, lane 5 versus lane 2) or cumene hydroperoxide (data not shown) than the single *oxyR* mutant. This indicates that OprL

seems to confer a level of resistance that is specific for only H₂O₂ and not for organic peroxides.

To clarify the H₂O₂ sensitivity differences in *oxyR* versus *oxyR oprL* mutant bacteria, we also examined survival profiles upon exposure of these bacteria to 5 mM H₂O₂ for 40 min in planktonic culture, except that we used stationary-phase planktonic cultures that have ~10⁹ CFU/ml. At these high cell titers, the *oxyR* mutant shows an H₂O₂ sensitivity profile identical to that of wild-type bacteria (Fig. 3C). Interestingly, the single *oprL* and the double *oxyR oprL* mutants showed increased sensitivity to H₂O₂. In fact, the *oxyR oprL* double mutant was nearly 2 logs more sensitive to H₂O₂ than the *oxyR* mutant. This reinforces the premise that the *oxyR* mutant alone at high cell densities on planktonic culture has enhanced resistance to H₂O₂.

FIG. 3. Sensitivity of *P. aeruginosa* compared to mutants strains to H₂O₂ or *t*-BOOH. All bacteria were grown aerobically in L broth until achieving stationary growth phase, and identical CFU were added to 0.8% molten top-agarose and poured onto L agar plates. Filter paper disks (7 mm) were impregnated with either 4.4 M H₂O₂ (A) or 1 M *t*-BOOH (B) and placed uniformly on the top-agarose. All cells were incubated aerobically at 37°C for 24 h prior to measuring the zones of growth inhibition. The results are from three independent experiments and expressed as the means ± the standard errors. Lane 1, wild type; lane 2, *oxyR*; lane 3, *oxyR-poxyR*; lane 4, *oprL*; lane 5, *oxyR oprL*; lane 6, *oxyR oprL* + pUCP19; lane 7, *oxyR oprL* + *pahpB*; lane 8, *oxyR oprL* + *pahpCF*; lane 9, *oxyR oprL* + *pkatB*. The bracketed asterisk (*) above panel A (lane 5 versus lane 2) indicates a significant difference between the kill zones ($P < 0.001$ at 95% confidence). (C) Determination of sensitivity to H₂O₂ in broth culture. The conditions of these experiments and calculation from three independent experiments are described in Materials and Methods. Symbols: PAO1 (●), *oprL* (○), *oxyR* (■), *oxyR oprL* (▽), *oxyR-poxyR* (▼).

FIG. 4. Confocal laser scanning microscopic images of H_2O_2 treated wild-type *P. aeruginosa* and *oxyR* mutant biofilms. Biofilms were grown on glass substrata for 3 days, and the wild-type and *oxyR* mutant were treated with H_2O_2 at 0, 30, 50, 70, and 100 mM. The biofilms were then stained with a viability stain as described in Materials and Methods, and live versus dead bacteria were observed by confocal laser scanning microscopy. The panels labeled “A” are top/bottom images; the panels labeled “B” are sagittal images.

Increased sensitivity of *oxyR oprL* versus *oxyR* mutants to H_2O_2 in biofilms. We have previously shown that *P. aeruginosa* biofilms are inherently resistant to concentrations of H_2O_2 that easily kill planktonic bacteria (26, 28). In particular, estimates range from 10 to 75 mM, depending on the growth and treatment parameters (7, 28, 45). Despite the sensitivity of the *oxyR oprL* mutant to H_2O_2 in free-living, planktonic culture, we tested the hypothesis that the biofilm mode of growth might still afford this severely compromised organism resistance to H_2O_2 -mediated killing. This could be due to the formation of a mature biofilm and the deposition of matrix-associated factors (e.g., the protease-resistant catalase KatA [25]) that could detoxify it, thereby protecting susceptible organisms in the immediate surroundings. Mature biofilms were grown for a period of 3 days, after which they were exposed to different concentrations of H_2O_2 . First, we exposed the wild type and the *oprL* mutant to H_2O_2 when cultured in biofilms. Mature, 3-day-old biofilms were then stained with a viability stain, where green cells are alive and red cells are dead (see Fig. S1 in the supplemental material, top view [A] and sagittal view [B]). In slight contrast to the results with planktonic cells, *oprL* mutant biofilms were no different in overall architecture or sensitivity to H_2O_2 compared to wild-type bacteria (see Fig. S1 in the supplemental material). Note that the biofilm mode of growth protected wild-type and *oprL* mutant bacteria from H_2O_2 concentrations of ~ 70 mM, a finding consistent with previous results (28).

We next performed similar experiments with wild-type and *oxyR* mutant bacteria using our flow biofilm system. We first observed that *oxyR* mutant biofilms were not nearly as thick and robust as those formed by wild-type organisms (Fig. 4, lower panel). Recall that the *oxyR* mutant cannot grow as single colonies on aerobic L agar plates because of ~ 1.2 mM H_2O_2 being generated in the media through autoxidation of

FIG. 5. Confocal laser scanning microscopic images of H_2O_2 -treated *oxyR* and *oxyR oprL* mutant biofilms. Biofilms were grown on glass substrata for 3 days as in Fig. 4. *oxyR* and *oxyR oprL* mutant biofilms were treated with 0.05, 0.5, and 5 mM H_2O_2 . The biofilms were then stained with a viability stain as described in Materials and Methods, and live versus dead bacteria were observed by confocal laser scanning microscopy. The panels labeled “A” are top/bottom images; the panels labeled “B” are sagittal images.

components in it (25). The medium used for our biofilm studies, TSB, also does not support the aerobic growth of *oxyR* mutant bacteria as isolated colonies (data not shown), which is similar to the results obtained on L agar (Fig. 1A, lane 2). Therefore, we believe that the *oxyR* mutant biofilm was not nearly as robust and differentiated as wild-type bacteria because of the low, yet significant peroxigenic stress in our flow biofilm system. To test this hypothesis experimentally, we performed flow biofilm analyses of the wild type and *oxyR* mutant in the presence or absence of added bovine liver catalase. Consistent with the dramatic H_2O_2 sensitivity of *oxyR* mutant bacteria, the addition of bovine liver catalase to the growth medium allowed for complete restoration of mature biofilm formation by the *oxyR* mutant (see Fig. S2 in the supplemental material).

Unlike wild-type and *oprL* mutant bacteria, the *oxyR* mutant was easily killed by 30 mM H_2O_2 (Fig. 4, lower panel) relative to wild-type organisms (Fig. 4, top panel). In addition, H_2O_2 caused a mechanical disruption of the biofilms because of the vigorous bubbling due to inherently high catalase activity in the biofilms (18). This is the reason behind the “web”-like biofilm structures observed in both wild-type and *oxyR* mutant biofilms treated with >30 mM H_2O_2 .

We next assessed the H_2O_2 sensitivity of *oxyR* relative to *oxyR oprL* mutants in biofilms using very low concentrations of H_2O_2 to help reveal potential subtle differences in biofilm susceptibility patterns between these two strains. Although the *oxyR* mutant was resistant to 5 mM H_2O_2 (Fig. 5, top panel), as little as 0.5 mM H_2O_2 killed the vast majority of *oxyR oprL* bacteria in biofilms (Fig. 5, bottom panel). Moreover, the dead/live ratios/ mm^3 of the *oxyR oprL* mutant biofilm (light gray bars, Fig. 6) were also higher than the *oxyR* mutant (black bars, Fig. 6) at the same concentration of H_2O_2 , while the *oxyR* dead/live ratio was greater than that of the wild-type strain PAO1 (dark gray bars, Fig. 6).

FIG. 6. Calculation of dead/live ratios of H₂O₂-treated *P. aeruginosa* biofilms. The ratio of live versus dead wild-type (dark gray bars), *oxyR* (black bars), and *oxyR oprL* (light gray bars) biofilm bacteria are calculated from optical sections of confocal images obtained from 3-day-old biofilms grown in 1% TSB as described by Lequette and Greenberg (36). The ratio is representative of 60 z-section slices with a 60- μ m depth. Each stained particle was assigned a value that represents an object with an average size of 1 μ m.

The sensitivity of the *oxyR oprL* versus *oxyR* mutant to H₂O₂ in both planktonic cells and biofilm is not based upon significant differences in catalase activity. Even though the *oxyR oprL* mutant was more sensitive to H₂O₂ than the *oxyR* mutant, we suspected that this could be due to overall differences in total catalase activity. Recall that the *oxyR* mutant possesses only the protective activity of the major catalase, KatA (45). However, in planktonic cells, the *oxyR oprL* mutant was found to have slightly increased catalase activity compared to the *oxyR* mutant (Fig. 7A, lane 5 versus lane 2), yet the double mutant was paradoxically more sensitive to H₂O₂ (recall Fig. 3A and C). However, during biofilm culture, we found no differences in catalase activity between the two mutant strains (Fig. 7C). Moreover, the catalase activity of both strains during anaerobic culture was also similar (Fig. 7B, lanes 5 versus lane 2). However, the catalase activity of all bacteria was inexplica-

bly higher (~5-fold) during anaerobic growth (Fig. 7B and A), and yet the *oxyR* and *oxyR oprL* strains possessed nearly 2,000 U less than did the wild-type bacteria (Fig. 7B, lanes 2 and 5 versus lane 1). We offer an explanation for this apparent paradox in the Discussion.

TEM evidence H₂O₂-mediated structural damage in *oxyR* and *oxyR oprL* mutant *P. aeruginosa*. The *oprL* gene was previously shown to be (i) regulated by RegA and iron levels (17), (ii) maximally expressed in the stationary growth phase (17), and (iii) overexpressed during long-term anaerobic pyruvate fermentation (51). It is also essential for normal cell shape in the related organism *P. putida* (49). We next tested the hypothesis that OxyR may play a role in the expression of the *oprL* gene. We based this hypothesis on the fact that the promoter region of the *oprL* gene possessed 10 of 13 of the canonical OxyR promoter recognition sequence (ATAG-N₆-CTAT-N₆-ATAG-N₆-CTAT) (65). To test this postulate experimentally, *oprL* gene expression was measured by RT-PCR. We found no difference in *oprL* gene expression in the wild type versus the *oxyR* mutant in both exponential- and stationary-phase organisms (data not shown), thereby refuting our hypothesis.

Because OprL has been shown to play a role in overall cell envelope structure in the related strain *P. putida* (49), we tested the hypothesis that the sensitivity of the *oxyR oprL* mutant to H₂O₂ compared to the *oxyR* mutant might be because of structural alterations in the membrane(s) between these two strains. To test this hypothesis, we elected to perform an ultrastructural analysis of wild-type, *oxyR* mutant, and *oxyR oprL* mutant bacteria using TEM to determine whether the *oxyR* or *oprL* mutations, either singly or in tandem, affected cell morphology in the presence versus the absence of H₂O₂. We used high levels of H₂O₂ (50 mM) in these studies because the morphology of wild-type organisms appeared to be unaltered when organisms were treated with 10 mM H₂O₂ (data not shown). Untreated bacteria were characterized by intact, smooth,

FIG. 7. Catalase activity of planktonic versus biofilm-grown bacteria. Planktonic bacteria were grown in aerobic L broth (A) or anaerobic L broth with 100 mM KNO₃ (B) at 37°C until the stationary phase. Cell extracts were then assayed for catalase activity in triplicate. (C) Biofilm organisms were grown in 1% TSB for 3 days and assayed for catalase activity. Lane 1, PAO1; lane 2, *oxyR*; lane 3, *oxyR* + *paxyR*; lane 4, *oprL*; lane 5, *oxyR oprL*.

FIG. 8. Ultrastructure of *P. aeruginosa* *oxyR* and *oxyR oprL* control and H_2O_2 -treated bacteria. All strains were grown aerobically in L broth until the stationary growth phase. The bacteria were then treated with 50 mM H_2O_2 for 30 min at room temperature prior to processing for TEM analysis. Electron micrographs of wild-type (A and B) and isogenic *oxyR* (C and D) and *oxyR oprL* (E and F) mutant cells at a magnification of $\times 100,000$ are shown. Adjacent to each cell are high-magnification images with black arrows outlining the overall membrane structure. In panel A, the arrow is directed at the periplasmic space. Panels G to L show the same strains as in panels A to F, but the magnification is $\times 40,000$.

and uninterrupted membranes with a clearly visible periplasmic space (Fig. 8A, arrow). Treatment with H_2O_2 caused a slight swelling of the bacteria, and yet the membranes remained intact (Fig. 8B). Similarly, the untreated *oxyR* mutant also had intact, yet not as clearly defined membranes (Fig. 8C). However, the H_2O_2 -treated *oxyR* mutant possessed an invaginated membrane organization with no clearly defined periplasmic space (Fig. 8D). In contrast, the *oxyR oprL* double mutant also possessed wavy, invaginated membranes, more ghost cells, and probably “peeling” of membranes (Fig. 8E). In contrast, H_2O_2 treatment caused intermittent membrane fusion and the periplasmic space to appear beaded, with numerous invaginations and evidence of membrane-membrane fusion (Fig. 8F).

We also collected pictures of wild-type, *oxyR* mutant, and *oxyR oprL* mutant strains in the absence (Fig. 8G, I, and K) versus the presence (Fig. 8H, J, and L) of H_2O_2 . Note that the majority of H_2O_2 -treated organisms are slightly swollen relative to control bacteria. Photographs of these bacteria were taken at $\times 40,000$ and $\times 100,000$ magnification for statistical purposes that are meant to define (i) differences in cell size and (ii) changes in membrane integrity, respectively. About 70 bacteria photographed at $\times 40,000$ magnification were used to compare the structural nature of the membranes under control and H_2O_2 -treated conditions. An evaluation of untreated PAO1 showed that ca. 77% of cells have an intact, smooth, and uninterrupted membrane with a clearly visible periplasmic

space (Fig. 8A and G). Similarly, nearly 86% of the untreated *oxyR* mutant also had intact membrane (Fig. 8C and I). In contrast, only 42% of the *oxyR oprL* mutant membranes had intact membranes, whereas the remainder possessed wavy, invaginated membranes and clear evidence of peeling (Fig. 8E and K). In contrast, treatment with H_2O_2 caused membrane alterations even in wild-type strain PAO1, for which $\sim 67\%$ of the treated cells have a wavy membrane (Fig. 8B and H). However, we found that 72% of H_2O_2 -treated *oxyR* mutants possessed invaginated membranes with no clearly defined periplasmic space (Fig. 8D and J), whereas 82% of the *oxyR oprL* mutant H_2O_2 revealed intermittent membrane fusion while the periplasmic space appeared beaded, with numerous invaginations and evidence of membrane-membrane fusion events (Fig. 8F and L).

DISCUSSION

Approximately 7 years ago, we demonstrated that an absence of the global H_2O_2 -responsive regulator OxyR in *P. aeruginosa* renders such organisms exquisitely susceptible to micromolar H_2O_2 (25, 45). Given that H_2O_2 is a significant component of the antimicrobial armament of human phagocytic cells (e.g., neutrophils and macrophages), an in-depth analysis of *oxyR* mutant phenotypes became a major research focus. Toward this end, one of the most peculiar and intriguing

phenotypes of an *oxyR* mutant is an inability to form isolated colonies on aerobic (Fig. 1A) but not anaerobic L agar (25) (Fig. 1B). This was found to be due to the fact that diluted organisms cannot cope with the paltry 1.2 μ M H₂O₂ produced per min in aerobic L broth by autooxidation (25), levels that are consistent with those produced by human erythrocytes (22, 60). Proteins in the *oxyR* mutant would predictably be most susceptible to H₂O₂-mediated oxidation at the level of outer/cytoplasmic membranes and periplasm, especially when diluted to $\leq 10^7$ cells per ml, but also affecting the overall metabolic properties of the organisms.

Effects of H₂O₂ on *P. aeruginosa* metabolism. In the face of potentially toxic levels of H₂O₂, bacteria such as *P. aeruginosa* encounter significant metabolic problems. For example, Tamarit et al. (58) showed that in *E. coli* H₂O₂ caused oxidation of the β -subunit of the F₁F_o-ATPase. In doing so, the ability of such organism to generate the vast majority of its cellular ATP is compromised. Furthermore, Farr et al. (20) experimentally reinforced this premise in demonstrating that an H₂O₂-mediated loss in ΔP membrane potential significantly impacts ATP-dependent anabolic activities that include the synthesis of essential cellular components (e.g., DNA, RNA, and protein). However, two of the oxidized proteins of the H₂O₂-treated *oxyR* mutant discovered in the present study were the β -subunit of the tricarboxylic acid cycle enzyme, SDH (SdhB) and DnaK, an Hsp70 molecular chaperone (1). Because the cytoplasmic membrane-bound SDH is involved in the production of reducing power in the form of FADH₂, its electrons could drive the formation of the vastly more reactive oxygen reduction product, HO $\cdot$. HO $\cdot$ reacts with virtually all known biomolecules at rates approaching the diffusion limit (62). SDH also has Fe-S clusters that have been shown to be involved in the production of superoxide (O₂ $^{\cdot -}$) in *E. coli* (39). However, formation of O₂ $^{\cdot -}$ by SDH is likely a result of the formation of a flavosemiquinone intermediate that can react with molecular oxygen (35), forming O₂ $^{\cdot -}$. Thus, in SOD-proficient organisms, of which virtually all *P. aeruginosa* express at least Fe-SOD (6), H₂O₂ would still be formed in aerobic bacteria. SdhB is also known to be Fur-activated in *E. coli* (24) and also inducible by nitric oxide (NO) (48). In contrast, DnaK is known to be involved in the H₂O₂-mediated stress response of *Salmonella enterica* serovar Typhimurium by a mechanism involving chaperone-like activity (41). However, *oxyR sdhB* and *oxyR dnaK* mutants were no more susceptible to H₂O₂ than the *oxyR* mutant.

One of the greatest surprises of this work was the dramatic increase in catalase activity in organisms grown anaerobically via respiratory NO₃ $^-$ reduction (Fig. 7B). We had initially shown increased catalase activity in anaerobically grown *P. aeruginosa* (21). In the present study, we show that the anaerobic induction of optimal anaerobic catalase activity actually requires OxyR (Fig. 7B, lane 2 versus lane 1). Being that multiple prokaryotic and eukaryotic catalases are multifunctional (e.g., catalases/oxidase) during aerobic growth (11, 12), there is no reason to believe that they may not also possess an anaerobic detoxification function of molecules that have a similar molecular structure, redox properties, and electronic state. Catalase has actually been shown to slowly detoxify NO (8). It is possible that the major *P. aeruginosa* KatA also possesses this function since we detected significant spectral

changes upon the exposure of purified KatA to NO gas (data not shown). However, further experimentation is required to precisely define the mechanism of NO binding to the core heme of KatA.

Role of OxyR-controlled gene products in H₂O₂ resistance in *oxyR oprL* mutant bacteria. One of the unique features of the *oxyR* mutant was that it paradoxically possessed near-wild-type catalase levels (Fig. 3) (25) and yet was exquisitely sensitive to H₂O₂ in diluted planktonic and biofilm culture. Thus, as discussed above, it would behoove *P. aeruginosa* to possess the capacity to deploy antioxidant enzymes such as the periplasmic alkyl hydroperoxide reductase/catalase AhpB (45) for protection from H₂O₂. The same paradigm holds true in gram-negative bacteria in the strategic deployment of β -lactamase to the periplasm when confronted with the β -lactam antibiotics. Surprisingly, it was another *P. aeruginosa* OxyR-regulated gene product, AhpCF, that offered some level of protection when provided in *trans* to the *oxyR oprL* mutant (Fig. 3A, lane 8 versus lane 5), and yet neither AhpCF, AhpB, nor KatB could exclusively restore wild-type H₂O₂ resistance levels. However, the overall protection by AhpCF alone was greater than that of AhpB and KatB against H₂O₂, and this is consistent with AhpCF protecting an *oxyR* mutant in the aerobic plate dilution assay (25). Although one of the oxidized proteins in the *oxyR* mutant was a thiol peroxidase known as Tpx, the *oxyR tpx* mutant was a “red herring” in the sense that it, too, was no more sensitive to H₂O₂ than was the *oxyR* mutant. This suggests that the *P. aeruginosa* Ahp enzyme class has a range of substrate specificities that include both H₂O₂ and organic peroxides.

Impact of OprL on biofilm sensitivity to H₂O₂ and associated structural abnormalities. However, three of the oxidized proteins in the *oxyR* mutant were the outer membrane proteins OprF, OprD, and the peptidoglycan-associated lipoprotein OprL. Because two of the aforementioned proteins are surface exposed (OprF and OprD), they are obviously the most readily accessible to H₂O₂, the diffusion rate of which is only limited by enzymes capable of detoxifying it (e.g., catalases, peroxidases, and AHPs) and certain porins of the eukaryotic aquaporin family (2). However, unlike OprF and OprD, OprL is not on the surface of *P. aeruginosa* but is reported to be linked to peptidoglycan, which resides between the cytoplasmic and outer membranes. *P. putida* OprL in *P. putida* has multiple functions, including the uptake of a number of carbon sources (37), as well as resistance to SDS, EDTA, and deoxycholate (49). However, far less is known of its function in *P. aeruginosa*. Interestingly, we found that OprL contributes significantly to the overall biofilm sensitivity of the *oxyR* mutant organism when we discovered a dramatically enhanced sensitivity of the *oxyR oprL* mutant in biofilms. In addition, we also observed greater structural alterations in *oxyR oprL* mutants treated with H₂O₂ relative to the *oxyR* mutant alone (Fig. 8). Finally, consistent with the theme of structural proteins contributing to H₂O₂ resistance in bacteria, *E. coli* porins were found to contribute to resistance to the lactoperoxidase-H₂O₂-SCN $^-$ antimicrobial system (15), while optimal H₂O₂ resistance in serovar Typhimurium requires a 59-kDa outer membrane protein (56).

The other unique feature of cells exposed to H₂O₂ is its capacity to induce swelling due to the oxygen produced by

catalase-mediated decomposition, despite the fact that oxygen diffusion through membranes is through free diffusion. Another classic example of cell swelling in the face of H_2O_2 -generating agents is in the case of paraquat-treated *E. coli* (40). When exposed to redox-active paraquat under aerobic conditions, the optical density of the suspension actually increased, an event that would possibly have led to the postulate that paraquat did not interrupt cell growth or kill *E. coli*. However, it was ultimately found that the bacteria were actually perishing, and the increase in optical density of the suspension was attributable to cell swelling (40).

Summary. In conclusion, we have shown that the limited ability of an *oxyR* mutant of *P. aeruginosa* to survive exogenous exposure to H_2O_2 under aerobic conditions requires not only the support of the putatively bifunctional catalase/Ahp AhpCF but also the structural support of the peptidoglycan-associated lipoprotein OprL. This dynamic is unique in the sense that a classical oxidative stress response (e.g., OxyR) has now been linked to a structural protein, OprL, especially during the biofilm mode of growth. This suggests that translational efforts to combat certain sensitive features of highly recalcitrant pathogens can be multifaceted, attacking transcription factors (e.g., OxyR), organic hydroperoxide reductases (e.g., AhpCF), and membrane or peptidoglycan-associated proteins (e.g., OprL).

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