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...
the context of extracellular versus intraphagosomal concentrations, to avoid death. However, the most robust genetic response to H$_2$O$_2$ 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 H$_2$O$_2$. The transcriptionally dormant OxyR becomes an active transactivator when two sulphhydril groups on cysteines 199 and 208 are oxidized in an H$_2$O$_2$-dependent fashion. This event allows OxyR and RNA polymerase (61) to transcribe genes whose products are specifically designed to cope with H$_2$O$_2$-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 µl 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 autoxidizable components that are capable of generating ~1.2 µM H$_2$O$_2$ per min (25), an amount that is sufficient to kill ~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 H$_2$O$_2$ under aerobic conditions. Using both proteomic and genetic approaches, we discovered that the peptidoglycan-associated lipoprotein OplL is involved in what modest protection is afforded an oxyR mutant when exposed to exogenous H$_2$O$_2$. Transmission electron microscopy (TEM) studies suggest that it is likely that the altered membrane integrity of the oxyR oplL double mutant compromises H$_2$O$_2$-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 KNO$_3$, 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% Bactoagar. Frozen bacterial stocks were stored at −80°C. The 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 µl of either 4.4 M H$_2$O$_2$ (Sigma), M (r-t-butyl hydrogen peroxide) (t-BOOH) were placed on the L-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 H$_2$O$_2$ susceptibility, the same titers of 17-h-old suspensions were treated with 5 mM H$_2$O$_2$ for 0, 20, 30, and 40 min, respectively. Serial dilutions were spotted on agar plates containing filter-sterilized bovine liver catalase (Boehringer Mannheim), which allows the oxyR mutant to survive the ~1.2 µM H$_2$O$_2$ produced in aerobic L-agar plates by autoxidation (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, KPi). Catalase activity was measured by the decomposition of 19.5 mM H$_2$O$_2$ in KPi at 240 nm. Band intensities were determined by using AlphaEase FC Stand Alone software (Alpha Innotech).

**Detection of protein carbonyl formation using DNPH derivatization and two-dimensional (2-D) gel electrophoresis after H$_2$O$_2$ exposure.** The oxidation of specific amino acids is considered an appropriate indicator of the level of oxidative damage mediated by other oxidants or transition metals (13). Exposure to such oxidants, carbonyl groups in specific proteins are formed which can react with 2,4-dinitrophenylhydrazine (DNPH). An antibody specific for DNPN can then bind to the adduct, and protein carbonyl formation can be quantified. First, the _P. aeruginosa_ oxyR mutant was grown aerobiologically 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 H$_2$O$_2$ for 30 min. The organisms were harvested by centrifugation at 13,000 × g for 10 min at 4°C and subsequently washed twice in 10 mM Tris-HCl (pH 7.8). Cells were then disrupted by lysis buffer (9.5 M urea, 2% CHAPS [3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), 0.8% ampholytes [Amerham], 1 mM EDTA, 50 mM dithiothreitol [DTT]) at room temperature for 30 min. Microfuge Dystrips (Amerham) 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 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 (pH6.8) 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-PAGE using the electrophoresis system (34). The electroblotting 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)
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.

### TABLE 1. Strains and plasmids in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>Wild type</td>
<td>31</td>
</tr>
<tr>
<td>oxR UM</td>
<td>oxR unmarked deletion mutant</td>
<td>25</td>
</tr>
<tr>
<td>oxR UM attB-oxR</td>
<td>oxR unmarked deletion mutant containing oxR at attB site</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM PA3716::Gm</td>
<td>Gm; oxR UM, PA3716::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM PA1011::Gm</td>
<td>Gm; oxR UM, PA1011::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM oprF::Gm</td>
<td>Gm; oxR UM, oprF::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM fkbP::Gm</td>
<td>Gm; oxR UM, fkbP::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM oprL::Gm</td>
<td>Gm; oxR UM, oprL::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM PA0700::Gm</td>
<td>Gm; oxR UM, PA0700::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM oprD::Gm</td>
<td>Gm; oxR UM, oprD::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM rpsA::Gm</td>
<td>Gm; oxR UM, rpsA::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM aqgA::Gm</td>
<td>Gm; oxR UM, aqgA::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM PA4632::Gm</td>
<td>Gm; oxR UM, PA4632::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM sdbh::Gm</td>
<td>Gm; oxR UM, sdbh::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM tpx::Gm</td>
<td>Gm; oxR UM, tpx::Gm</td>
<td>This study</td>
</tr>
<tr>
<td>oxR UM dnaK::Gm</td>
<td>Gm; oxR UM, dnaK::Gm</td>
<td>This study</td>
</tr>
</tbody>
</table>

| E. coli DH5α-MCR | mcrA (mrr-hsdRMS-mcrBC) Δ80ΔlacZΔM15 (lacZYA-argF)U169 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1 | Protein Express, Inc. |

### Plasmids

- pEX100T: Ap⁺; oriT mob sacB gene replacement vector
- pEX100T PA3716::Gm: pEX100T carrying a PA3716::Gm gene replacement construct
- pEX100T PA1011::Gm: pEX100T carrying a PA1011::Gm gene replacement construct
- pEX100T oprF::Gm: pEX100T carrying a oprF::Gm gene replacement construct
- pEX100T fkbP::Gm: pEX100T carrying a fkbP::Gm gene replacement construct
- pEX100T oprL::Gm: pEX100T carrying a oprL::Gm gene replacement construct
- pEX100T PA0700::Gm: pEX100T carrying a PA0700::Gm gene replacement construct
- pEX100T oprD::Gm: pEX100T carrying a oprD::Gm gene replacement construct
- pEX100T rpsA::Gm: pEX100T carrying a rpsA::Gm gene replacement construct
- pEX100T aqgA::Gm: pEX100T carrying an aqgA::Gm gene replacement construct
- pEX100T PA4632::Gm: pEX100T carrying a PA4632::Gm gene replacement construct
- pEX100T sdbh::Gm: pEX100T carrying a sdbh::Gm gene replacement construct
- pEX100T tpx::Gm: pEX100T carrying a tpx::Gm gene replacement construct
- pUCP22-ahpB: pUCP22 with ahpB
- pUCP22-ahpCF: pUCP22 with ahpCF
- pUCP22-katB: pUCP22 with katB

### Results

Remarkable sensitivity of oxR mutant bacteria to merely aerobic growth on rich media and protection by anaerobic growth. We have previously shown that oxR mutant bacteria...
...under aerobic conditions (anaerobic chamber for 48 h. Lane 1, PAO1; lane 2, oxyR bacterial suspension. filtered supernatant; lane 5, oxyR katA complemented strain; lane 4, oxyR 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 H_2O_2 generation, the bacteria grew as wild-type organisms with negligible differences (Fig. 1B, −O_2).

Because of the remarkable aerobic sensitivity of the *P. aeruginosa* oxyR mutant to H_2O_2, our next goal was to identify proteins that protect the aerobic oxyR mutant from H_2O_2. 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 H_2O_2-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 H_2O_2. Realizing that the oxidation of specific amino acids is considered an appropriate indicator of the level of oxidative stress mediated by H_2O_2 and other oxidants in aerobic cells, we embarked on the determination of carbonyl groups in proteins that can be formed upon oxidation by H_2O_2 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 be specific for DNP, protein carbonyl formation can be quantified.

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 H_2O_2-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 H_2O_2 than in untreated bacteria (data not shown). In contrast, the oxyR mutant lacks (i) the H_2O_2-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 H_2O_2 (25, 45). Therefore, the oxyR mutant strain was used in the following experiments. First, the organism was exposed to H_2O_2 at a concentration that would minimally affect wild-type bacteria (10 mM H_2O_2, 30 min) to determine carbonylated proteins in H_2O_2-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 H_2O_2. 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-
tively (also refer to Fig. 2E and F). Upon observation of distinct differences in oxidized protein profiles between control and H₂O₂-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, agmatine 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 H₂O₂-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 H₂O₂ resistance of an oxyR mutant. First, isogenic mutants were constructed in each of the 13 candidate genes encoding the oxidized proteins detected in the H₂O₂-treated oxyR mutant (Fig. 2B and D) to assess their possible contribution to H₂O₂ resistance or susceptibility. There were no differences in the H₂O₂ sensitivity of each of these mutants compared to wild-type bacteria (data not shown). Therefore,

**FIG. 2.** Protein oxidation profile of *P. aeruginosa* oxyR mutant bacteria exposed to H₂O₂. (A to D) AgNO₃-stained 2-D gels of control (A) and H₂O₂-treated (10 mM for 30 min) (C) bacteria; Western blot and DNP staining of control (B) and H₂O₂-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.
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

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Gene</th>
<th>Protein</th>
<th>PA no.</th>
<th>Molecular mass (kDa)/pI</th>
<th>MOWSE score</th>
<th>Masses matched</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Hypothetical protein</td>
<td>PA3716</td>
<td>60.50/4.4</td>
<td>1.15E + 08</td>
<td>18 (26)</td>
<td></td>
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<tr>
<td>2</td>
<td>Hypothetical protein</td>
<td>PA1011</td>
<td>43.05/6.9</td>
<td>1.72E + 10</td>
<td>19 (50)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>oprF</td>
<td>Outer membrane protein OprF precursor</td>
<td>PA1777</td>
<td>37.64/5.0</td>
<td>3.67E + 05</td>
<td>10 (32)</td>
</tr>
<tr>
<td>4</td>
<td>fkbP</td>
<td>Probable peptidyl-prolyl cis-trans isomerase, FkbP-type</td>
<td>PA3262</td>
<td>26.84/5.1</td>
<td>14882</td>
<td>9 (47)</td>
</tr>
<tr>
<td>5</td>
<td>oprL</td>
<td>Outer membrane protein OprL precursor</td>
<td>PA0973</td>
<td>17.92/5.9</td>
<td>1.84E + 04</td>
<td>6 (8)</td>
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<tr>
<td>6</td>
<td>Hypothetical protein</td>
<td>PA0700</td>
<td>31.71/8.9</td>
<td>474</td>
<td>4 (10)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>oprD</td>
<td>Outer membrane porin protein OprD precursor</td>
<td>PA0958</td>
<td>48.36/5.0</td>
<td>4.26E + 04</td>
<td>11 (23)</td>
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<tr>
<td>8</td>
<td>rpsA</td>
<td>30S ribosomal protein S1</td>
<td>PA3162</td>
<td>61.87/4.8</td>
<td>1.25E + 08</td>
<td>17 (26)</td>
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<tr>
<td>9</td>
<td>agaA</td>
<td>Agmatine deiminase</td>
<td>PA0292</td>
<td>41.19/4.8</td>
<td>251</td>
<td>9 (12)</td>
</tr>
<tr>
<td>10</td>
<td>Hypothetical protein</td>
<td>PA4632</td>
<td>29.15/7.0</td>
<td>22385</td>
<td>10 (36)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>sdhB</td>
<td>Succinate dehydrogenase (β-subunit)</td>
<td>PA1584</td>
<td>25.15/5.6</td>
<td>2.81E + 04</td>
<td>7 (7)</td>
</tr>
<tr>
<td>12</td>
<td>tpx</td>
<td>Thiol peroxidase</td>
<td>PA2532</td>
<td>17.23/5.2</td>
<td>2117</td>
<td>15 (53)</td>
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<tr>
<td>13</td>
<td>dnaK</td>
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<td>PA4716</td>
<td>68.40/4.8</td>
<td>3.20E + 06</td>
<td>15 (53)</td>
</tr>
</tbody>
</table>

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

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we next constructed 13 isogenic double mutants in an unmarked oxyR mutant background. Surprisingly, we found that 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 mutant shows an H₂O₂ sensitivity profile identical to that of wild-type bacteria (Fig. 3C). Interestingly, the single oprL 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.

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 ~10⁵ 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 growth 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 (▲).
Increased sensitivity of oxyR oprL versus oxyR mutants to H₂O₂ in biofilms. We have previously shown that *P. aeruginosa* biofilms are inherently resistant to concentrations of H₂O₂ 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 H₂O₂ in free-living, planktonic culture, we tested the hypothesis that the biofilm mode of growth might still afford this severely compromised organism resistance to H₂O₂-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 H₂O₂. First, we exposed the wild type and the *oprL* mutant to H₂O₂ 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 different in overall architecture or sensitivity to H₂O₂ 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 H₂O₂ 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 H₂O₂ being generated in the media through autoxidation of 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 H₂O₂ 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 H₂O₂ (Fig. 4, lower panel) relative to wild-type organisms (Fig. 4, top panel). In addition, H₂O₂ 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 H₂O₂.

We next assessed the H₂O₂ sensitivity of *oxyR* relative to *oxyR oprL* mutants in biofilms using very low concentrations of H₂O₂ to help reveal potential subtle differences in biofilm susceptibility patterns between these two strains. Although the *oxyR* mutant was resistant to 5 mM H₂O₂ (Fig. 5, top panel), as little as 0.5 mM H₂O₂ killed the vast majority of *oxyR oprL* bacteria in biofilms (Fig. 5, bottom panel). Moreover, the dead/live ratios/mm² 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 H₂O₂, while the *oxyR* dead/live ratio was greater than that of the wild-type strain PAO1 (dark gray bars, Fig. 6).
The sensitivity of the *oxyR oprL* versus *oxyR* mutant to H$_2$O$_2$ 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 H$_2$O$_2$ 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 H$_2$O$_2$ (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 inexplicably 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 H$_2$O$_2$-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-N$_6$-CTAT-N$_6$-ATAG-N$_6$-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 H$_2$O$_2$ 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 H$_2$O$_2$. We used high levels of H$_2$O$_2$ (50 mM) in these studies because the morphology of wild-type organisms appeared to be unaltered when organisms were treated with 10 mM H$_2$O$_2$ (data not shown). Untreated bacteria were characterized by intact, smooth,
and uninterrupted membranes with a clearly visible periplasmic space (Fig. 8A, arrow). Treatment with H₂O₂ 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 H₂O₂-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, H₂O₂ 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 H₂O₂. Note that the majority of H₂O₂-treated organisms are slightly swollen relative to control bacteria. Photographs of these bacteria were taken at ×40,000 and ×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% of the bacteria photographed at ×40,000 magnification were used to compare the structural nature of the membranes under control and H₂O₂-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 H₂O₂ caused membrane alterations even in wild-type strain PAO1, for which ~67% of the treated cells have a wavy membrane (Fig. 8B and H). However, we found that 72% of H₂O₂-treated oxyR mutants possessed invaginated membranes with no clearly defined periplasmic space (Fig. 8D and J), whereas 82% of the oxyR oprL mutant H₂O₂ 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 H₂O₂-responsive regulator OxyR in *P. aeruginosa* renders such organisms exquisitely susceptible to micromolar H₂O₂ (25, 45). Given that H₂O₂ 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 μM 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 ∼10⁷ 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 F₁F₀-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· HO· 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⁻) in E. coli (39). However, formation of O₂⁻ by SDH is likely a result of the formation of a flavosemiquinone intermediate that can react with molecular oxygen (35), forming O₂. 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/peroxidase) 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$_2$O$_2$-generating agents is in the case of parquat-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 persisting, 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$_2$O$_2$ under aerobic conditions requires not only the support of the putatively bifunctional catalase/AhpAhpCF 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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