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Pseudomonas aeruginosa Thiol Peroxidase Protects against Hydrogen Peroxide Toxicity and Displays Atypical Patterns of Gene Regulation

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The *Pseudomonas aeruginosa* PAO1 thiol peroxidase homolog (Tpx) belongs to a family of enzymes implicated in the removal of toxic peroxides. We have shown the expression of *tpx* to be highly inducible with redox cycling/superoxide generators and diamide and weakly inducible with organic hydroperoxides and hydrogen peroxide (H_2O_2). The PAO1 *tpx* pattern is unlike the patterns for other peroxide-scavenging genes in *P. aeruginosa*. Analysis of the *tpx* promoter reveals the presence of a putative IscR binding site located near the promoter. The *tpx* expression profiles in PAO1 and the *iscR* mutant, together with results from gel mobility shift assays showing that purified IscR specifically binds the *tpx* promoter, support the role of IscR as a transcriptional repressor of *tpx* that also regulates the oxidant-inducible expression of the gene. Recombinant Tpx has been purified and biochemically characterized. The enzyme catalyzes thioredoxin-dependent peroxidation and can utilize organic hydroperoxides and H_2O_2 as substrates. The Δ*tpx* mutant demonstrates differential sensitivity to H_2O_2 only at moderate concentrations (0.5 mM) and not at high (20 mM) concentrations, suggesting a novel protective role of *tpx* against H_2O_2 in *P. aeruginosa*. Altogether, *P. aeruginosa tpx* is a novel member of the IscR regulon and plays a primary role in protecting the bacteria from submillimolar concentrations of H_2O_2.

Thiol peroxidase (Tpx), formerly known as p20 or scavengase (9), is a member of the peroxiredoxin family that is widely distributed among prokaryotic organisms (20). Tpx contains thiol-dependent peroxidase activity and is capable of reducing either organic hydroperoxides or H_2O_2 by using reduced thioredoxin (Trx) as an electron donor. The resultant oxidized thioredoxin is cycled back to the reduced form by the thioredoxin reductase reaction, which utilizes NADPH as a reductant (Fig. 1A) (3, 9, 29). Tpx belongs to a group of atypical 2-Cys peroxiredoxins, as it contains two redox-active cysteine residues that correspond to Cys-61 and Cys-95 of the *Escherichia coli* Tpx (3, 9, 46). In the presence of peroxides, the peroxidatic Cys-61 reacts with the peroxide substrates and is oxidized to the sulfenic acid intermediate, which in turn reacts with the resolving Cys-95 to form an intermolecular disulfide bond (3).

An *E. coli tpx* mutant displays sensitive phenotypes to both H_2O_2 and organic hydroperoxides (t-butyl and cumene hydroperoxides) and a superoxide generator, paraquat (PQ) (8). This picture strongly supports the physiological role of Tpx in the oxidative stress protection of *E. coli*.

Pseudomonas aeruginosa, a Gram-negative nonfermentative bacterium, is one of the leading causes of lethal hospital-acquired infections (7) and of chronic pulmonary infections in patients with cystic fibrosis (26). Upon invasion of a human host, the bacteria encounter an innate immune response that is the first line of defense against the infecting pathogens. The bacteria also have to confront reactive oxygen species (ROS) generated in the phagolysosome, including superoxide anions, H_2O_2, and peroxynitrite. In addition, the bacteria are also exposed to ROS from their own by-products of aerobic metabolism, during exposure to chemicals and when interacting with other microbes in the environment. *P. aeruginosa* has evolved multiple strategies to overcome these deleterious conditions. A number of ROS-scavenging enzymes have been reported to play a physiological role in oxidative stress protection. *P. aeruginosa* produces several isozymes of superoxide dismutases to dismutate superoxide anions, catalases to detoxify H_2O_2, and peroxiredoxins to detoxify peroxides, including both organic hydroperoxides and H_2O_2 (2, 6, 24, 25, 39, 41, 42). Here, we report the biochemical, genetic, and physiological characterization of *tpx* and reveal it to be an important member of a group of peroxide removal genes that protect *P. aeruginosa* from oxidative stress.

MATERIALS AND METHODS

Bacterial growth conditions. *P. aeruginosa* PAO1 and mutant strains were cultured aerobically in LB medium at 37°C with continuous shaking (150 rpm). Exponential-phase cells were used routinely in all experiments.

Purification of *P. aeruginosa* Tpx, thioredoxin (TrxA), and thioredoxin reductase (TrxB). Tpx protein from *P. aeruginosa* was purified using the *E. coli* expression system. A putative *tpx* gene (PA2532) was amplified from the PAO1 genomic DNA with primers BT2864 (5′-ATC AACGCCATGGCTCAAG-3′) and BT2865 (5′-GGAGAGCCGCCAGGG CCG-3′). The PCR product was digested with NcoI before being cloned into the pETBlue-2 vector (Novagen) digested with NcoI and XhoI (blunt ended) to produce pET-tpx for high expression of Tpx with a C-terminal His_6 tag. An *E. coli* BL21(DE3)pLysS strain harboring pET-tpx was grown

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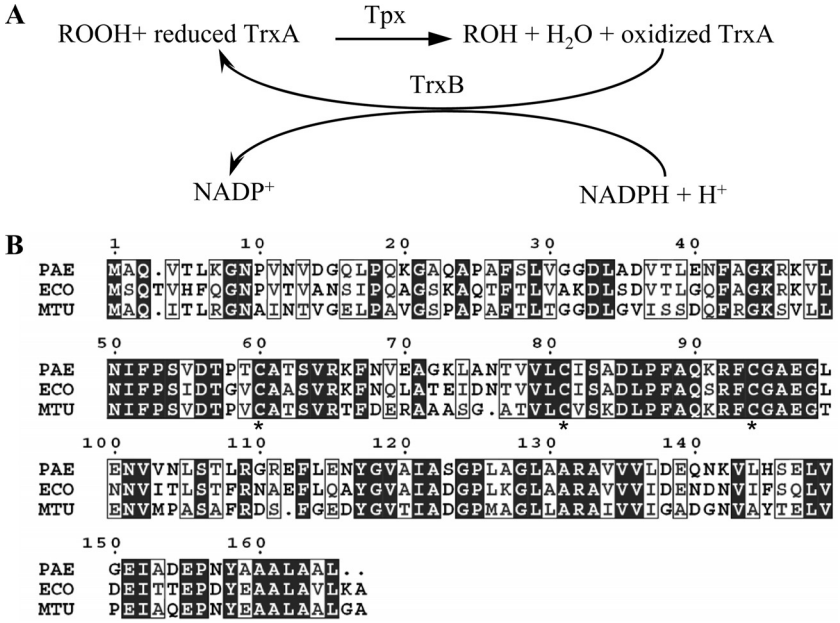

FIG 1 Peroxide reduction by Tpx and alignment of Tpx enzymes. (A) Schematic diagram showing the reduction of peroxide (ROOH) to its corresponding alcohol (ROH) as summarized from previous reports (3, 9, 29). (B) Alignment of putative Tpx from *P. aeruginosa* (PAE), *E. coli* (ECO), and *M. tuberculosis* (MTB) as performed using Clustal W2 (36). Asterisks indicate conserved cysteine residues that were mutated.

aerobically in LB medium containing 100 μg ml^{-1} ampicillin at 37°C until the culture reached an optical density of approximately 0.6 to 0.8 at 600 nm (OD_{600}). The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to grow for an additional 3 h. Cells were harvested, resuspended in binding buffer (50 mM sodium phosphate buffer, 300 mM NaCl, and 10 mM imidazole, pH 7.0), and sonicated intermittently until being completely lysed. The clear lysate was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Invitrogen) equilibrated with the binding buffer. The unbound proteins were washed with washing buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 7.0). The Tpx protein was eluted with elution buffer (50 mM phosphate buffer, 300 mM NaCl, 250 mM imidazole, pH 7.0), and the eluent was dialyzed against the dialysis buffer (50 mM phosphate buffer, 100 mM NaCl, pH 7.0). The purity of Tpx was estimated to be 90% based on a Coomassie blue-stained SDS-polyacrylamide gel.

The full-length *trxB* (PA2616) was PCR amplified from the PAO1 genomic DNA with primers BT2982 (5′-CTTTCCATGGGTGAAGTCA AGCAT-3′) and BT2983 (5′-GTCCTCGAGATGGTCGTCGAGGTA-3′). The product was cut with NcoI prior to cloning into pETBlue-2 digested with NcoI and XhoI (gap filled with Klenow fragment), yielding pETtrxB for high expression of TrxB with C-terminal His_6-tag. Purification of TrxB from *E. coli* harboring pETtrxB was similar to that described for Tpx purification.

To purify *P. aeruginosa* TrxA (PA5240), pET11a-trxA (L. B. Poole, unpublished data), a plasmid for high-level expression of nontagged TrxA protein, was introduced into *E. coli* BL21(DE3). Bacteria were harvested, and the cell pellet was resuspended in lysis buffer (5 mM EDTA, 100 μM phenylmethylsulfonyl fluoride [PMSF], 25 mM phosphate buffer, pH 7.0) before being lysed using a French press. The clear lysate was loaded onto a DEAE cellulose column equilibrated with 25 mM phosphate buffer, 0.5 mM EDTA, pH 7.0. The unbound proteins were washed with 250 mM phosphate buffer, 0.5 mM EDTA, pH 7.0. The bound proteins were eluted with a linear phosphate gradient from 250 to 500 mM containing 0.5 mM EDTA, pH 7.0. The partially purified TrxA was precipitated with 80% ammonium sulfate prior to loading onto the Superdex G-75 column pre-equilibrated with 100 mM phosphate buffer, 0.5 mM EDTA, pH 7.0. The column was eluted with the same buffer to obtain the purified TrxA.

Steady-state kinetic analysis of Tpx. Michaelis constants (K_m) and turnover numbers (k_{cat}) of *P. aeruginosa* Tpx with peroxide substrates were determined by measuring initial reaction velocities at various concentrations of peroxides and at saturating concentrations of TrxA (100 μM). The initial Tpx reaction velocities were measured from the absorbance decrease at 340 nm due to the oxidation of NADPH in the TrxB-coupled reaction as described for *E. coli* Tpx (3) (Fig. 1A). The reaction mixture typically consists of 50 mM phosphate buffer, 0.5 mM EDTA (pH 7), 150 μM NADPH, 100 μM purified *P. aeruginosa* TrxA, 1.5 μM purified *P. aeruginosa* TrxB, 0.05 μM purified Tpx, and various concentrations of peroxides (H_2O_2, cumene hydroperoxide [CHP], and t-butyl hydroperoxide [BHP]).

Construction of *P. aeruginosa* mutants. The Δ*tpx* mutant was constructed using the allelic exchange method with a *cre-lox* antibiotic marker recycling system (40). A 1,390-bp DNA fragment containing the *tpx* gene plus the sequences flanking both *tpx* termini was PCR amplified from PAO1 genomic DNA with the primers BT2387 (5′-ACTACATCCTGTC GCTGG-3′) and BT2388 (5′-GGAAAGCCTGCGTGACGA-3′) and subsequently cloned into a pUC18 plasmid cut with HincII, yielding pUCtpx. The SalI and EcoRI (blunt-ended) fragment containing a gentamicin resistance (Gm^r) cassette flanked with *lox* sequences from pUC18Gm (pUC18 containing *lox*-flanked Gm^r, which is constructed by inserting SacI-EcoRI fragments containing *lox*-flanked Gm^r from pCM351 [40] into pUC18 cut with the same enzymes) was cloned into pUCtpx digested with BstXI (blunt ended) and XhoI, yielding pUCΔtpx::Gm. Digestion with BstXI-XhoI deleted 109 bp of the internal sequence of *tpx*. pUCΔtpx::Gm was transferred into PAO1, and the putative *tpx* mutants that arose from the double crossover were selected for the Gm^r and carbenicillin sensitivity (Cb^s) phenotype. An unmarked Δ*tpx* mutant was created using the *cre-lox* system to excise the Gm^r gene as previously described (40), and deletion of *tpx* was confirmed by PCR and Southern blot analysis.

The *iscR* mutant was constructed by insertional inactivation using pKNOCK, a suicide vector system (1). The 236-bp *iscR* (PA3815) fragment was amplified from the genomic DNA with the primers BT3186 (5′-TATCTCCGAACGCCAAGG-3′) and BT3187 (5′-GGTGGTGGGTC AGACAGG-3′). The PCR product was cloned into pKNOCK-Gm digested with SmaI, generating pKNOCK-iscR. This recombinant plasmid

was introduced into *E. coli* BW20767 prior to being transferred into PAO1 by conjugation (1). The Gm-resistant transconjugants were selected and confirmed to be the *iscR* mutants by Southern analysis.

Construction of pTpx, pTpx$_{C60S}$, pTpx$_{C81S}$, pTpx$_{C94S}$, and pIscR. The full-length *tpx* gene was amplified from the PAO1 genome with the primers BT2647 (5′-GAAGGATCAACGCAATGG-3′) and BT2648 (5′-GGTCATGGGACGAAGCGG-3′). A 544-bp PCR product was cloned into the low-copy-number and broad-host-range vector pUFR047 (18) at the EcoRI and BamHI sites, yielding pTpx.

PCR-based site-directed mutagenesis was performed as previously described to generate mutant Tpx that has the conserved cysteine residue replaced with serine (45). The mutagenic forward primers and reverse primers used to generate the mutant Tpx were BT3411 (5′-CGACCTCCGCCACCTCGGTGCGCAA-3′) and BT3412 (5′-GTGGCGGAGGTCGGGGTGTCGACGC-3′) for C60S, BT3413 (5′-TGCTGTCCATCTCCGCCGACCTGCC-3′) and BT3414 (5′-GAGATGGACAGCACCACGGTGTTGG-3′) for C81S, and BT3415 (5′-GCTTCTCCGGCGCCGAAGGCCTGGA-3′) and BT3416 (5′-GCGCCGGAGAAGCGCTTCTGCGCGA-3′) for C94S. pTpx was used as a DNA template to allow PCR amplification with a mutagenic primer pair. PCR products were digested with BamHI and EcoRI and cloned into pUFR047 (18), which was then cut with the same enzymes. The resultant plasmids harboring inserted DNA were designated pTpx$_{C60S}$, pTpx$_{C81S}$, and pTpx$_{C94S}$. The plasmids were sequenced to verify the accuracy of site-directed mutagenesis.

The full-length *iscR* gene was amplified from PAO1 genomic DNA with the primers BT3209 (5′-AAGAGCATAATCCGCGTC-3′) and BT3210 (5′-CGAGGTAGATCGGCAATT-3′). A 591-bp PCR product was cloned into expression vector pBBR1MCS4 (34) that had been digested with SmaI, generating pIscR.

Construction of the *tpx-phoA* and *tpx-lacZ* fusions. The *phoA* and *lacZ* fusions were performed as previously described (52). A pTpx$_{39}$-PhoA plasmid was constructed by in-frame fusion of the 5′-end fragment of *tpx* with a leaderless *phoA* from *E. coli*. The *tpx* fragment amplified from PAO1 genomic DNA with the primers BT3674 (5′-GCTTCTCACTGACTAGAA-3′) and BT3617 (5′-AGGGTACCAGGGTCACGTCGGCG-3′) was cut with KpnI before being cloned into pPhoA (52), whereas pBBR1MCS4 containing *phoA* was digested with SmaI and KpnI, generating pTpx$_{39}$-PhoA.

pTpx$_{39}$-LacZ was constructed by amplifying the *tpx* fragment with the BT3674 and BT3628 (5′-AGGGATCCAGGGTCACGTCGGCG-3′) primers using PAO1 genomic DNA as the template. The PCR product was cut with PstI prior to cloning into pLacZ (pBBR1MCS4 containing *lacZ*) and then digested with Acc65I (filled in with the Klenow fragment) and PstI, yielding pTpx$_{39}$-LacZ.

Northern blot analysis. Total RNA isolation, agarose formaldehyde gel electrophoresis, blotting, and hybridization were performed as previously described (12). A 250-bp fragment of the *tpx* coding region amplified from pTpx using primers BT2647 (5′-GAAGGATCAACGCAATGG-3′) and BT2649 (5′-ACCACGGTGTTGGCCAGC-3′) was used as a radioactive probe. The labeling of the DNA probe with [α-^{32}P]dCTP was performed using a DNA labeling bead (Amersham, GE Healthcare). Results from one representative experiment out of three independent experiments that were performed are shown. Densitometric analysis of the blot using ImageScanner III with LabScan 6.0 software (GE Healthcare) was performed to determine fold induction above the untreated level.

RT-PCR. Endpoint reverse transcription-PCR (RT-PCR) was carried out as previously described (31) to determine the expression level of *tpx* in *P. aeruginosa* under exposure to oxidative stress. Total RNA samples were isolated from the exponential-phase cultures of PAO1 induced with 250 μM H_2O_2, CHP, menadione (MD), plumbagin (PB), or 100 μM N-ethylmaleimide (NEM) for 20 min. The RT reaction was performed as described previously (31). After reverse transcription, the cDNA concentration was measured using a NanoDrop spectrophotometer. The cDNA (100 ng) was used as a template for PCR amplification with primers specific to *tpx*, BT2647 (5′-GAAGGATCAACGCAATGG-3′) and BT2649 (5′-ACCACGGTGTTGGCCAGC-3′). The 16S rRNA gene was used as an internal control along with the primers BT2781 (5′-GCCCGCACAAGCGGTGGAG-3′) and BT2782 (5′-ACGTCATCCCCACCTTCCT-3′). To allow quantification within the linear range of the assay, PCR was performed under the following cycling conditions: 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s; this cycle was repeated 25 times for *tpx* and 22 times for the 16S rRNA.

Primer extension. Total RNA was isolated from uninduced and PB-induced cultures. Primer extension was performed using [^{32}P]-labeled BT3590 (5′-GCCTTTCTGCGGGAGCT-3′) primer, 20 μg of total RNA, and 200 U of Superscript III reverse transcriptase (Invitrogen). The extension products were sized on a 6% acrylamide-7 M urea sequencing gel next to dideoxy sequencing ladders generated using a PCR sequencing kit (Applied Biosystems), with the labeled BT3590 primer and putative *tpx* promoter fragment used as the templates.

Purification of *P. aeruginosa* IscR and gel mobility shift assay. His-tagged IscR from *P. aeruginosa* was purified using the pET-Blue2 expression system. The full-length *iscR* gene was amplified from PAO1 genomic DNA with primers EBI6 (5′-CGTCTGACCACCAAAGGCCGCTACGC-3′) and EBI7 (5′-CCGCTCGAGGTCGATGGCGGACGCTTCAATC-3′). A 495-bp PCR product was digested with XhoI before ligation into pET-Blue-2 digested NcoI (blunt ended) and XhoI to generate pET-iscR for high-level expression of IscR containing a C-terminal His$_6$ tag. An *E. coli* BL21(DE3)pLysS strain harboring pET-iscR was grown in LB medium containing 100 μg ml^{-1} ampicillin at 37°C to an OD$_{600}$ of 1.0 before being induced with 1 mM IPTG for 15 min. IscR purification was carried out as previously described (43). The purity of IscR protein was more than 95% as judged by SDS polyacrylamide gel electrophoresis.

Gel mobility shift assays were performed essentially as previously described (43). Briefly, a labeled probe (194 bp) containing the *tpx* promoter was prepared by amplifying PAO1 genomic DNA with ^{32}P-labeled BT3712 (5′-TCGGGGGACGGCGATGCT-3′) and BT3590 primers. Binding reactions were conducted using 3 fmol of labeled probe in 25 μl of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.02 mg ml^{-1} bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), 10% (vol/vol) glycerol, and 200 ng of poly(dI-dC). Various amounts of purified IscR were added, and the reaction mixture was incubated at 25°C for 20 min. Protein-DNA complexes were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 4°C and visualized by exposure to X-ray film.

Determination of oxidant resistance levels. The resistance level of *P. aeruginosa* strains against low concentrations of oxidant was determined using the plate sensitivity assay (15). Serial dilutions of the exponential-phase cells were made in LB medium, and 10 μl of each dilution was spotted onto the LB agar plates alone or containing 0.5 mM H_2O_2, 1.5 mM CHP, 1.0 mM BHP, 1.0 mM diamide, 1.0 mM PB, or 0.5 mM PQ. The plates were incubated at 37°C overnight, and the bacterial colonies were counted. The resistance level was expressed as the surviving fraction, defined as the number of CFU on a plate containing oxidant divided by the number of CFU on a plate without oxidant.

The resistance level against lethal concentrations of H_2O_2 was determined as previously described (11), with some modifications. The exponential-phase cultures in LB medium were treated with 20 and 50 mM H_2O_2 for 30 min. Cells surviving the treatment were scored using viable cell counts by plating appropriate cell dilutions on LB agar plates and incubating them overnight at 37°C. The surviving fraction was defined as the ratio of CFU recovered after the treatment to the CFU existing prior to the treatment.

Enzyme activity assays. Bacterial cell lysate preparation, protein assay, and catalase activity determination were performed as described previously (13). One unit of catalase was defined as the amount of enzyme capable of catalyzing the turnover of 1 μmol of substrate per min under assay conditions. Alkaline phosphatase activity was determined as described earlier (5). One unit of enzymatic activity was defined as the

amount of enzyme capable of catalyzing 1 μmol of *o*-nitrophenol per min at room temperature. β-Galactosidase was assayed as described previously (41).

Nematode killing assays. The pathogenicity of *P. aeruginosa* strains was determined using a *Caenorhabditis elegans* animal model (51). Both slow and fast killing experiments were performed as previously described (51). Nematodes in the fourth larval (L4) stage (30 to 40 animals per plate) were used in all experiments. Nematode killing was scored with a dissecting microscope after 3, 5, and 9 h for fast killing and 1, 2, 3, and 4 days for slow killing. Three biological replicates were carried out.

RESULTS AND DISCUSSION

***P. aeruginosa* Tpx belongs to the atypical 2-Cys peroxiredoxin class.** Analysis of the PAO1 genome (49) reveals the presence of an open reading frame, PA2532, encoding a thiol peroxidase (Tpx) homolog. This 165-amino-acid protein with a theoretical molecular mass of 17.23 kDa shares 66% sequence identity with *E. coli* Tpx, an atypical 2-Cys peroxiredoxin, and 60% identity with *Mycobacterium tuberculosis* Tpx (Fig. 1B). Analysis of the *P. aeruginosa* Tpx sequence using the SignalP 3.0 algorithm (http://www.cbs.dtu.dk/services/SignalP/) and TatP 1.0 (http://www.cbs.dtu.dk/services/TatP/) did not reveal a signal peptide. However, reanalysis of the protein for subcellular localization using PSORTb v3.0.2 (http://www.psort.org/psortb/) suggested that *P. aeruginosa* Tpx is a periplasmic enzyme similar to the previously described *E. coli* Tpx (9). To verify this *in silico* assumption experimentally, *phoA* and *lacZ* reporter gene fusions were used to determine the location of Tpx. The Tpx N-terminal fragment containing the first 39 codons of *tpx* was translationally fused either to alkaline phosphatase (PhoA), which is active only in the periplasm, where disulfide bonds are correctly formed, or to β-galactosidase (LacZ), which is active in the cytoplasm. PAO1 harboring pTpx$_{39}$-PhoA that had an in-frame fusion of *tpx39* to *phoA* and PAO1 carrying pPhoA, a plasmid expressing unfused *phoA*, produced an undetectable level of alkaline phosphatase activity. However, PAO1 harboring pTpx$_{39}$-LacZ produced 5.86 ± 1.12 U mg^{-1} protein, whereas PAO1 harboring pLacZ (a plasmid expressing unfused *lacZ*) produced 0.11 ± 0.02 mU mg^{-1} protein. If Tpx was a typical periplasmic protein that was secreted from the cytosol via an N-terminal signal sequence, then we would expect a fusion to alkaline phosphatase to be active when expressed as a fusion to the first 39 amino acids of Tpx. Since we did not find any alkaline phosphatase activity in a strain expressing this fusion protein, we conclude that the protein is not secreted to the periplasm via a typical signal sequence, greatly decreasing the likelihood that this protein is located in the periplasm. Of note, a recent reanalysis of *E. coli* Tpx localization suggested that this protein also localizes to the cytoplasm (53).

Expression analysis of *P. aeruginosa tpx*. In *P. aeruginosa*, the expression of several genes encoding peroxide-scavenging enzymes, such as alkyl hydroperoxide reductase (AhpC), catalase, and Ohr (a thiol peroxidase), is inducible by peroxides (6, 41, 42). This adaptive expression in response to stress is a crucial general strategy for bacteria to survive under conditions of environmental stress, including oxidative stress. Previous observations have revealed contrasting *tpx* expression profiles. *E. coli tpx* is constitutively expressed and does not respond to treatments with peroxides, superoxide generators, or thiol-depleting agents (10). However, a proteomic analysis of *Mycobacterium* Tpx levels revealed increasing amounts of protein expression after exposure to thiol stress (19). Here, endpoint RT-PCR and Northern analysis

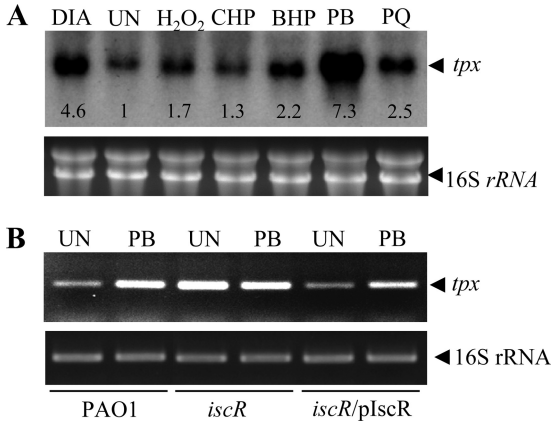

FIG 2 Expression analysis of *tpx*. (A) Autoradiogram of a Northern blot analysis of *tpx* expression in an RNA sample extracted from exponential-phase PAO1 cells left untreated (UN) or treated with 0.25 mM H_2O_2, 0.5 mM cumene hydroperoxide (CHP), 0.5 mM t-butyl hydroperoxide (BHP), 0.5 mM plumbagin (PB), 0.25 mM paraquat (PQ), and 0.5 mM diamide (DIA) for 15 min. The number below each hybridized band represents the fold change in band intensity relative to the untreated culture. The RNA gel is shown below the autoradiogram to illustrate equal quantities of the RNA loaded. (B) The expression level of *tpx* in PAO1 strains was determined using endpoint RT-PCR. The exponential-phase cells of the PAO1 wild type, *iscR* mutant, and a complemented strain (*iscR*/pIscR) were treated with 0.25 mM PB for 15 min prior to RNA extraction. PCR was performed as described in Materials and Methods.

were employed to determine the expression profile of *P. aeruginosa tpx* in response to oxidative stress. Exponential-phase PAO1 cells were treated with various oxidants for 15 min prior to RNA isolation. Northern analysis was performed using a *tpx*-specific probe labeled with [^{32}P]dCTP. Exposure of the cultures to redox-cycling agents/superoxide generators, such as plumbagin (PB), paraquat (PQ), and the thiol-depleting agent diamide, substantially induced *tpx* expression by 7.3-, 2.5-, and 4.6-fold, respectively, as judged from densitometric analysis of the blot (Fig. 2A). Treatment of the cultures with either organic hydroperoxides (cumene hydroperoxide [CHP] or *t*-butyl hydroperoxide [BHP]) or H_2O_2 resulted in less *tpx* induction (1.3-fold for CHP, 2.2-fold for BHP, and 1.7-fold for H_2O_2). A similar pattern of *tpx* expression in response to oxidative stress was observed in the RT-PCR analysis (data not shown). The *P. aeruginosa tpx* expression profile revealed a novel pattern of oxidant-inducible gene expression that differs from other bacterial examples of *tpx*. In addition, the *tpx* expression pattern differs from that of other *P. aeruginosa* genes encoding peroxide-scavenging enzymes, such as *ahpCF*, *katA*, *katB*, *gpx*, and *ohr* (6, 27, 35, 41, 42). The expression of *ahpCF*, *katA*, and *katB* is regulated by the global H_2O_2-sensing regulator OxyR, while that of *gpx* and *ohr* is controlled by the repressors OspR and OhrR, respectively, both of which sense and respond to organic hydroperoxides (6, 27, 35, 41, 42). This unique expression pattern is unexpected, in that peroxide substrates of Tpx such as organic hydroperoxides are not strong inducers, whereas redox-cycling agents/superoxide generators and diamide, which are not substrates of the enzyme, act as strong inducers.

***tpx* expression is regulated by IscR.** Numerous transcriptional regulators in *P. aeruginosa*, such as OxyR, SoxR, OhrR, OspR, MexR, and IscR, have been shown to be involved in the regulation of oxidative stress-inducible gene expression for a variety of genes

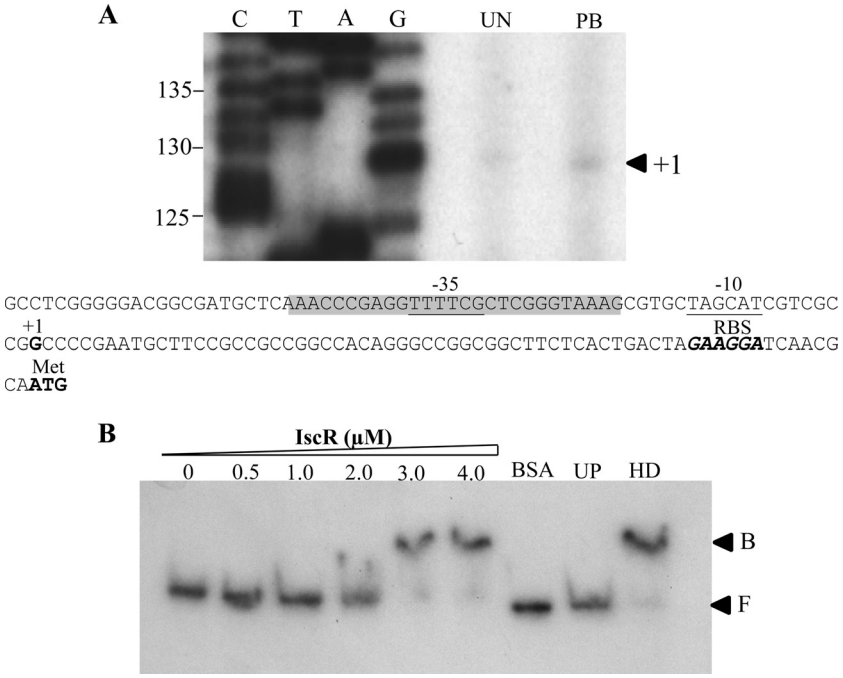

FIG 3 Analysis of the *tpx* promoter. (A) Primer extension was carried out using the ^{32}P-labeled BT3590 primer and RNA extracted from the PAO1 wild type, *iscR* mutant, and a complemented strain (*iscR*/pIscR) either uninduced (UN) or induced with 0.25 mM plumbagin (PB). C, T, A, and G represent the DNA sequence ladders prepared using a labeled primer identical to that used in primer extension and a *tpx* promoter fragment as the template. Numbers at the left indicate fragment lengths in base pairs. The arrowhead indicates the transcription start site (+1). Putative −35 and −10 motifs are underlined, and the ribosome binding site (Rbs) is in boldface and italics. Nucleotides that match the *E. coli* consensus sequence for the IscR binding box are shaded. The conserved bases are indicated by asterisks. The boldfaced ATG represents the translational initiation codon. (B) Gel mobility shift assay of reaction mixtures containing ^{32}P-labeled *tpx* promoter fragment and the indicated concentrations of purified IscR. UP and HD represent the binding reaction mixtures containing 3.0 μM IscR and an additional 1 μg of unlabeled *tpx* promoter and 2.5 μg of heterologous DNA (pUC28 plasmid), respectively. F and B indicate free and bound probes, respectively.

(14, 23, 33, 35, 41, 44). These regulators are thought to be sensing and responding to different types of reactive oxygen species. The strong induction of *tpx* by superoxide generators/redox-cycling agents (Fig. 2A) suggests that OxyR, a peroxide sensor/transcriptional regulator, and/or transcription regulators containing the Fe-S cluster, such as SoxR or IscR, are involved in the regulation of the gene (32). Fe-S clusters containing transcriptional regulators are thought to be susceptible to oxidation by superoxide anions generated from redox-cycling drugs. In *P. aeruginosa*, SoxR has been shown not to be involved in the sensing of superoxide anions, and it has no role in the regulation of genes involved in the protection of superoxide stress. Nonetheless, RT-PCR analysis of *tpx* expression was performed on RNA samples from uninduced and plumbagin-induced wild-type PAO1 and *oxyR* and *soxR* mutants. RNA samples from *oxyR* and *soxR* mutants demonstrated plumbagin-inducible expression of *tpx* to levels similar to those attained in PAO1 samples (data not shown). This finding suggests that neither OxyR nor SoxR regulates plumbagin-inducible *tpx* expression.

IscR is generally known as a transcriptional regulator of genes that participate in Fe-S cluster biogenesis. Experiments in *P. aeruginosa* PA14 have revealed a link between IscR regulation and KatA catalase, a gene that protects against oxidative stress (33). The *iscR* mutant shows reduced KatA activity and H_2O_2 hypersensitivity (33). IscR exerts its regulatory function by binding to the specific DNA sequence located in close proximity to the target gene promoters. Therefore, the putative *tpx* promoter was localized using the primer extension method.

Total RNA extracted from uninduced and PB-induced cultures was reverse transcribed using ^{32}P-labeled BT3590 primer. The 129-base extension products mapped the transcriptional start site (+1) to the G residue located 66 bases upstream of the ATG translational start codon (Fig. 3A). The two conserved regions of the *tpx* promoter regions are TTTTCG and TAGCAT for the −35 and −10 regions, respectively, and are separated by 16 bp (Fig. 3A). As expected, no sequence similar to the consensus sequence for the *E. coli* SoxR binding site, 5′-CCTCAAGTTAACTT GAGG-3′ (28), could be identified in the region of the putative *tpx* promoter. This finding concurs with the *tpx* expression analysis in the *soxR* mutant. Interestingly, the sequence 5′-AAACCCGAGG TTTTCGCTCGGGTAAA-3′, which shares a high degree of homology (18 out of 26) with the consensus sequence for the *E. coli* IscR binding site 5′-AWARCCCYTSNGTTTGMNGKKKTKWA-3′ (22), was identified at positions −46 to −21 of the *tpx* promoter region. The presence of a putative IscR box in the promoter region of *tpx* suggests that IscR participates in transcriptional regulation of *tpx* gene expression. Thus, an *iscR* knockout mutant was constructed and used to monitor *tpx* expression. Northern blot analysis was performed using RNA samples prepared from uninduced and PB-induced *iscR* mutants, in addition to its complemented strain harboring pIscR plasmid (*iscR*/pIscR) and a PAO1 strain. As expected, PB-treated PAO1 showed a 6.5-fold induction in *tpx* expression based on densitometric analysis. The PB induction of *tpx* expression was abolished in the *iscR* mutant. The *tpx* expression showed constitutively high expression levels in the *iscR* mutant. The uninduced level of *tpx* transcripts in the *iscR* mutant was

10.5-fold higher than the uninduced level in PAO1 and 1.4-fold higher than the levels attained in the PB-induced sample from PAO1 (Fig. 2B). In the *iscR*/pIscR strain, the uninduced *tpx* transcript level was 0.7-fold lower than the uninduced level in the PAO1 strain. As expected, the exposure to PB induced a 2.5-fold increase in the *tpx* transcription level. PB-induced *tpx* expression was restored in the complemented mutant strain.

In other bacteria, IscR has been shown to function as a transcriptional repressor or activator, depending on the binding site and the form of transcription regulator that binds to the operator. Here, *tpx* expression patterns in PAO1 and in the *iscR* mutant indicate that IscR functions as a transcription repressor on the *tpx* promoter. This notion is supported by observations that *tpx* expression is constitutively high in the *iscR* mutant and the documented decrease in the uninduced *tpx* expression levels when large amounts of IscR were produced from an expression plasmid in the *iscR*-complemented strain. One possible mechanism for IscR regulation of *tpx* expression is that, in uninduced conditions, the IscR binding site overlaps with the *tpx* promoter regions. This situation would prevent RNA polymerase from binding to the promoter and would result in repression of gene transcription. The 2Fe-2S center of IscR is known to be highly susceptible to oxidation by oxidants, i.e., redox-cycling drugs/superoxide generators and thiol-depleting reagents and peroxides (17, 38, 47, 54). Thus, with exposure to redox-cycling drugs/superoxide anions, such as PB, the 2Fe-2S center of IscR is likely to be oxidized, leading to disruption of the 2Fe-2S and a possible change in IscR conformation, thus rendering the regulator unable to bind to the operator site of the *tpx* promoter. This situation would allow RNA polymerase to bind to the promoter and enable full expression of the gene. To definitively prove this model, a gel mobility shift assay was performed using purified His-tagged IscR protein. Purified IscR specifically bound to the *tpx* promoter fragment (Fig. 3B). The binding specificity of IscR was illustrated by the ability of the unlabeled *tpx* promoter fragment, but not an excess of heterologous DNA (pUC18 plasmid), to compete with IscR for binding to the labeled promoter fragment, and the presence of an excess amount of unrelated protein (2.5 μg BSA) failed to enable binding to the *tpx* promoter (Fig. 3B). Thus, IscR directly binds the *tpx* promoter and regulates its expression.

Biochemical properties of *P. aeruginosa* Tpx. As a member of the peroxiredoxin enzyme family, Tpx uses reducing equivalents from the thioredoxin (TrxA)/thioredoxin reductase (TrxB) system to reduce peroxides (4). The peroxidase activity of *P. aeruginosa* Tpx was measured by coupling the Tpx reaction with the TrxA/TrxB system in which TrxB catalyzes the reduction of TrxA by NADPH (4). The His-tagged *P. aeruginosa* Tpx, TrxA (PA5240), and His-tagged TrxB (PA2616) proteins were purified using the *E. coli* expression system (see Materials and Methods). The oxidation of NADPH to $NADP^+$ was monitored by the absorbance decrease at 340 nm. The results showed that H_2O_2 and organic hydroperoxides (CHP and BHP) can be used as enzyme substrates. The apparent kinetic parameters, including the Michaelis constant [$K_{m\ (app)}$], turnover number [$k_{cat\ (app)}$], and catalytic efficiency [$k_{cat\ (app)}/K_{m\ (app)}$], were determined for each peroxide substrate and are shown in Fig. 4. The $K_{m\ (app)}$ values of the *P. aeruginosa* Tpx for H_2O_2, CHP, and BHP (determined at 100 μM TrxA) were 375 ± 28, 8 ± 1, and 59 ± 10 μM, respectively, and the $k_{cat\ (app)}$ values were 26.6 ± 0.5, 25.7 ± 1.3, and 22.2 ± 0.5 s^{-1}, respectively. Therefore, these data indicate that *P. aeruginosa* Tpx is more efficient at reducing organic hydroperoxides [CHP and BHP, with $k_{cat\ (app)}/K_{m\ (app)}$ of 3.2×10^6 and 4×10^5 $M^{-1} \cdot s^{-1}$, respectively] than H_2O_2 [with $k_{cat\ (app)}/K_{m\ (app)}$ of 1×10^5 $M^{-1} \cdot s^{-1}$]. Clearly, Tpx can use both organic hydroperoxides and H_2O_2 as substrates, albeit with different efficiencies. In this respect, *P. aeruginosa* Tpx and *E. coli* Tpx are similar, in that their K_ms for organic peroxide substrates are lower than that of H_2O_2 (3). Interestingly, the results of Fig. 4 show similar turnover numbers for all peroxides, implying that the binding or reaction of peroxide with the enzyme is not the rate-limiting step in the overall reaction. When the assays were carried out in the absence of either TrxA or TrxB, no oxidation of NADPH to $NADP^+$ occurred, confirming that *P. aeruginosa* Tpx is a thioredoxin-dependent peroxidase.

Physiological functions of *P. aeruginosa tpx*. The physiological role of *tpx* in *P. aeruginosa* PAO1 was evaluated using a Δ*tpx* mutant. The resistance levels against peroxides, including CHP (1.5 mM), BHP (1.0 mM), and H_2O_2 (0.5 mM), and superoxide generators of the mutant, including PB (1.0 mM) and PQ (0.5 mM), were determined using the plate sensitivity assay and were compared to the PAO1 wild type. No significant differences in the resistance levels against organic hydroperoxides (CHP and BHP) and PB between the Δ*tpx* mutant and the PAO1 wild type were observed (data not shown). This finding is quite unexpected, because purified *P. aeruginosa* Tpx showed high catalytic efficiency for organic hydroperoxides, particularly for CHP. This point raises the question of whether Tpx functions as an organic hydroperoxide thiol peroxidase *in vivo*. *P. aeruginosa* has evolved multiple systems to efficiently detoxify organic hydroperoxides (41, 42). It produces four organic hydroperoxide reductase enzymes, AhpA, AhpB, AhpCF, and Ohr. Thus, the absence of Tpx alone has no obvious effect on the bacterium's ability to detoxify organic hydroperoxides. Moreover, the resistance levels of the Δ*tpx* mutant against redox-cycling drugs/superoxide-generating substances (plumbagin and paraquat) were comparable to the corresponding levels in PAO1 (data not shown).

In many bacteria, the *tpx* mutant has decreased resistance to H_2O_2 (8, 16, 30). Hence, the resistance to moderate (0.5 mM) and high (20 and 50 mM) concentrations of H_2O_2 in the Δ*tpx* mutant and in the PAO1 wild type were determined. The Δ*tpx* mutant was more than 10-fold more sensitive to 0.5 mM H_2O_2 than the PAO1 wild type (Fig. 5). This altered phenotype could be complemented in the Δ*tpx* mutant carrying pTrxA for *trans*-expression of *tpx* from the pUFR047 plasmid (18). Surprisingly, when the Δ*tpx* mutant and PAO1 wild type were treated with lethal concentrations of H_2O_2 (20 and 50 mM) for 30 min and surviving cells were scored using viable cell counts, no significant changes in the resistance levels against H_2O_2 were observed (data not shown). In general, *P. aeruginosa* has redundant systems to scavenge H_2O_2 generated within cells or from exogenous sources. It produces several isozymes of catalases and peroxiredoxins that are capable of degrading H_2O_2 (2, 24, 39, 42). Total catalase activity in the Δ*tpx* mutant and in the parental PAO1 wild type were measured to test whether the H_2O_2-sensitive phenotype of the Δ*tpx* mutant arose from changes in the total catalase activity levels. The total catalase activity of the Δ*tpx* mutant was 347.3 ± 8.5 U mg^{-1} protein, and that for the PAO1 wild type was 356.1 ± 16.7 U mg^{-1}. Thus, the H_2O_2-sensitive phenotype of the Δ*tpx* mutant was not due to a lowering of total catalase activity. Based on currently available data, *P. aeruginosa* possesses three different monofunctional cata-

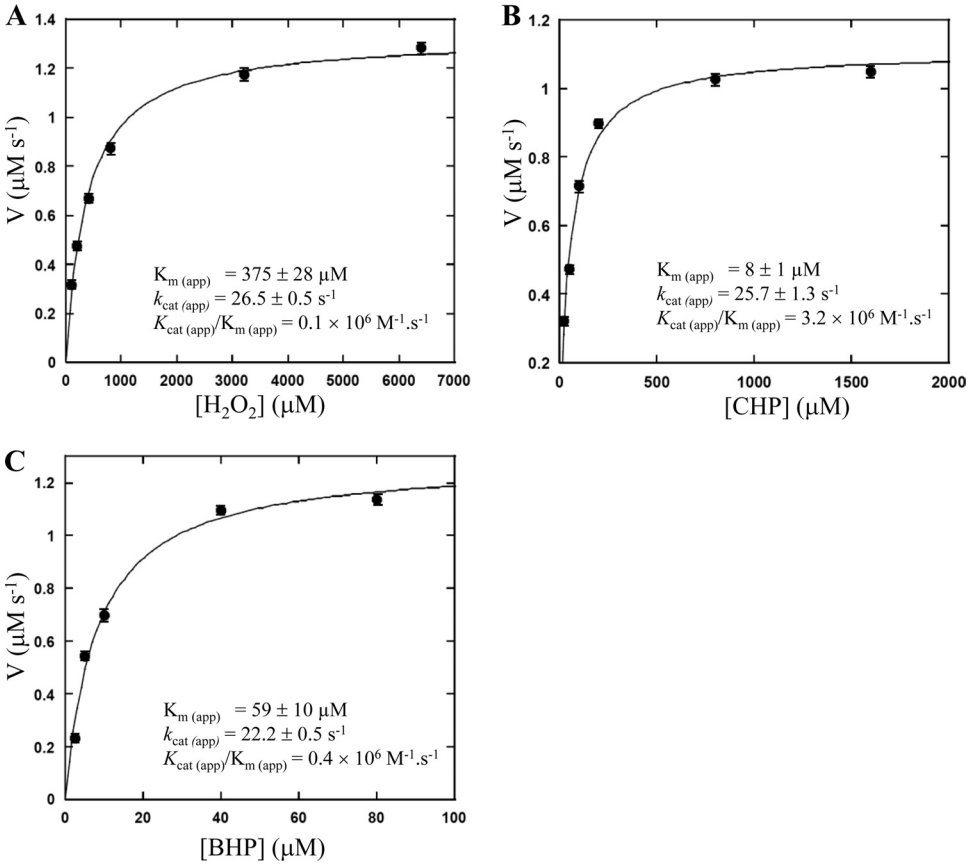

FIG 4 Michaelis-Menten plots of *P. aeruginosa* Tpx activity versus peroxide substrates. The reaction mixtures contain 100 μM TrxA, 1.5 μM TrxB, 150 μM NADPH, 0.05 μM Tpx, and indicated concentrations of H_2O_2 (A), cumene hydroperoxide (CHP) (B), or t-butyl hydroperoxide (BHP) (C). The $K_{m\ (app)}$, $k_{cat\ (app)}$, and $k_{cat\ (app)}/K_{m\ (app)}$ values were calculated using KaleidaGraph.

lases, namely, KatA, KatB, and KatE (37, 39, 42), but lacks a homolog of a bifunctional catalase-peroxidase (KatG) (49). Monofunctional catalases typically have relatively high apparent K_m values for the reduction of H_2O_2 (38 to 200 mM) compared to bifunctional catalase-peroxidases (~5 mM) (48, 50). Interestingly, our data indicate that the $K_{m\ (app)}$ for H_2O_2 of Tpx is much lower than the K_m for H_2O_2 of catalases (Fig. 4). For many metabolic enzymes, K_m values are similar to *in vivo* concentrations of substrates. It can be envisaged that Tpx is the main enzyme that defends against H_2O_2 at ~0.5 mM because its K_m value is in this range. This notion is also supported by the finding that the Δ*tpx* mutant is only sensitive to 0.5 mM H_2O_2 and not to 20 or 50 mM concentrations (Fig. 5 and data not shown). Our data suggest that Tpx plays a disparate role in the protection of *P. aeruginosa* against H_2O_2. Tpx may offer protection against submillimolar levels of H_2O_2 in *P. aeruginosa*, whereas other systems, such as catalases, may play a role at higher mM concentrations. The question remains as to why, if *tpx* is important in H_2O_2 protection, the mutation results in only a small reduction in the peroxide resistance level. The small reduction in the resistance level to 0.5 mM H_2O_2 in the *tpx* mutant (10-fold) is most likely due to the presence of multiple *P. aeruginosa* alkyl hydroperoxide reductase (AhpC) enzymes which have lower K_m values for H_2O_2 than for catalases. These AhpC enzymes contribute to the protection of the bacteria against lower concentrations of H_2O_2. The results clearly show that *P. aeruginosa* Tpx has a novel physiological role in H_2O_2 protection that has not been observed in other bacteria.

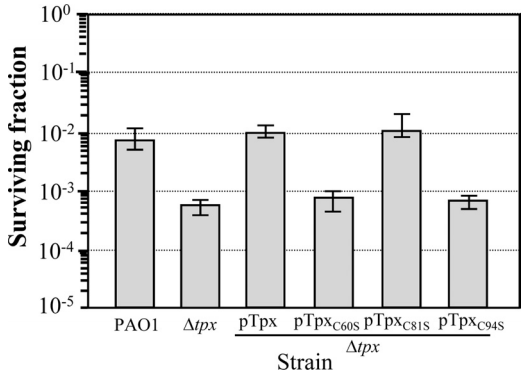

FIG 5 Functional analysis of *tpx* in *P. aeruginosa.* The resistance level of the PAO1 wild type, Δ*tpx* mutant, or the mutants harboring pTpx, $pTpx_{C60S}$, $pTpx_{C81S}$, or $pTpx_{C94S}$ was determined using a plate sensitivity assay in the presence of 0.5 mM H_2O_2. The surviving fraction was calculated by counting the CFU on the LB plates containing H_2O_2 divided by the CFU on the LB plates without H_2O_2. Error bars indicate the means ± SD from three independently performed experiments.

Tpx has been shown to contribute to the pathogenicity of some bacteria. In *P. aeruginosa* PAO1, Tpx may provide the first line of defense against host-generated peroxides. Thus, the effect of *tpx*

inactivation on the pathogenicity of *P. aeruginosa* was assessed using the *C. elegans* nematode model (51). Fast killing of *C. elegans* is believed to be based on cyanide poisoning, which leads to inhibition of mitochondrial cytochrome oxidase, while slow killing requires proliferation of live bacteria in the worm gut (21, 51). We found that the percent survival of nematodes fed the Δ*tpx* mutant under both fast-killing and slow-killing conditions was not significantly different from that of nematodes fed the PAO1 wild-type strain at all time points (data not shown). The results suggest that *tpx* was not required for *P. aeruginosa* pathogenicity when *C. elegans* was used as an animal model.

Role of conserved cysteine residues in Tpx. *P. aeruginosa* Tpx contains three cysteine residues at positions Cys-60, Cys-81, and Cys-94. The importance of these three cysteine residues in terms of H_2O_2 detoxification was evaluated through single-site-directed mutagenesis to replace Cys-60, Cys-81, and Cys-94 with serine residues. The mutated tpx_{C60S}, tpx_{C81S}, and tpx_{C94S} genes were cloned into the broad-host-range expression vector pUFR047, yielding $pTpx_{C60S}$, $pTpx_{C81S}$, and $pTpx_{C94S}$, respectively. The ability of these recombinant plasmids to complement the H_2O_2-sensitive phenotype of the *P. aeruginosa* Δ*tpx* mutant was tested. The expression of tpx_{C81S} was able to complement the phenotype of the Δ*tpx* mutant to a level similar to that of wild-type *tpx* complementation, whereas tpx_{C60S} and tpx_{C94S} failed to increase the H_2O_2 resistance level of the Δ*tpx* mutant (Fig. 5). Thus, *P. aeruginosa* Tpx is a 2-Cys peroxiredoxin that requires Cys-60 and Cys-94 residues for its function as an H_2O_2 scavenger. However, we cannot rule out that a change in protein stability resulting from a point mutation occurred.

P. aeruginosa Tpx Cys-60 and Cys-94 correspond to Cys-61 and Cys-95 in the *E. coli* Tpx, which function as peroxidatic and resolving cysteines, respectively (3). The enzyme catalyzes the reduction of peroxide, which first entails oxidation of the thiol group of the peroxidatic cysteine, which then reacts with the thiol group of the resolving cysteine located within the same polypeptide, thereby forming an intramolecular disulfide bond (3).

Conclusion. We present here the gene regulation, biochemical properties, and physiological roles of a *P. aeruginosa* antioxidant enzyme, Tpx. The presented data suggest that *tpx* is a novel member of the IscR regulon whose expression was induced in response to thiol-depleting agents and redox-cycling drugs. Cytoplasmic Tpx has thiol-dependent peroxidase activity with higher substrate affinity for organic hydroperoxides than for H_2O_2. Phenotypic analysis of the *tpx* mutant reveals differential patterns of H_2O_2 sensitivity with different H_2O_2 concentrations. The mutant is sensitive to submillimolar but not millimolar concentrations of H_2O_2. Incidentally, this sensitivity to submillimolar concentrations of H_2O_2 is in the range of the K_m value of Tpx for H_2O_2. It is likely that Tpx offers a major defense against H_2O_2 in the submillimolar but not the millimolar range. Based on the *C. elegans* results, Tpx does not contribute to the virulence of *P. aeruginosa*.

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