Supporting Information

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Materials and Methods

DTNB Assay. The free thiol contents of reduced and oxidized wild-type and mutant MexR were determined by using the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay (1). The excess β-mercaptoethanol in purified protein was removed by buffer exchange column (Bio-Rad) with 2× DTNB assay buffer [200 mM KH_2PO_4·K_2HPO_4, 200 mM NaCl, 2 mM EDTA (pH 7.0)]. The MexR samples were treated with 3 equivalents of cumene hydroperoxide (CHP) for 1 h at room temperature to obtain the oxidized form. Excess CHP was removed by buffer exchange. The reduced or oxidized protein sample was denatured by mixing with the same volume of 8 M guanidine HCl and heating at 95°C for 20 min followed by quenching on ice. After addition of DTNB, the spectrum of the sample was taken against a blank without the protein. The $A_{412\,nm}$ was recorded, and free thiol concentrations were calculated based on the absorbance.

Construction of *mexR* Mutant Complemented by a Low-Copy Plasmid Encoding a FLAG-Tagged *mexR*. A DNA fragment of C-terminal FLAG-tagged *mexR* with its upstream promoter (500 bp) was amplified by PCR from *Pseudomonas aeruginosa* PAO1 genomic DNA (5′ primer, 5′-CAAGCTGAATTCTGAGGATGATGC-CGTTC-3′; and 3′ primer, 5′-TCTAGAGGATCCTTACTT-GTCATCGTCGTCCTTGTAGTCAATATCCTCAAGCGG-3′) and subcloned into a shuttle plasmid pMM67HE (low copy) (2) between EcoRI and BamHI sites in *Escherichia coli* DH5α (Fig. S6). The cloned vector was transformed into *mexR* mutant (3) and selected for the complementary strain with antibiotics.

RNA Isolation and Northern Blot Analysis. The overnight *P. aeruginosa* cultures were diluted 100-fold into LB medium, grown at 37°C for 2.5 h to an A_{600} of ≈0.8, then incubated with H_2O_2, CHP, or different antibiotics at 37°C for 30 min (Fig. S5 *A* and *B*), 60 min (Fig. S5*C*), or 75 min (Fig. S5*D*) before being harvested. Total RNAs were extracted according to ref. 4 (Fig. S5*A*) or by a Qiagen RNeasy mini kit (Fig. S5 *B–D*). For Northern blot analysis, 15 μg of RNA (Fig. S5*A*) or 11 μg of RNA (Fig. S5 *B–D*) was loaded onto a formaldehyde–agarose (1%) gel. RNA samples were separated by electrophoresis and blotted onto a nylon membrane (Hybond N; Amersham). Northern blots were hybridized with a DNA probe [PCR from genomic DNA of *P. aeruginosa* PAO1: 571-bp *oprM* (left primer, 5′-AGTTGCAGCTGACCAAGGAC-3′; right primer, 5′-GTCT-GGATCGCCTTCTCGTA-3′), 585-bp *mexB* (left primer, 5′-CAA CATCCAGGACCCACTCT-3′; right primer, 5′-ATCACCAGGAACACGAGGAG-3′), and 583-bp *mexA* (left primer, 5′-GCAGACGGTGACCCTGAATA-3′; right primer, 5′-GTATTGGCTACCGTCCTCCA-3′)] labeled with [α-^{32}P]dCTP by using a random priming method. After washes, hybridization signals were exposed and quantified by a PhosphorImager (Bio-Rad).

Mass Spectrometric Characterization of Oxidized MexR. Protein digestion and ESI-Q-TOF mass spectrometric analysis of oxidized MexR was performed as follows. The covalently linked dimer band was excised from an SDS/polyacrylamide gel, and a 50 mM ammonium bicarbonate/50% acetonitrile solution was used to destain the band before digestion by 12-h incubation at 37°C in 35 μl of 5.9 ng/μl trypsin (Promega). Peptide extraction was performed in two rounds; first with 30 μl of 2% acetonitrile, 1% formic acid, then with 24 μl of 60% acetonitrile. Extractions were lyophilized, resuspended in 15 μl of 0.1% trifluoroacetic acid, and subjected to C18 Zip-Tip (Millipore) purification according to the manufacturer's protocol for desalting before analysis by mass spectrometry. Sample analysis proceeded with an ABI QSTAR XL (Applied Biosystems/MDS Sciex) hybrid Q-TOF MS/MS mass spectrometer equipped with a nanoelectrospray source (Protana XYZ manipulator). Positive-mode nanoelectrospray was generated from borosilicate nanoelectrospray needles at 1.5 kV. TOF mass spectra were obtained by using the Analyst QS software with an m/z range of 100–2,000. The m/z response of the instrument was calibrated daily with standards from the manufacturer. Species of interest were selected by using the quadrupole and subjected to collisionally activated dissociation (MS/MS) for confirmation of peptide identification. The fragmentation data were manually interpreted and correlated to the peptide primary amino acid sequence.

Prediction of the Structure of Oxidized MexR. Protein Data Bank (PDB) ID code 1LNW (5) contains four structures of the MexR dimer with cysteines 30 and 62 in their reduced forms. We independently prepared each of these structures for molecular dynamics simulations for prediction of the oxidized structure as follows. We represented the protein in aqueous solution with the CHARMM all-hydrogen (c22) topology and parameter sets (6, 7) together with the GBSW implicit solvent model (8); dihedral angle terms were treated with the CMAP parameterization appropriate for use with GBSW (9, 10). Using CHARMM version c34a1, coordinates missing because of disorder (residues 85–92) were constructed from idealized internal coordinate values, hydrogens were added, and the protonation states of histidines were set based on a grid-based Poisson–Boltzmann procedure (11). The structure was then relaxed by minimizing the energy with a combination of the steepest descents (SD) and adopted basis Newton–Raphson (ABNR) algorithms until the root mean square of the gradient components reached 0.01 kcal/mol Å. All subsequent calculations used Langevin dynamics at 300 K. The friction coefficients of the protein backbone heavy atoms were set to the corresponding values as described previously (12), and those of the side chain heavy atoms were set uniformly to the value of C^β in that work (84.38 ps^{-1}); the random force was not applied directly to any of the hydrogen atoms. SHAKE (13) was used to constrain the lengths of bonds to hydrogen atoms, and an integration time step of 1 fs was used.

We initially used a steered molecular dynamics (SMD) procedure (11) to bring cysteines 30 and 62 close enough to each other to form the disulfide bonds. In this procedure, we apply harmonic restraints of the form $k[r(t) - r_0(t)]^2$, where k is a force constant, r is the separation of two atoms at time t, and r_0 is their target separation; r_0 is decreased by an amount Δr_0 immediately after satisfaction of the criterion $r(t) \langle r_0(t)$. The pairs of atoms considered were the S^γ atoms of cysteines 30 and 62 and the C^β atoms of those same residues. The minimum r_0 value was 2.1 Å for the former pair of atoms and 3.9 Å for the latter; k = 100 kcal/mol $Å^2$ and Δr_0 = 0.1 Å in both cases. During these simulations, the secondary structure of the monomers was maintained by applying harmonic restraints with force constants of 200 kcal/mol $Å^2$ to minimize the mass-weighted rmsd values of the backbone heavy-atom coordinates of residues 7 to 72, 37 to 49, 53 to 61, and 65 to 78 with respect to their relative positions in the minimized structures. The total length of each such simulation was 100 ps. Once cysteines 30 and 62 were close to each other, we introduced a disulfide bond and parameters appropriate for the oxidized form. The system was then equil-

ibrated for an additional 100 ps with the rmsd restraints on the α-helices mentioned above. Finally, sequential simulations of 20-ps length were performed with the force constants for these restraints progressively reduced to 100, 50, 20, 10, and 0 kcal/mol Å^2.

Plate Assay for Antibiotic Sensitivity. Four *P. aeruginosa* strains (wild-type PAO1 with empty plasmid pAK1900 as control, *mexR* mutant PAO1 with empty pAK1900, *mexR* mutant PAO1 complemented with wild-type *mexR* in pAK1900, and *mexR* mutant PAO1 complemented with *mexRC30SC62S* (C30S/C62S double mutant) in pAK1900) were grown at 37°C overnight in LB with 150 μg/ml carbenicillin to ensure plasmid maintenance. Overnight cultures were diluted 100-fold into the same medium and further grown at 37°C to $A_{600\,\mathrm{nm}} \approx 0.8$. Then, the cultures were subjected to a five times 10-fold dilution with LB. The 5-μl portion of bacterium was spotted to 2% agar LB plate with 150 μg/ml carbenicillin and corresponding antibiotics or CHP. The plates were grown at 37°C for 16–24 h before examination.

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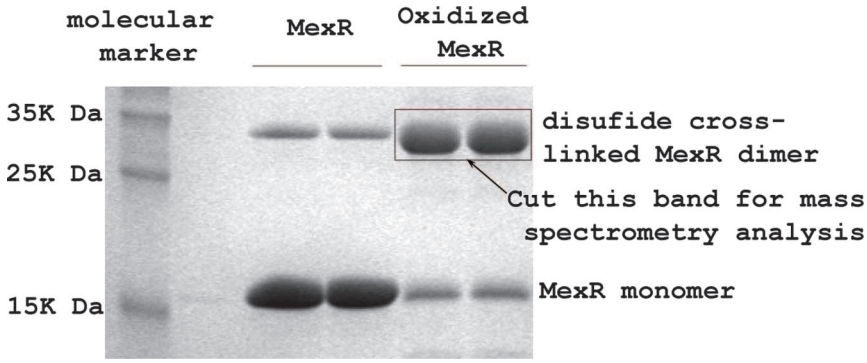

Fig. S1. Formation of cross-linked MexR dimer. MexR (35 μM) was treated with and without 350 μM CHP at 37°C for 1.5 h before 100 μM iodoacetamide was added to alkylate-free thiols. Protein samples were analyzed by nonreducing, denaturing SDS/PAGE. The covalently linked dimer band was excised, in-gel digested, and used for mass spectrometry experiment.

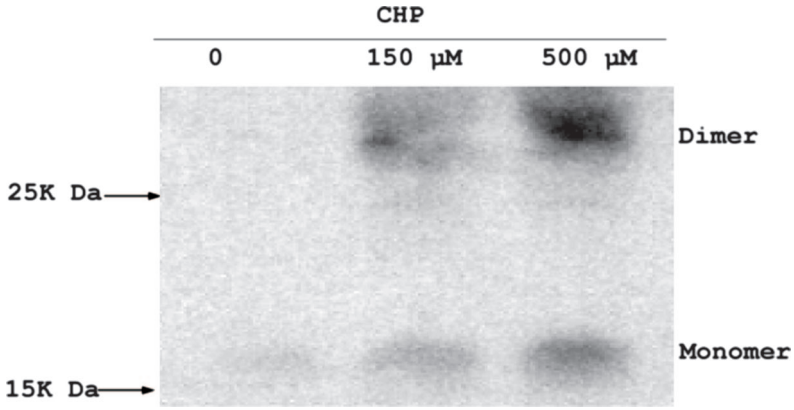

Fig. S2. Formation of covalently linked MexR dimer *in vivo*. Cells of *mexR* mutant complemented with a FLAG-tagged *mexR* were grown and treated with 1, 0.15, or 0.5 mM CHP at 37°C for 1 h before harvesting. Proteins were separated on nonreducing, denaturing SDS/PAGE, and MexR bands were visualized by Western blotting with an anti-FLAG antibody.

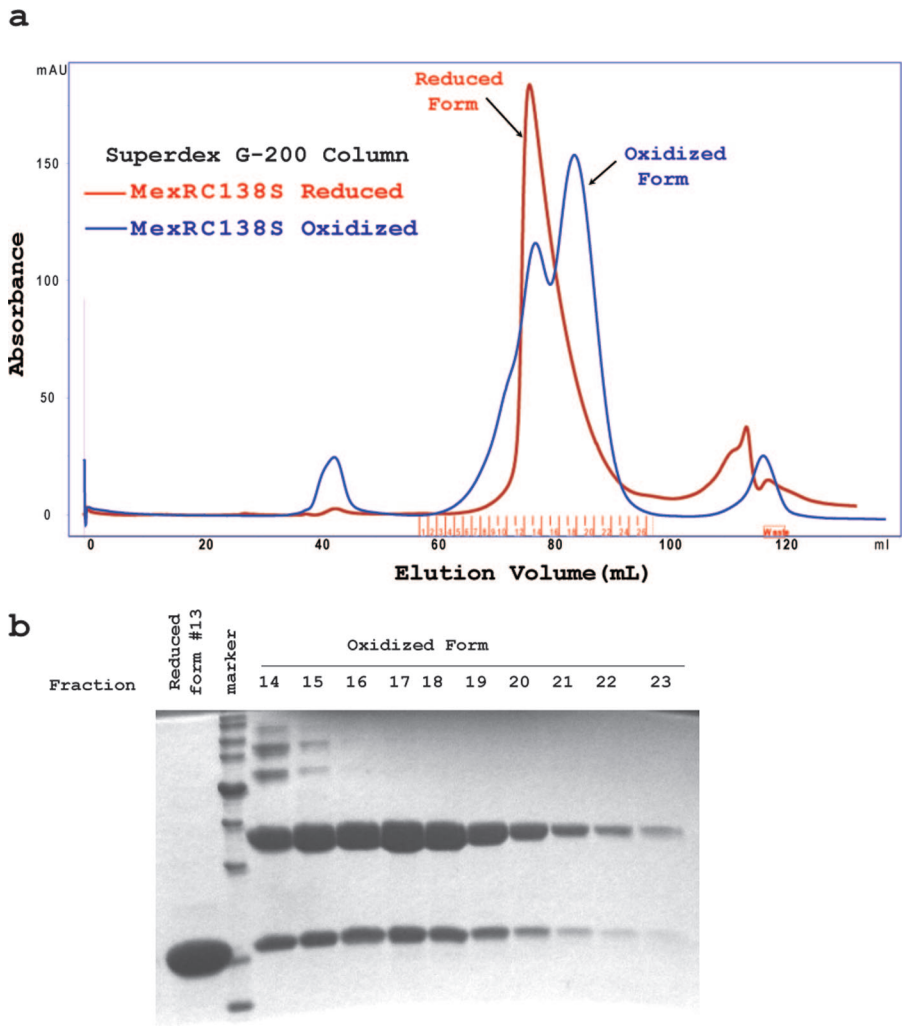

Fig. S3. Characterization of reduced and oxidized MexRC138S by gel filtration chromatography. (*a*) The reduced and oxidized MexR C138S eluted differently on Supperdex G-200 gel filtration column with 10 mM Tris·HCl (pH 7.4) and 100 mM NaCl. (*b*) Fractions of protein samples were analyzed by nonreducing, denaturing SDS/PAGE.

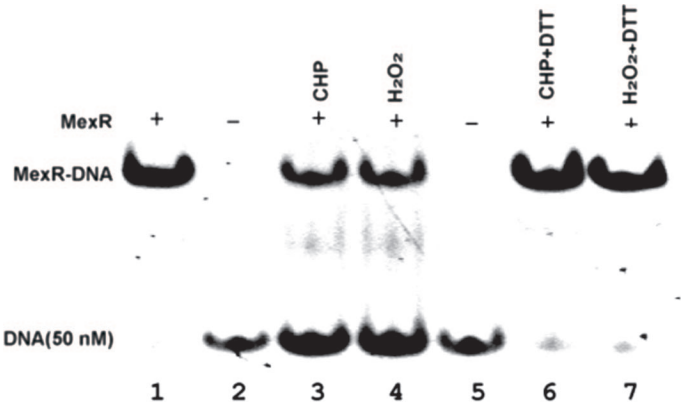

Fig. S4. The oxidation-induced dissociation of MexR from DNA is reversible. Oxidation of MexR (3 μM, dimer) with CHP (125 μM, lane 3) or H_2O_2 (125 μM, lane 4) led to dissociation of MexR from DNA. Addition of excess of DTT (2.5 mM) to oxidized MexR samples restored the protein binding to DNA (lanes 6 and 7).

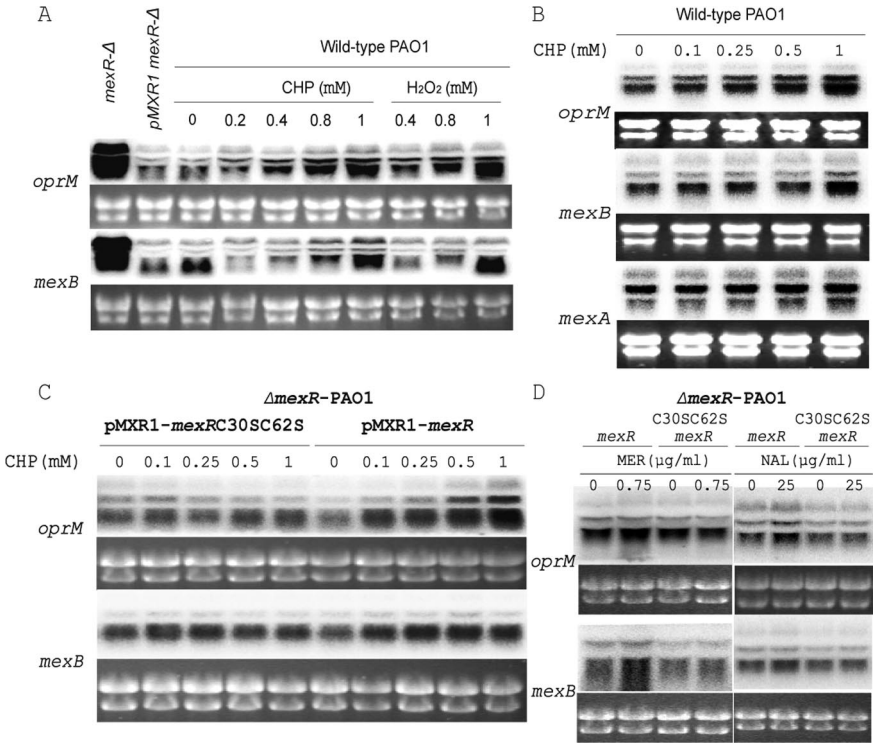

Fig. S5. Effects of peroxide stress and antibiotics on the activation of the *mexAB–oprM* operon. Northern blot analysis was performed on RNA (with the ethidium bromide gel picture of the loaded RNA sample shown below each lane) isolated from PAO1, *mexR* mutant (*mexR*-Δ), *mexR* mutant complemented with pMXR1-*mexR* (*mexR*-Δ–pMXR1–*mexR*), and mutant complemented with pMXR1–*mexRC30SC62S* (*mexR*-Δ-pMXR1-*mexRC30SC62S*). (*A*) Northern blots of RNA samples (15 μg) isolated from the wild-type PAO1, *mexR*-Δ, and *mexR*-Δ–pMXR1–*mexR* strains treated with various concentrations of CHP or H_2O_2 for 30 min before harvesting. (*B*) Northern blots of RNA samples (11 μg) isolated from the wild-type PAO1 strain treated with various concentrations of CHP for 30 min before harvesting. (*C*) Northern blots of RNA samples (11 μg) isolated from the *mexR*-Δ–pMXR1–*mexR* and *mexR*-Δ–pMXR1–*mexRC30SC62S* strains treated with various concentrations of CHP for 60 min. (*D*) Northern blots of RNA samples (11 μg) isolated from the *mexR*-Δ–pMXR1–*mexR* and *mexR*-Δ–pMXR1–*mexRC30SC62S* strains treated with different concentrations of MER and NAL for 75 min.

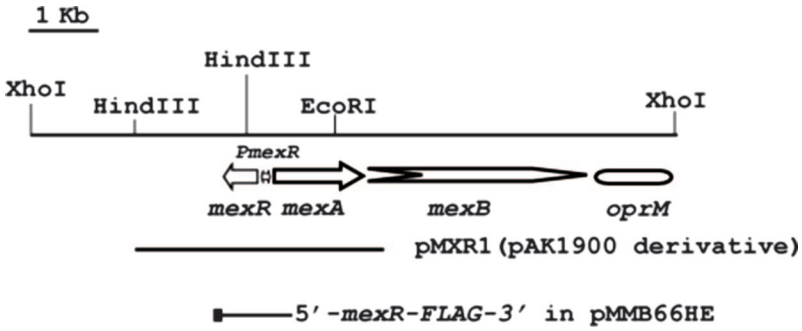

Fig. S6. Genomic sketch map of *mexAB–oprM* operon (14). The C-terminal FLAG-*mexR* was subcloned into a low-copy plasmid pMMB66HE between the EcoRI and BamHI sites.