Supporting Information

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SI Experimental Procedures

Protein Expression and Purification. All of the PaOxyR proteins and its derivatives were expressed in *Escherichia coli* strain BL21 (DE3). The *E. coli* that was transformed with each plasmid was cultured in LB medium at 37 °C until OD_{600} reached 0.5–1.0, after which the protein was induced by adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 6 h at 30 °C. For the selenomethionyl-labeled PaOxyR RD, *E. coli* strain B834(DE3) was cultured in M9 medium containing L-(+)-selenomethionine, 100 μg/mL ampicillin and other cofactors and was induced by 0.5 mM IPTG for 1 d at 30 °C. The cells were harvested by centrifugation at 1,380 × *g* for 10 min and resuspended in 50 mL of lysis buffer containing 20 mM Tris·HCl (pH 8.0), 150 mM sodium chloride, and 2 mM 2-mercaptoethanol. The resuspended cells were disrupted by one pass through a French press at a pressure of 20 kpsi. The cell lysate was centrifuged for 30 min at 10,000 × *g* to remove the cell debris. All of the proteins were purified by Ni-NTA affinity chromatography. The FL proteins were directly subjected to a HiLoad Superdex 16/60 200 column (GE Healthcare) that was equilibrated with 20 mM Tris·HCl (pH 7.5), 200 mM NaCl, and 2 mM TCEP. For PaOxyR RDs, the hexahistidine tag was eliminated by overnight treatment of the TEV protease at room temperature after Ni-NTA chromatography. Then, PaOxyR RD proteins were further purified using a HiTrap Q anion-exchange column (GE Healthcare) and a HiLoad Superdex 16/60 200 column (GE Healthcare) equilibrated with 20 mM Tris·HCl (pH 7.5), 200 mM NaCl, and 2 mM TCEP. The purified proteins were concentrated to 15 mg/mL and then frozen at −173 °C.

Crystallization, Data Collection, and Structural Determination of the OxyR RD. The PaOxyR RDs were crystallized by the hanging-drop vapor-diffusion method at 14 °C, and each precipitation solution was cryoprotected by adding 20% (vol/vol) glycerol. The X-ray diffraction data were collected at −173 °C in a liquid nitrogen stream. The Se-PaOxyR RD WT protein was crystallized in precipitation solution containing 0.1 M Tris·HCl (pH 8.5), 24% (wt/vol) PEG 4k, and 2 mM TCEP. A single-wavelength anomalous diffraction (SAD) dataset was collected at Pohang Accelerator Laboratory beamline 5C and processed with the HKL2000 package (1). The crystals belong to space group $P3_112$ with unit-cell dimensions of a = 151.3 Å, b = 151.3 Å, and c = 218.8 Å. Phase calculation, density modification, and model building were performed by the program PHENIX AutoSol (2). The initial model from AutoSol was rebuilt by the program Coot and refined by the program phenix.refine (3, 4). The structure was refined at a 2.3-Å resolution, resulting in an R factor of 21.7% and an R_{free} of 27.1% (Table S1). The PaOxyR RD (C199D) mutant protein was crystallized in precipitation solution containing 0.1 M Tris·HCl (pH 9.0), 22% (wt/vol) PEG 4k, 5 mM DTT, and 5 mM TCEP. The crystals belong to space group $P6_1$ with unit-cell dimensions of a = 129.9 Å, b = 129.9 Å, and c = 135.7 Å. The coordinate of PaOxyR RD WT was used as a search model for the molecular replacement method. The structure was refined at a 3.0-Å resolution, resulting in an R factor of 22.9% and an R_{free} of 26.0% (Table 1).

Measurement of Stress Sensitivity. To determine peroxide stress sensitivity, a spotting assay was performed as previously described (5). Briefly, the cells were grown at 37 °C to the logarithmic phase (OD_{600} 0.7). Aliquots (3 μL) of cultures that were serially diluted 10-fold in LB broth were spotted onto LB agar with or without 200 or 500 μM H_2O_2.

Thiol Trapping by 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic Acid. For the in vivo thiol trapping of OxyR, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was used as previously described (6). Briefly, *oxyR* mutant bacteria with either FLAG-tagged PaOxyR or FLAG-tagged PaOxyR (H198A) were grown at 37 °C to the logarithmic phase (OD_{600} 0.7). For the oxidation-susceptibility experiment, aliquots of the bacterial cultures (1 mL) were treated with H_2O_2 of various concentrations for 10 s and mixed with 110 μL of ice-cold 100% trichloroacetic acid (TCA) followed by centrifugation. For the rereduction experiment, the bacterial cultures were pulse-treated with 1 mM H_2O_2, and their aliquots (1 mL) were mixed with TCA as above at various time points and harvested. The cell pellets were resuspended in 400 μL of 10% TCA and disrupted by sonication. After centrifugation, the pellets were mixed with 20 μL of AMS buffer [10 mM AMS, 0.5 M Tris·HCl, pH 8.0, 2% (wt/vol) SDS, 100 mM NaCl, 1 mM EDTA, and 5% (vol/vol) glycerol] and incubated in the dark for 1 h, followed by Western blot analysis using an anti-FLAG M2 antibody (Sigma).

1. Rice LM, Earnest TN, Brunger AT (2000) Single-wavelength anomalous diffraction phasing revisited. *Acta Crystallogr D Biol Crystallogr* 56(Pt 11):1413–1420.
2. Terwilliger TC, et al. (2009) Decision-making in structure solution using Bayesian estimates of map quality: The PHENIX AutoSol wizard. *Acta Crystallogr D Biol Crystallogr* 65(Pt 6):582–601.
3. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60(Pt 12 Pt 1):2126–2132.
4. Afonine PV, et al. (2010) Joint X-ray and neutron refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* 66(Pt 11):1153–1163.
5. Bae HW, Cho YH (2012) Mutational analysis of *Pseudomonas aeruginosa* OxyR to define the regions required for peroxide resistance and acute virulence. *Res Microbiol* 163(1):55–63.
6. Heo YJ, et al. (2010) The major catalase gene (katA) of *Pseudomonas aeruginosa* PA14 is under both positive and negative control of the global transactivator OxyR in response to hydrogen peroxide. *J Bacteriol* 192(2):381–390.

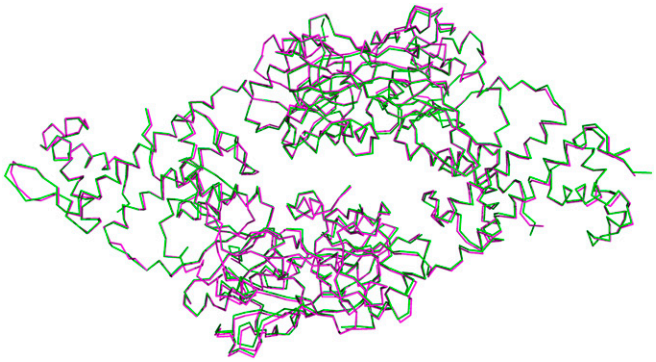

Fig. S1. Structural superposition of FL PaOxyR and PaOxyR (C199D). PaOxyR is displayed in magenta, and PaOxyR (C199D) is in green.

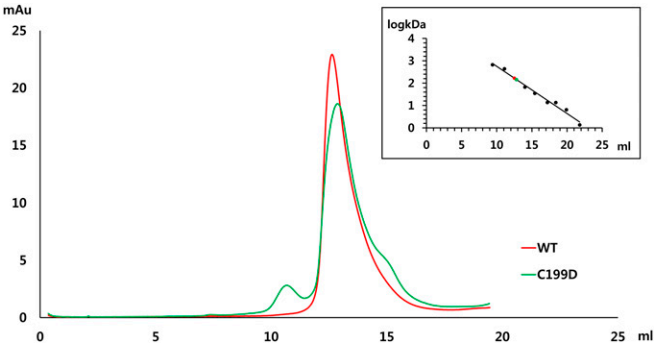

Fig. S2. Elution profiles of the full-length proteins [PaOxyR and PaOxyR (C199D)]. To confirm the molecular masses of FL PaOxyR (wild type) and FL PaOxyR (C199D), size-exclusion chromatography was performed using a Superdex S-200 HR 10/30 column (GE Healthcare) that was equilibrated with 20 mM Tris·HCl (pH 7.5), 200 mM NaCl, and 2 mM TCEP at a flow rate of 0.5 mL/min. The peaks indicate the tetramer [157 kDa for wild-type PaOxyR (red) and 140 kDa for PaOxyR (C199D) (green)], which was calculated based on the standard proteins. The molecular masses were calculated using a standard curve (*Inset*). Au is absorbance at 280 nm.

Fig. S3. Structural superposition of the RD in the FL PaOxyR (C199D) structure (cyan) on the RD structure of wild-type PaOxyR (red).

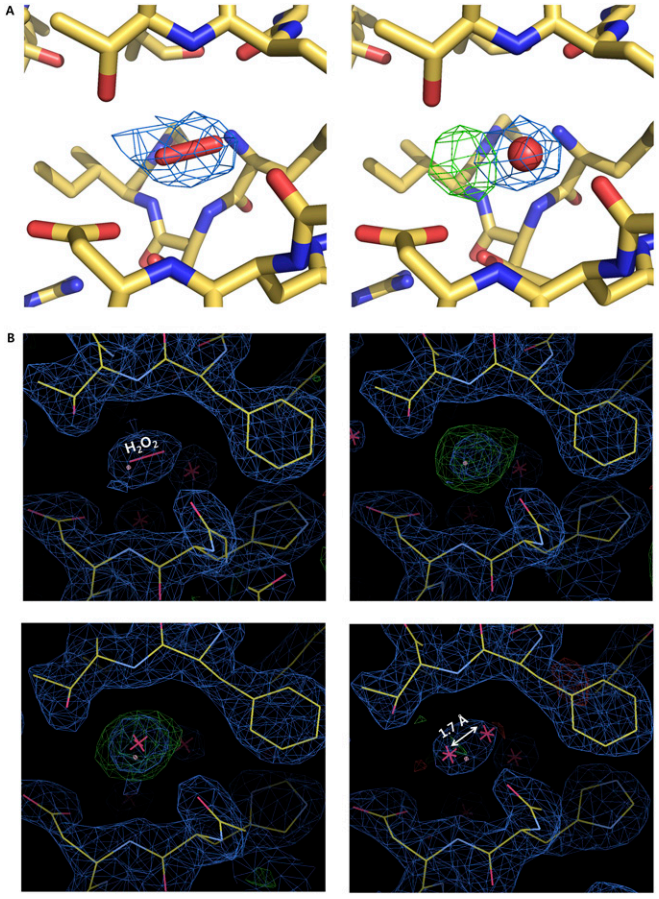

Fig. S4. Electron density map of the putative H_2O_2-binding site. (*A*) The 2.3-Å-resolution structures of FL PaOxyR (C199D) around the putative H_2O_2, superposed onto 2*Fo* − *Fc* (blue) and *Fo* − *Fc* (green) electron density maps contoured at 1.0 and 3.0 σ, respectively, when assuming an H_2O_2 molecule (*Left*) and when assuming a water molecule instead of H_2O_2 (*Right*). (*B*) The 2.0-Å-resolution structure of H_2O_2 vapor-exposed FL PaOxyR (C199D) around the H_2O_2-binding site with *2Fo* − *Fc* and *Fo* − *Fc* electron density maps contoured at 1.9 and 3.0 rmsd, respectively. One H_2O_2 molecule was fitted to the electron density map (*Upper Left*). The electron density maps were refined with three other candidate structures: no molecule is assigned (*Upper Right*), one water molecule is assigned (*Lower Left*), and two water molecules are assigned coordinates (*Lower Right*) at the H_2O_2-binding site. When two water molecules were assigned, the distance between the two water molecules was 1.7 Å (*Lower Right*).

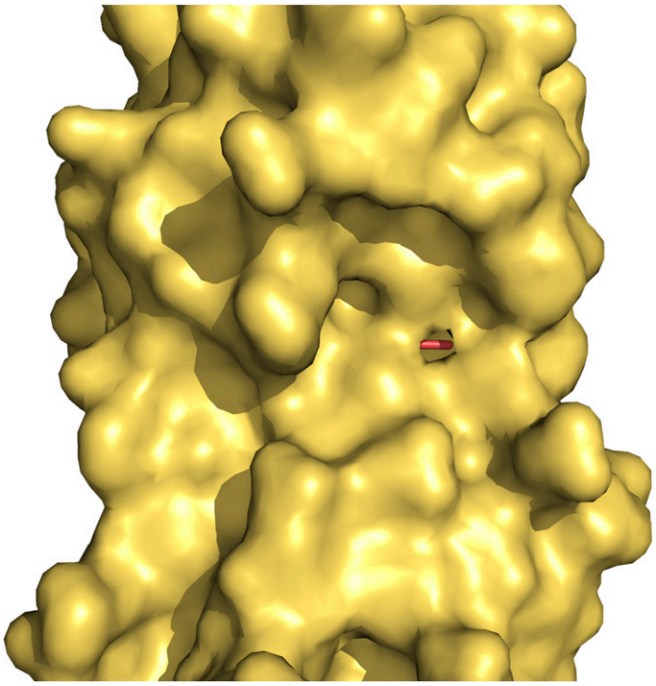

Fig. S5. Surface representation of the PaOxyR (C199D) RD. The H_2O_2 is shown as a red stick in a small pocket between RD-I and RD-II, which is close to Asp199.

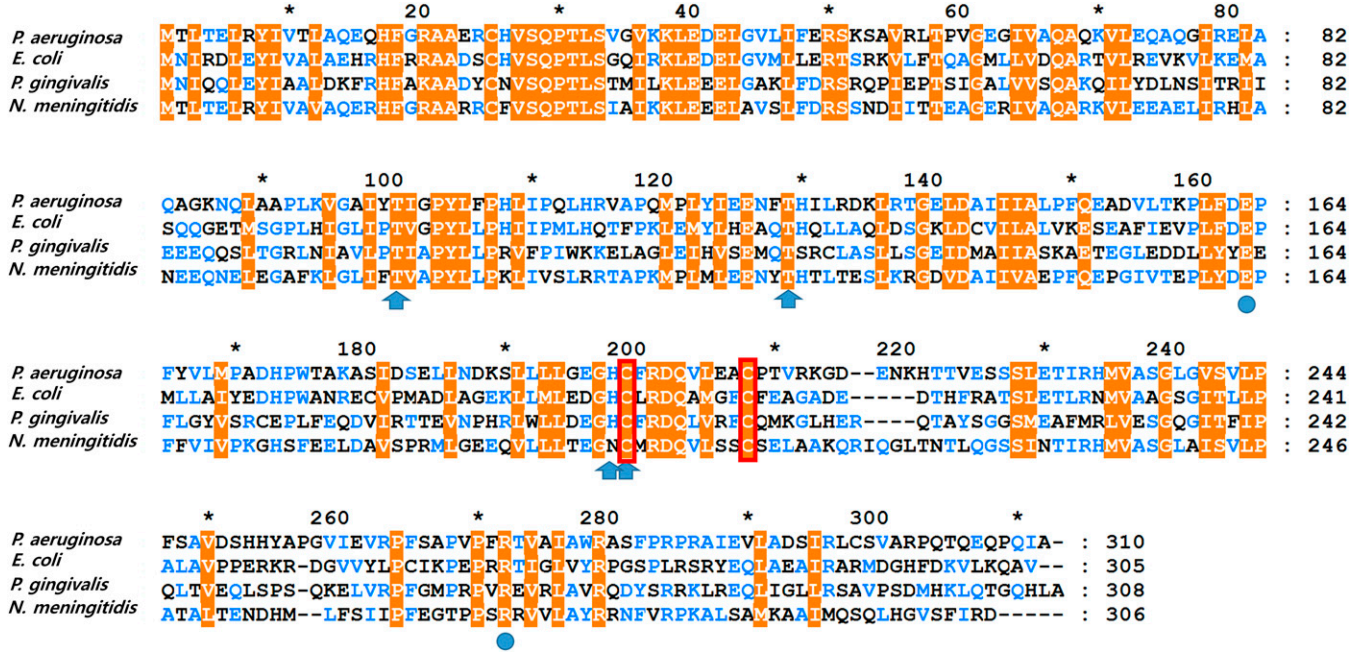

Fig. S6. Amino acid sequences of the OxyR proteins of *P. aeruginosa*, *E. coli*, *Porphyromonas gingivalis*, and *Neisseria meningitidis*. The blue arrows indicate the residues that are involved in binding to H_2O_2 and water molecules (Thr100, Thr129, His198, and Cys199). The red boxes indicate the conserved cysteine residues (Cys199 and Cys208). The blue circles indicate the conserved residues holding the position of Thr100 (Glu163 and Arg270). The strictly conserved amino acid residues are indicated in orange boxes, the moderately conserved amino acid residues are indicated in blue letter, and the nonconserved amino acid residues are indicated in black letter. The gap of sequence alignment is indicated with a dash.

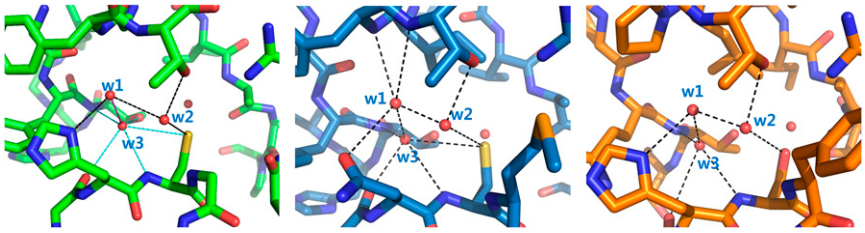

Fig. S7. Water-binding sites near Cys199. PaOxyR RD (*Left*), *N. meningitidis* OxyR (*Middle*), and *P. gingivalis* OxyR (*Right*). In the FL PaOxyR (C199D) structure, H_2O_2 is bound in place of water molecule w3. Water molecules w1 and w2 are commonly observed in the PaOxyR structure.

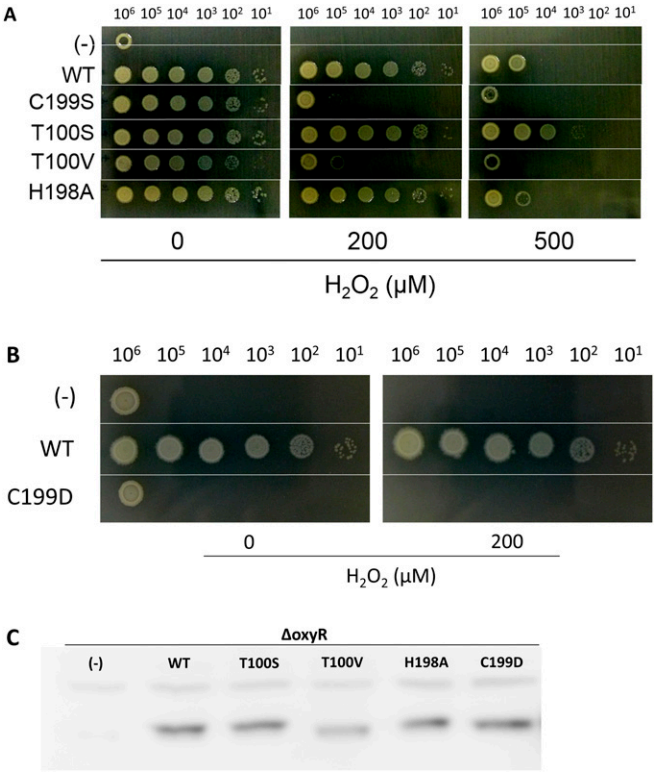

Fig. S8. Susceptibility of *oxyR* mutants to H_2O_2. (*A*) *oxyR* null mutant bacteria with either PaOxyR (WT) or PaOxyR mutant proteins (C199S, T100S, T100V, and H198A) were grown to the logarithmic growth phase. The negative control (-) with the integrated mini-Tn7 vector was included. Tenfold serial dilutions of each sample were spotted onto an LB agar plate containing either nothing (-) or 200 or 500 μM H_2O_2. The numbers (10^1 to 10^6) indicate the cfu of the cell spots. (*B*) Susceptibility of the *oxyR* C199D mutant to H_2O_2. *P. aeruginosa oxyR* null mutant bacteria expressing either PaOxyR (WT) or PaOxyR (C199D) were grown to the logarithmic growth phase. The negative control (-) with the integrated mini-Tn7 vector was included. Tenfold serial dilutions were spotted onto an LB agar plate containing either nothing (-) or 200 μM H_2O_2. The numbers (10^1 to 10^6) indicate the cfu of the cell spots. (*C*) Expression level of the OxyR variant in *P. aeruginosa*. *P. aeruginosa oxyR* null mutant bacteria (Δ*oxyR*) expressing either PaOxyR (WT), PaOxyR (T100S), PaOxyR (T100V), PaOxyR (H198A), or PaOxyR (C199D) were grown to the logarithmic growth phase. The negative control (-) with the integrated mini-Tn7 vector was included. The expression level of each OxyR protein was measured by immunoblotting.

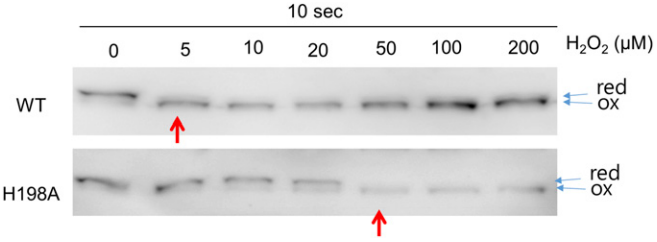

Fig. S9. Oxidation susceptibility of PaOxyR and PaOxyR (H198A). *P. aeruginosa oxyR* mutant bacteria expressing either PaOxyR (WT) or PaOxyR (H198A) were grown to the logarithmic growth phase and subjected to AMS trapping as described in *SI Experimental Procedures*. Bacterial cultures that had been pulse-treated with H_2O_2 at the indicated concentrations (5–200 μM) for 10 s were harvested. The reduced (red) and oxidized (ox) species of OxyR proteins are indicated by blue arrows. The red arrows indicate the lowest concentration of H_2O_2 at which oxidized OxyR is generated.

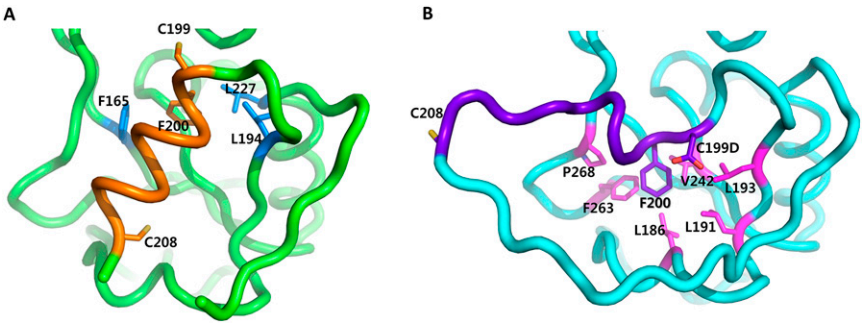

Fig. S10. Structural comparison between the reduced (WT) and intermediate forms (C199D). (*A*) In the reduced form of PaOxyR RD WT, the region of residues 199–208 (orange) formed an α-helix, and Phe200 is located in a hydrophobic pocket consisting of Phe165, Leu194, and Leu227 (blue). (*B*) In the intermediate form of the PaOxyR RD (C199D), the region of residues 199–208 (purple) formed a loop, not an α-helix. In the contrast to the reduced form, Phe200 is in another hydrophobic pocket (magenta), consisting of Leu186, Leu191, Leu193, Val242, and Phe263.

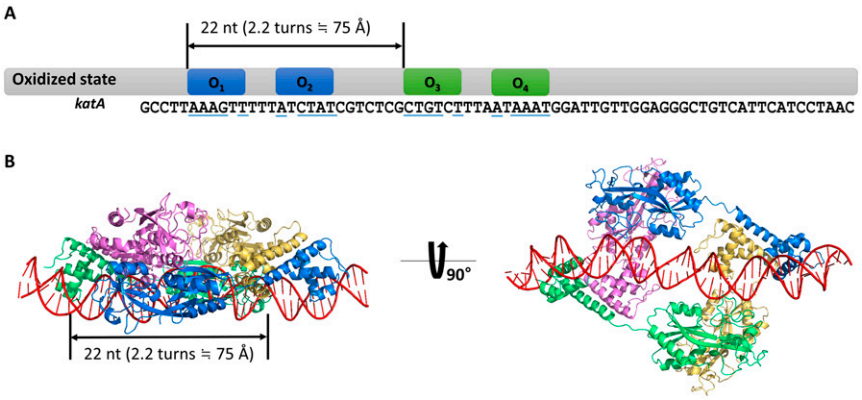

Fig. S11. Modeling of the DNA-binding structures of PaOxyR in the reduced and oxidized states. (*A*) The promoter region of the *P. aeruginosa katA* gene and schematic diagram for the OxyR-binding sites. The putative binding sites of OxyR in the oxidized state are indicated by O1, O2, O3, and O4. The spacings between O1 and O3 (for the oxidized form) are indicated, where the DNA helical turns were calculated based on 10 bp per helical turn. The OxyR-binding nucleotides of *katA* promoter are indicated with blue underline. (*B*) The DNA-docking models of PaOxyR in the oxidized states. Side views (*Left*); bottom views (*Right*). Each protomer is in a different color, and the DNA is shown in red.

Table S1. X-ray diffraction and refinement statistics for the SeMet-labeled PaOxyR RD, wild-type PaOxyR FL, and PaOxyR FL (C199D) variant

Statistics	Se-PaOxyR RD WT	PaOxyR FL WT	PaOxyR FL (C199D)
Data collection			
Space group	$P3_112$	$P12_11$	$P12_11$
Cell dimensions			
a, *b*, *c*, Å	151.4, 151.4, 218.8	70.8, 308.7, 96.2	81.3, 151.0, 141.5
α, β, γ, °	90, 90, 120	90, 99.74, 90	90, 97.69, 90
Resolution, Å	50.0–2.3 (2.34–2.30)	50–4.5 (4.58–4.50)	20.0–2.3 (2.34–2.30)
R_{merge}	0.114 (0.396)	0.157 (0.402)	0.072 (0.379)
$I/\sigma I$	28.7 (3.3)	7.9 (2.7)	20.1 (2.5)
Completeness, %	99.5 (97.8)	88.3 (83.7)	96.4 (92.5)
Redundancy	7.4 (4.2)	2.8 (2.2)	3.7 (2.6)
Refinement			
Resolution, Å	37.89–2.3	37.29–5.0	20.07–2.303
No. of reflections	125,942	15,744	143,413
R_{work}/R_{free}	0.2167/0.2710	0.2574/0.2816	0.2079/0.2510
No. of total atoms	19,492	27,303	18,691
No. of ligand molecules	0	0	24
No. of water molecules	316	0	237
Average *B* factor, Å	39.80	20.00	48.70
Rms deviations			
Bond lengths, Å	0.008	0.018	0.004
Bond angles, °	1.166	2.19	0.81
Ramachandran plot			
Favored, %	97.67	95.97	97.84
Allowed, %	2.29	3.39	2.03
Outliers, %	0.04	0.64	0.13
PDB ID code	4Y0M	—	—

Values in parentheses are for the highest resolution shell.