An EmrB multidrug efflux pump in Burkholderia thailandensis with unexpected roles in antibiotic resistance

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An EmrB multidrug efflux pump in *Burkholderia thailandensis* with unexpected roles in antibiotic resistance

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The antibiotic trimethoprim is frequently used to manage *Burkholderia* infections, and members of the resistance-nodulation-division (RND) family of efflux pumps have been implicated in multidrug resistance of this species complex. We show here that a member of the distinct *Escherichia coli* multidrug resistance B (EmrB) family is a primary exporter of trimethoprim in *Burkholderia thailandensis*, as evidenced by increased trimethoprim (EmrB) family. We also found that the *emrB* gene is up-regulated following the addition of gentamicin and that this up-regulation is due to repression of the gene encoding OstR, a member of the multiple antibiotic resistance regulator (MarR) family. The addition of the oxidants H$_2$O$_2$ and CuCl$_2$ to *B. thailandensis* cultures resulted in OstR-dependent differential *emrB* expression, as determined by qRT-PCR analysis. Specifically, OstR functions as a rheostat that optimizes *emrB* expression under oxidizing conditions, and it senses oxidants by a unique mechanism involving two vicinal cysteines and one distant cysteine (Cys$^3$, Cys$^5$, and Cys$^{169}$) per monomer. Paradoxically, *emrB* inactivation increased resistance of *B. thailandensis* to tetracycline, a phenomenon that correlated with up-regulation of an RND efflux pump. These observations highlight the intricate mechanisms by which expression of genes that encode efflux pumps is optimized depending on cellular concentrations of antibiotics and oxidants.

Bacteria experience diverse environmental challenges during host infection. To survive, they must withstand host-derived stresses, such as changes in temperature, pH, generation of reactive oxygen species (ROS), and nutrient scarcity. In addition, they may encounter antibiotics, disinfectants, and other harmful chemicals. To proliferate in the face of such challenges, bacteria employ diverse transcription factors, which respond to the environmental changes and modulate gene expression. Multiple antibiotic resistance regulator (MarR) family transcriptional regulators often participate in regulating expression of virulence genes in response to environmental cues (1). First characterized in *Escherichia coli* K-12, MarR was shown to play critical roles in conferring resistance to antibiotics, household disinfectants, and oxidative stress (2). Members of the protein family named for *E. coli* MarR are ubiquitous in bacterial species, and the most common mode of transcriptional regulation involves binding to the intergenic DNA between the gene encoding the MarR homolog and divergently or adjacently oriented genes, thereby hindering access of RNA polymerase to the promoter. DNA binding by a given MarR family protein may be modified upon binding to small-molecule ligands or oxidants, a result of which is altered expression of genes in its regulon (1).

Many pathogenic *Burkholderia* species exhibit inherent resistance to a wide range of antibiotics and disinfectants (3). Efflux systems involved in antibiotic resistance include the resistance-nodulation-division (RND) efflux systems and the *E. coli* multidrug resistance B (EmrB) family transporters (3–5). Extensively studied pathogens include *Burkholderia pseudomallei*, a soil saprophyte causing melioidosis, and its clonal derivative *Burkholderia mallei*, a host-adapted pathogen, which is the causative agent of glanders (6). Both are categorized as category B priority pathogens because of the low infectious dose, capability for aerosol transmission, and resistance to antibiotics (3).

By contrast, *Burkholderia cenocepacia* is an opportunistic pathogen that primarily infects immunocompromised patients (7). *Burkholderia thailandensis* is particularly closely related to *B. pseudomallei* and *B. mallei*, and these species are founding members of the *B. pseudomallei* (Bpc) group (8, 9). *B. thailandensis* is much less virulent despite conservation of many genes involved in virulence (10). One of the very early host responses to a bacterial infection is the production of ROS. For example, NADPH oxidase generates superoxide radicals (O$_2^-$), which give rise to H$_2$O$_2$. H$_2$O$_2$ may in turn oxidize ferrous iron to generate highly reactive hydroxyl radicals (OH$^-$) (11, 12). ROS can target bacterial DNA, protein, or membrane lipids and thus can attenuate bacterial survival and proliferation in the host environment (13). Transition metals, such as Cu(II) and Zn(II), can also cause microbial poisoning when in excess (14). In addition, Cu(II) can participate in the Fenton reaction and cause elevated intracellular ROS production.

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This article contains Tables S1–S4.

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The abbreviations used are: ROS, reactive oxygen species; EmrB, *E. coli* multidrug resistance B; EMSA, electrophoretic mobility shift assay; MarR, multiple antibiotic resistance regulator; MIC, minimal inhibitory concentration; OstR, oxidant-sensing transport regulator; RND, resistance-nodulation-division; PAR, 4-(2-pyridylazo) resorcinol; LB, lysogeny broth; LSLB, low-salt LB; qPCR, quantitative PCR.

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Efflux pump with unexpected roles in antibiotic resistance

Bacterial responses to ROS by transcriptional regulators such as MarR family proteins have been described, including in *Burkholderia* species (15, 16); oxidation of cysteine residues, usually by reversible formation of disulfide linkages, leads to conformational changes that allow such transcriptional regulators to promote expression of genes involved in detoxification of ROS or repair of ROS-mediated damage (17). The redox-active Cu(II) has also been implicated in modification of MarR proteins by inducing formation of disulfide linkages between separate dimers, but with distinct outcomes (15, 18).

*B. thailandensis* encodes 12 annotated MarR homologs, all of which are conserved in *B. pseudomallei* and *B. mallei* (19). One of these is encoded by *BTH_I0021*, which is upstream of a gene encoding an EmrB/QacA family drug resistance transporter (Fig. 1A). A model of OstR was predicted using SwissModel and 4AIK (*Yersinia pseudotuberculosis* RovA (20)) as a template, one of the structures with highest sequence identity to OstR (28%). This model shows the expected homodimer, which consists of a dimerization region formed by N- and C-terminal α1, α5, and α6 helices from both subunits and a DNA-binding region that includes recognition helices α4/α4’ (arrow, Fig. 1B). In addition, each monomer contains N- and C-terminal extensions beyond the conserved core fold that is represented in the modeled structure, with the core fold spanning residues Ser28–Ala164 (Fig. 1B; terminal residues in *gray space-filling representation*). The electrostatic surface potential of the OstR core was calculated by Swiss PDB Viewer. Notably, one lobe of the dimeric protein is predicted to be highly positively charged (Fig. 1C). The other lobe features a more usual distribution of charges, being electropositive surrounding the recognition helix and the wing regions, a common feature that would promote interaction with the negatively charged cognate DNA. The unusual surface charge distribution within one protein lobe appears to derive from a constellation of five Arg and Lys residues, which is not balanced by equivalent negative charge (Fig. 1D); salt bridges between Glu75 and Arg77/Arg78 are predicted, whereas the side chain of Glu27 is predicted to be >8 Å from the other positively charged side chains (Lys36, Arg67, and Lys79). Another notable feature of the OstR protein lobe is the presence of a positively charged patch, which consists of a dimerization region formed by N- and C-terminal α1, α5, and α6 helices from both subunits and a DNA-binding region that includes recognition helices α4/α4’ (arrow, Fig. 1B). In addition, each monomer contains N- and C-terminal extensions beyond the conserved core fold that is represented in the modeled structure, with the core fold spanning residues Ser28–Ala164 (Fig. 1B; terminal residues in *gray space-filling representation*). The electrostatic surface potential of the OstR core was calculated by Swiss PDB Viewer. Notably, one lobe of the dimeric protein is predicted to be highly positively charged (Fig. 1C). The other lobe features a more usual distribution of charges, being electropositive surrounding the recognition helix and the wing regions, a common feature that would promote interaction with the negatively charged cognate DNA. The unusual surface charge distribution within one protein lobe appears to derive from a constellation of five Arg and Lys residues, which is not balanced by equivalent negative charge (Fig. 1D); salt bridges between Glu75 and Arg77/Arg78 are predicted, whereas the side chain of Glu27 is predicted to be >8 Å from the other positively charged side chains (Lys36, Arg67, and Lys79). Another notable feature of the OstR

![Image](https://example.com/image_url)
sequence is the presence of three cysteine residues per monomer, two vicinal cysteines (Cys³ and Cys⁴) in the N-terminal extension that precedes the core MarR fold and Cys¹⁶⁹ in the C-terminal extension (Fig. 1E). With six cysteines per dimer, regulation of OstR function by oxidation would be possible.

**OstR regulates emrB differentially in response to oxidants**

To assess whether OstR participates in regulation of *emrB*, we obtained and verified *B. thailandensis* E264 mutant strains in which ostR or *emrB* genes were disrupted by the insertion of transposons at position 98 of *emrB* and position 89 of *emrB* open reading frames, respectively, to generate ΔostR and ΔemrB strains (Fig. 1A) (21). Relative transcript levels of *emrB* were measured in both WT and ΔostR strains. *emrB* was up-regulated 25.2 ± 0.7-fold in the ΔostR strain (Fig. 2A), suggesting that OstR is a repressor of *emrB*. Complementation of ΔostR was attempted using plasmid-encoded *ostR*. Because a gentamicin-resistant derivative of the broad host range vector pBBR1-MCS5 was used for this purpose, WT cells were transformed with empty pBBR1-MCS5 (generating strain WTe) to control for any effects of gentamicin on gene expression. However, restoration of WT levels of *emrB* expression was not achieved in ΔostR transformed with pBBR1-MCS5-ostR (ΔostRc) and grown with gentamicin, only an ~20% reduction in expression compared with WTe. To address the possible reason, expression of *ostR* was determined in WTe grown with gentamicin and found to be significantly reduced (Fig. 2B). Consistent with a gentamicin-mediated reduction in *ostR* expression, *emrB* expression was ~7-fold higher in WTe compared with WT cells (Fig. 2A). This suggests that gentamicin negatively regulates the expression of *ostR*, a consequence of which is increased *emrB* expression.

Many MarRs are autoregulatory (1). To determine whether any autoregulation of *ostR* by OstR occurs, we analyzed expression of the gene fragment that is upstream of the point of transposon insertion (position 98 of the *ostR* ORF). There was no significant difference in expression between WT and ΔostR, indicating that expression of *ostR* is not regulated by OstR (Fig. 2B). By contrast, analysis of *ostR* expression using primers, which amplify a fragment that is downstream of the transposon insertion, showed no expression in ΔostR consistent with transposon-mediated gene disruption (Fig. 2B, gel inset); combined with the ~25-fold increase in *emrB* expression in ΔostR, this experiment also shows that *emrB* is expressed under the control of its own promoter.

To determine the effect of oxidants on the expression of *emrB*, WT and ΔostR strains were grown to mid-log phase followed by incubation with a 2 mM concentration of the oxidant H₂O₂ or CuCl₂ for 30 min. RNA was isolated, and cDNA was made using gene-specific primers for analysis of relative transcript levels by quantitative RT-PCR. In the presence of 2 mM H₂O₂, *emrB* expression was increased 3.5 ± 0.4-fold, whereas incubation with 2 mM CuCl₂ resulted in significant reduction in *emrB* expression (0.4 ± 0.01-fold; Fig. 2C). To determine whether redox-inactive metals also regulate *emrB* expression, we assessed Zn²⁺-dependent transcriptional regulation of *emrB*. Gene expression analysis revealed an ~2-fold increase in *emrB* expression in the presence of 2 mM ZnCl₂. No significant change in *emrB* expression was observed in the ΔostR strain in the presence of oxidants or ZnCl₂, suggesting that the observed

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**Figure 2. Regulation of gene expression.** A, relative transcript level of *emrB* in WT, ΔostR, WTe (containing empty vector), and ΔostRc (complemented with *ostR*) strains. †-fold changes are reported relative to the reference gene encoding glutamate synthase large subunit (*BTH* J. Biol. Chem. (2019) 294(6) 1891–1903 1893

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changes in gene expression in WT cells depend on OstR (Fig. 2D).

**Gene disruption affects sensitivity to antibiotics and oxidants**

For clues to the substrate for EmrB, the WT, ΔostRX, and ΔemrBX strains (in which antibiotic resistance cassettes were removed from transposons) were serially diluted and spotted on LB-agar plates containing antibiotics. It was previously reported that expression of the corresponding *B. cepacia* EmrB protein (named BcrA) in *E. coli* resulted in resistance to tetracycline and nalidixic acid (22). Nalidixic acid was seen to be toxic to WT cells and to ΔemrBX, whereas the ΔostRX strain was more resistant (Fig. 3B). A minimal inhibitory concentration (MIC) was estimated by growing the respective strains in liquid culture containing serially diluted antibiotics; this analysis indicated a similar MIC for WT and ΔemrBX cells, whereas the MIC for ΔostRX cells was higher (Table 1). The increased resistance exhibited by ΔostRX would be consistent with up-regulation of *emrB* and more efficient export of nalidixic acid.

Resistence to tetracycline was likewise elevated in the ΔostRX strain, consistent with export through EmrB; however, resistance was also increased in the ΔemrBX strain (Fig. 3C and Table 1). Expression of two operons encoding RND efflux systems (BpeEF-OprC and AmrAB-OprA) was previously reported to be induced by the tetracycline derivative doxycycline (23); a possible explanation for the increased resistance to tetracycline in the ΔemrBX strain is therefore that failure to export tetracycline through EmrB might lead to elevated intracellular concentrations and induction of one or both of these RND efflux systems. We therefore grew ΔemrBX and ΔostRX strains in the presence of different concentrations of tetracycline and measured expression of *amrB* and *bpeF* (Fig. 4). Expression of *amrB* was greater in ΔemrBX cells grown with at least 10 μg/ml tetracycline, whereas expression of *bpeF* was reduced. In contrast, expression of both *amrB* and *bpeF* was reduced in ΔostRX cells grown with tetracycline. This is consistent with the inference that the increased resistance to tetracycline characteristic of ΔemrBX cells is due to induction of the operon that encodes AmrAB-OprA.

High concentrations of gentamicin were tolerated by WT cells, with a further increase in resistance in ΔostRX cells (Fig. 3D and Table 1). Because increased resistance was also observed in ΔemrBX cells, one possibility is that failure to export gentamicin by EmrB may result in induction of an alternate exporter, as seen for tetracycline. Notably, EmrB appears to be a primary efflux system for trimethoprim; at a concentration of the antibiotic where growth of WT and ΔostRX was largely unaffected, as estimated by plate assays, growth of ΔemrBX was severely compromised (Fig. 3E). Consistent with EmrB being important for trimethoprerm efflux, the MIC for ΔemrBX cells was lower than the MIC for WT cells (Table 1).

Because *emrB* expression was sensitive to redox state (Fig. 2C), the strains were exposed to either 5 mM H₂O₂ or CuCl₂ and plated on LB-agar plates. Whereas the ΔemrBX strain exhibited similar sensitivity to either oxidant as WT cells, ΔostRX was more sensitive to H₂O₂ but less sensitive to CuCl₂ (Fig. 3, F and G). This differential sensitivity is intriguing, and it parallels the opposite effects of either oxidant on *emrB* expression, suggesting that gene regulation by oxidized OstR depends on the identity of the oxidant. Exposure to ZnCl₂, however, resulted in a phenotype similar to that observed on treatment with tetracycline and gentamicin (Fig. 3H).

**Interaction of OstR with Zn²⁺ and oxidants in vitro**

OstR was examined in *vitro*, focusing on responses to the inducers of differential *emrB* expression in *vivo*. The gene encoding OstR was cloned, and the protein was expressed in *E. coli* and purified using a nickel-nitrilotriacetic acid–agarose column to apparent homogeneity (Fig. 5E and F, lanes 2). The calculated molecular mass of monomeric OstR is ~21 kDa. Far-UV CD spectroscopy showed that the predicted secondary structure composition of OstR is about 37% α-helix, 25%
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Figure 4. Expression of genes encoding AmrAB-OprA and BpeEF-OprC efflux pumps in the presence of tetracycline. A and B, expression of *amrB* (*BTH_I2444*) in ΔemrBX and ΔostRX, respectively. C and D, expression of *bpeF* (*BTH_I2105*) in ΔemrBX and ΔostRX, respectively. Expression is reported relative to unsupplemented cultures. Asterisks represent statistically significant differences in expression compared with unsupplemented cultures based on Student’s *t* test (*, *p* < 0.05; **, *p* < 0.001). Horizontal axes identify the concentration of tetracycline (in μg/ml). Note that the ordinate scale for A differs from that in B–D.

Error bars, S.D.

Figure 5. Characterization of OstR. A, far-UV CD spectrum of OstR. Ellipticity is presented in machine units (millidegrees). B, thermal denaturation of OstR determined by differential scanning fluorimetry. Fluorescence intensity reflects binding of SYPRO Orange to hydrophobic regions of denatured protein as a function of temperature. C, prediction of intrinsically disordered regions (blue) and likelihood of disordered regions participating in protein interactions (orange). The y axis reflects confidence score, with values >0.5 considered relevant. D, release of metal ion from denatured OstR determined by absorbance of PAR. Absorbance at 416 nm corresponds to uncomplexed PAR, whereas absorbance at 520 nm reflects formation of PAR–metal ion complex. E and F, OstR oxidation by H₂O₂ (E) and CuCl₂ (F). In both images, the left lanes show protein marker (kDa), lanes 2 show protein incubated with DTT (Rd; species migrating at ~43 kDa is residual oxidized protein), and lanes 3 show air-oxidized protein (O₂). Lanes 4–8, increasing concentration of H₂O₂ (10 μM to 2 mM). F, lanes 4–7, increasing concentration of CuCl₂ (10 μM to 2 mM).

β-sheet, and 38% random coils (Fig. 5A). By comparison, the structure of *Deinococcus radiodurans* HucR, which also has an N-terminal extension beyond the conventional MarR fold, reveals ~55% α-helix, a helical content that is also reflected in its CD spectrum; the lower α-helical content for OstR may reflect that its unique N- and C-terminal extensions are not helical (24, 25). The secondary structure of OstR was predicted using the PSIPRED Protein Analysis Workbench (26); disorder prediction using DISOPRED3 revealed that the extensions beyond the core MarR fold represent intrinsically disordered...
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A thermal stability assay in which SYPRO Orange was used as a fluorescent reporter of protein unfolding as a function of temperature showed that reduced OstR exhibited a one-step melting transition with a relatively low \( T_{m} \) = 34.1 ± 0.1 °C (Fig. 5B). Protein stability was found to increase in the presence of DNA (\( T_{m} \) = 39.3 ± 0.2 °C) and Zn\(^{2+}\) (\( T_{m} \) = 37.2 ± 0.1 °C) or upon oxidation (\( T_{m} \) ~ 39 °C; Table S1). Incubation of OstR with bipyridyl prior to measurement of thermal stability did not affect the \( T_{m} \), indicating that purified OstR had no metal ions already bound. To verify binding to metal ions, OstR was incubated with 2 mM ZnCl\(_2\) followed by treatment with 4-(2-pyridylazo) resorcinol (PAR). The metallochromic indicator PAR chelates zinc ions released from the protein upon its denaturation, which results in an absorbance peak at ~520 nm, whereas uncomplexed PAR has an absorbance maximum at 416 nm (27). As shown by the marked absorbance at 520 nm, OstR binds Zn\(^{2+}\) (Fig. 5D).

Each OstR monomer harbors three cysteine residues, all within the N- and C-terminal extensions (Fig. 1E). Reduced OstR was therefore incubated with increasing concentration of H\(_2\)O\(_2\) and CuCl\(_2\), and oxidation products were analyzed by electrophoresis on SDS-polyacrylamide gels. When oxidized with H\(_2\)O\(_2\), OstR formed dimers and oligomeric species as well as intramolecular disulfide bonds, as indicated by a species with a faster migration than reduced monomeric protein. In addition, OstR formed three dimeric forms with distinct mobility, likely reflecting different disulfide-bonded species (Fig. 5E).

OstR was also oxidized by CuCl\(_2\). Whereas the appearance of multiple dimeric species was more evident on H\(_2\)O\(_2\)-mediated oxidation, intramolecular disulfide bonds and oligomeric species were also seen upon oxidation with CuCl\(_2\).

OstR binds the emrB promoter

Electrophoretic mobility shift assays (EMSAs) were performed to determine OstR binding upstream of emrB. Reduced OstR bound the 146-bp DNA spanning emrB and emrB forming multiple complexes (Fig. 6A). The apparent dissociation constant \( K_{d} \) was 6.7 ± 0.8 nM with Hill coefficient \( n_{H} \) = 1.9 ± 0.3, indicating positive cooperativity of binding (Table S2 and Fig. 6C). At lower protein concentration, the faster-migrating complex C1 was visible, whereas the slower-migrating complex C3 remained predominant across a greater range of concentration. Nonspecific binding may occur at even higher protein concentration, leading to slower migration of the complexes. To address whether OstR bound specifically to the emrB promoter, increasing concentration of competitor DNA was added in the reaction with specific labeled DNA and a constant concentration of the protein. Specific unlabeled DNA competed efficiently for OstR binding (Fig. 6F, lanes 10–15). Nonspecific DNA did not compete as efficiently for OstR binding (Table S2 and Fig. 6C). At lower protein concentration, the faster-migrating complex C1 was visible, whereas the slower-migrating complex C3 remained predominant across a greater range of concentration. Nonspecific binding may occur at even higher protein concentration, leading to slower migration of the complexes. To address whether OstR bound specifically to the emrB promoter, increasing concentration of competitor DNA was added in the reaction with specific labeled DNA and a constant concentration of the protein. Specific unlabeled DNA competed efficiently for OstR binding (Fig. 6F, lanes 10–15). Nonspecific DNA did not compete as efficiently for OstR binding (Table S2 and Fig. 6C). At lower protein concentration, the faster-migrating complex C1 was visible, whereas the slower-migrating complex C3 remained predominant across a greater range of concentration. Nonspecific binding may occur at even higher protein concentration, leading to slower migration of the complexes.

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oxidized OstR for cognate DNA was not markedly different from that of reduced protein \((K_d = 9.7 \pm 1.1)\). Incubation of increasing concentration of 2 mM CuCl2-oxidized OstR with emrB promoter DNA also showed no significant change in DNA-binding affinity, \(K_d = 6.5 \pm 1.0\) (Fig. 6 and Table S2).

**Substitution of cysteine residues leads to altered binding affinity for promoter DNA**

To determine the role of cysteines in oxidant sensing by OstR, we replaced each cysteine with alanine and created three individual cysteine mutants (OstR-C3A, OstR-C4A, and OstR-C169A). Each variant was expressed in *E. coli* and purified to apparent homogeneity (Fig. 7, lanes 2). The yield of OstR-C169A mutant was lower compared with the other variants, with precipitation observed after purification, indicating that the absence of Cys169 may cause instability or improper folding.

Increasing concentrations of OstR-C3A, OstR-C4A, or OstR-C169A were incubated with 146-bp emrB promoter DNA followed by analysis of protein–DNA complexes by EMSA (Fig. 8, A, C, and E). OstR-C3A showed a modest reduction in DNA-binding affinity with \(K_d = 17.5 \pm 2.3\) and Hill coefficient, \(n_H = 1.6 \pm 0.3\) (Fig. 8B). OstR-C4A exhibited a comparable reduction in DNA-binding affinity with \(K_d = 23.4 \pm 3.2\) and \(n_H = 1.2 \pm 0.3\) (Fig. 8D). Replacing Cys169 with alanine, however, caused DNA binding to be significantly impaired, with \(K_d = 539 \pm 58\) nM and \(n_H = 1.9 \pm 0.2\) (Fig. 8E and Table S2). Because the cysteine residues all reside in extensions beyond the core MarR fold, these data suggest that modulation of these extensions leads to structural rearrangements that impact the DNA-binding region, an impact that implies an interaction between the extensions and the protein core.

**Cys169 participates in intramolecular disulfide bond formation**

The role of individual cysteines in disulfide bond formation was investigated by incubation of OstR variants with increasing concentration of H2O2 (Fig. 7, A, C, and E) or CuCl2 (Fig. 7, B, D, and E), followed by analysis by SDS-PAGE. All variants were readily oxidized on exposure to air (lanes 3). Intramolecular disulfide bonds were seen for both OstR-C3A and OstR-C4A, as evidenced by a product that migrated faster than reduced, monomeric OstR (Fig. 7, A–D). By contrast, such faster-migrating monomeric species were absent with oxidized OstR-C169A (Fig. 7, E and F), suggesting that Cys169 is required for intramolecular disulfide bond formation. Oxidized OstR-C3A and OstR-C4A also formed two prominent dimeric species, whereas oxidized WT OstR featured three dimeric species and oxidized OstR-C169A formed only one. Higher-order oligomeric species were observed in the presence of CuCl2, but not in the case of H2O2-mediated oxidation.

**All cysteines play a structural role**

Differential scanning fluorometry showed that OstR-C3A and OstR-C4A are thermally more stable \((T_m = 44.7 \pm 0.1\) and 50.6 ± 0.1 °C, respectively) than WT OstR (Fig. 9A (red dotted line) and Fig. 7B (blue dotted line), respectively). The addition of oxidants (H2O2 or CuCl2), however, resulted in significant destabilization of these mutant variants, as reflected in high initial fluorescence and the absence of a melting transition (Fig. 9, A (blue dashed line) and B (purple dashed line), respectively, and Table S3). Upon binding to ligands such as DNA or zinc, both OstR-C3A and OstR-C4A variants showed significant increase in thermal stability.
By contrast, OstR-C169A exhibited a high initial fluorescence and a decrement in fluorescence as a function of temperature and no identifiable melting transition (Fig. 9C, orange dotted line). Such a pattern of interaction with SYPRO Orange suggests that OstR-C169A exposes hydrophobic residues, indicating improper folding and a feature reminiscent of molten globule formation. The addition of oxidant had no effect on this pattern. In the presence of zinc, OstR-C169A behaved as a globular protein with a clear melting transition (Fig. 9C, green dashed line). Incubation of OstR-C169A with DNA also resulted in protein stabilization (Table S3), consistent with the ability of this protein variant to bind DNA (albeit with low affinity; Fig. 8 (E and F)). That substitution of either cysteine impacted thermal stability is consistent with the inference that both N- and C-terminal extensions contact the protein core.

All three cysteine variants bind to zinc

The Zn^{2+}-mediated changes in thermal stability of OstR cysteine variants suggested metal binding. All OstR variants were incubated with 2 mM ZnCl_{2}, followed by denaturation and measurement of released Zn^{2+} by chelation to PAR. Significant absorbance peaks at ~520 nm were observed for all three variants, indicating release of Zn^{2+} from denatured proteins (Fig. 10, A–C). This suggests that none of the three cysteines were required to coordinate Zn^{2+} in OstR. Based on this information, we opted to assess whether Zn(II) binding modulated cysteine oxidation in vitro. The OstR variants were incubated with 2 mM ZnCl_{2} for 30 min, followed by oxidation with 12 mM H_{2}O_{2} for another 30 min. SDS-PAGE analysis showed that both OstR-C3A and OstR-C4A formed intramolecular disulfide bonds, dimeric species, and higher-order oligomers...
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By contrast, the increased resistance to tetracycline in the ΔemrBX strain points to a more intricate cross-talk between efflux systems. The tetracycline derivative doxycycline was previously shown to induce expression of two RND efflux systems, AmrAB-OprA at intermediate concentrations of doxycycline, followed by BpeEF-OprC at very high drug concentrations (23). It was also noted that the addition of an RND inhibitor resulted in a 2–3-fold increase in expression of amrB, an overexpression that was associated with elevated resistance to certain antibiotics. Consistent with these observations, we find that amrB expression is induced in ΔemrBX cells grown with tetracycline, an induction that does not occur in ΔostRX cells. This is consistent with the interpretation that failure to export tetracycline through EmrB leads to induction of amrB (Fig. 4). Taken together, these observations point to a complex mechanism for induction of optimal efflux systems, which should be considered when attempting to restore multidrug resistance by controlling activity of specific efflux pumps.

Redox-dependent differential regulation of emrB by OstR

OstR represses expression of emrB and modulates its expression under conditions of oxidative stress (Fig. 2). Preferred binding of OstR to the emrB promoter is consistent with the observed regulation. Reduced and H2O2-oxidized OstR bind to emrB promoter DNA with comparable affinity (Fig. 6, A–D), yet the presence of oxidant affects gene expression (Fig. 2C). Changes in gene expression due to features other than altered DNA-binding affinity of the cognate transcription factor have been reported previously. For example, the redox-sensitive MarR homolog HypR in Bacillus subtilis binds to DNA with similar affinity regardless of redox state, but only oxidized HypR activates target gene expression (29). Similarly, PecS from the plant pathogen Pectobacterium atrosepticum binds to promoter DNA with comparable affinity at pH 7.4 and 8.3, but it represses gene expression only at alkaline pH; in the case of PecS, the differential ability to repress gene activity was ascribed to its pH-dependent ability to alter DNA topology (30). Reduced and oxidized OstR may likewise impose distinct co-

(Fig. 10D, lanes 3 and 5, respectively). The OstR-C169A variant also formed dimers (faint dimeric species in Fig. 10D, lane 7) upon oxidation, but due to the presence of both metal and oxidant, the protein precipitated significantly. That Zn2+ did not preclude cysteine oxidation is consistent with the interpretation that neither Cys3, Cys4, nor Cys169 is required for Zn2+ coordination.

Discussion

Antibiotic efflux by EmrB

Trimethoprim inhibits dihydrofolate reductase, and it is frequently used in combination with sulfamethoxazole (as co-trimoxazole) to treat Burkholderia infections. In B. pseudomallei, the RND efflux pump BpeEF-OprC has been shown to extrude trimethoprim (28). Our data identify the EmrB drug transporter encoded by BTH_10022 as another primary transporter of trimethoprim, as evidenced by the increased sensitivity imposed by its inactivation (Fig. 3E and Table 1).

Expression of the B. cepacia EmrB ortholog (BcrA) in E. coli resulted in increased resistance to nalidixic acid and tetracycline (trimethoprim was not tested) (22). The emrB expression is increased in the ΔostRX strain that shows enhanced resistance to both nalidixic acid and tetracycline (trimethoprim was not tested) (22). The emrB expression is increased in the ΔostRX strain that shows enhanced resistance to both nalidixic acid and tetracycline (Fig. 3, B and C) and Table 1), consistent with facilitated efflux. However, we cannot rule out that genes encoding other drug transporters are up-regulated as a result of inactivation of ostR, thereby conferring or contributing to the observed resistance. Because inactivation of the emrB gene did not result in increased sensitivity to nalidixic acid, we also infer that other mechanisms for efflux of this compound exist.

By contrast, the increased resistance to tetracycline in the ΔemrBX strain points to a more intricate cross-talk between efflux systems. The tetracycline derivative doxycycline was previously shown to induce expression of two RND efflux systems,
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formational changes in promoter DNA that manifest as differential ability to compete with RNA polymerase for binding.

OstR also senses Cu^{2+}, which results in further repression of emrB (Fig. 2C). The opposite effects of H_{2}O_{2} and Cu^{2+} suggest that the different oxidants result in different OstR oxidation products and therefore distinct effects on gene expression; such opposite effects also manifest in the distinct changes in sensitivity to these oxidants imposed by deletion of ostR, consistent with differential control of genes in the OstR regulon (Fig. 3, F and G). Reduced and Cu^{2+}-oxidized OstR also bind to promoter DNA with equivalent affinity (Table S2). By comparison, the \textit{B. thailandensis} MarR homolog BfrR, which is also subject to Cu^{2+}-mediated oxidation, forms a dimer of dimers when oxidized and acts as a "superrepressor" to attenuate gene expression further even though oxidation has little effect on DNA-binding affinity (15). Taken together, our data suggest that reduced OstR represses \textit{emrB} and that it operates as a rheostat in the presence of oxidants to optimize \textit{emrB} expression.

Modification of cysteines affects stability of OstR

Oxidation of OstR and the Cys-to-Ala mutants results in formation of dimeric species (Figs. 5 (E and F) and 7). As evidenced by the formation of an oxidized species that migrates faster than reduced, monomeric OstR, we also infer that Cys^{169} can participate in intramolecular disulfide linkages with either Cys^{3} or Cys^{4} (Fig. 7). The presence of three dimeric species upon oxidation of WT protein (two dimeric species with OstR-C3A and OstR-C4A and one dimer with oxidized OstR-C169A) could be explained by the formation of intermolecular disulfide bonds involving either Cys^{3} or Cys^{4} from two OstR dimers alone (as seen for OstR-C169A) or in combination with intramolecular disulfide linkages between Cys^{169} and either Cys^{3} or Cys^{4}, resulting in oxidized species with distinct mobilities.

Differential scanning fluorometry reveals that OstR-C169A exposes hydrophobic residues (Fig. 9C), suggesting that it is intrinsically unfolded or that it exists in a molten globule state (25); considering that a closely packed protein conformation with a distinct melting transition is restored in the presence of Zn^{2+} or DNA, we speculate that it may be the latter. By contrast, OstR-C3A and OstR-C4A mutants are thermally more stable compared with WT OstR (Tables S1 and S3). That thermal stability is altered as a result of either mutation indicates that neither N- nor C-terminal extensions contact the core fold of the protein, in the process modulating protein stability and responses to oxidants. Substrates for EmrB include commonly used antibiotics; whereas inactivation of \textit{emrB} leads to trimethoprim sensitivity, it results in markedly increased resistance to tetracycline, a resistance that correlates with induced expression of the RND efflux pump AmrAB-OprA, illustrating the intricate mechanisms by which expression of genes encoding efflux pumps is optimized depending on cellular concentrations of inducing antibiotic.

Experimental procedures

Protein modeling and purification

PyMOL was used to visualize the model of OstR, which was generated by homology modeling using SwissModel in automated mode and Protein Data Bank entry 4AIK as template (selected as allowing the most residues of OstR to be included in the model).

The gene encoding \textit{B. thailandensis} E264 OstR was amplified from genomic DNA using primers OstR\textsubscript{trans}\textsubscript{Fw} and OstR\textsubscript{trans}\textsubscript{Rv} containing NdeI and EcoRI restriction sites, respectively (Table S4). The PCR product was cloned into pET28b after restriction digestion with NdeI and EcoRI to express N-terminal His\textsubscript{6}-tagged protein. The construct was verified by sequencing and then transformed into \textit{E. coli} BL21(DE3). Cells were grown until mid-exponential phase at 37 °C in LB with 50 μg/ml kanamycin, followed by induction using 1 mM isopropyl β-D-1-thiogalactopyranoside. After 1 h, cells were pelleted at 4 °C and stored at −80 °C. Cell pellets were thawed, and cells were resuspended in wash buffer (50 mM sodium phosphate (pH 7.0), 250 mM NaCl, 5% glycerol). Lysozyme (final concentration 1 mg/ml) and 2 μl of DNase I reaction buffer were added to each 5 ml cell suspension, which was incubated at 4 °C for 60 min.
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Lysate was obtained by centrifugation at 10,000 x g for 1 h at 4 °C and incubated with 1 ml of HIS-Select nickel affinity beads (previously washed with two volumes of distilled H2O and one volume of wash buffer) at 4 °C for 1 h. The beads were then transferred to a gravity flow column to elute protein by the addition of increasing concentrations of imidazole (10–200 mM). Pure protein fractions were detected by SDS-PAGE, pooled, and concentrated using Amicon centrifugal filter units (Millipore). Protein was dialyzed overnight against 50 mM sodium phosphate (pH 7.0), 250 mM NaCl, 10% glycerol, and 5 mM 2-mercaptoethanol. Concentrations were determined based on the absorbance at 280 nm, using a calculated extinction coefficient of 7,450 M⁻¹ cm⁻¹. To create cysteine-to-alanine substitutions, overhanging primers (Table S4) were used to amplify the whole plasmid harboring the OstR gene, reactions were incubated with DpnI, and the DNA was transformed into E. coli TOP10 (Invitrogen); plasmids were sequenced for verification. Mutant proteins were purified as described above.

To determine secondary structure of the protein, the far-UV CD spectrum was measured using a Jasco J-815 CD spectrophotometer (32). In brief, 0.2 mg/ml protein in CD buffer (20 mM sodium phosphate (pH 7.0), 20 mM NaCl, 0.8% glycerol, and 1.5 mM DTT) was used to measure ellipticity. A quartz cuvette with 0.1-cm path length was used after equilibrating the protein with 0.1 M NaCl and 2 mM 2-mercaptoethanol. Concentrations were determined by subtracting control values. SigmaPlot version 9 was used for determination of melting temperature by regression analysis using a four-parameter sigmoidal equation. The Tₘ was calculated from the averages of three technical replicates obtained from each of three independent experiments and is presented as mean ± S.D.

**Metal binding**

Proteins were incubated with 50 mM 2,2′-bipyridyl at 4 °C to remove any bound metals. Bipyridyl-treated proteins were dialyzed overnight against buffer A (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM 2-mercaptoethanol, and 0.1% glycerol) and then incubated with 1 mM ZnCl₂ on ice for 30 min and again dialyzed overnight against buffer A to remove unbound metal. Proteins were denatured by adding 1% SDS in assay buffer (20 mM Tris-HCl (pH 7.5), 50 mM sodium chloride) followed by heating at 90 °C for 10 min. 100 μM PAR was added to each sample and buffer control, and an Agilent 8453 spectrophotometer was used to measure absorbance from 320 to 630 nm. Fluorescence data are reported as the mean of three technical replicates from representative experiments.

**Confirmation of transposon mutants and removal of antibiotic resistance cassettes**

OstR (BTH_J0021) and emrB (BTH_J0022) were disrupted by the insertion of transposon T8 at position 98 of the ostR ORF to generate strain BTH_J0021–153:SlacZ-hah-Tc and T23 at position 89 of emrB (strain BTH_J0022–124:SlacZ-PraBo-Tp/FRT). Transposon inserted mutants (Δostr and ΔemrB) were obtained from the Manoli laboratory and grown on LB-agar plates with 80 μg/ml tetracycline and 50 μg/ml trimethoprim, respectively (21). Single colonies were grown overnight after inoculating in LB medium with the respective antibiotics. To verify correct insertion of transposons, PCR was performed using primers BTH_21_Fw, BTH_22int_Fw, and LacZ_148 (Table S4).

Transposon insertion mutants ΔemrB and Δostr harbor trimethoprim and tetracycline antibiotic cassettes, respectively. Plasmids pFLPe4 or pCRE3 (a generous gift from H. Schweizer) were used to remove the antibiotic resistance cassettes from ΔemrB or Δostr, respectively (36, 37). In brief, Flp/Cre recombinase-encoding pFLPe4 or pCRE3 plasmid was transformed into E. coli TOP10 cells. Tri-parental mating was performed to transfer plasmids harboring Flp/Cr recombine into ΔemrB.
or ΔostR strains, respectively. Selection was done on LSLB/kanamycin (for pFlpe4) or LSLB/ampicillin (for pCRE3) plates at 30 °C (where LSLB denotes low-salt LB). To induce flp or cre expression, selected colonies were streaked on LSLB/kanamycin/rhamnose (for pFlpe4) or LSLB/ampicillin/rhamnose (for pCRE3) plates. Corresponding antibiotic-sensitive clones were grown at 37 °C to induce loss of the temperature-sensitive pFlpe4/ pCRE3. Removal of antibiotic cassettes was confirmed by PCR using primers (Tra_delE_Fw, Tra_delE_Rv; Cre_del21_Fw, Cre_del21_Rv; Table S4). ΔemrB and ΔostR strains in which antibiotic resistance cassettes were removed were named ΔemrBX and ΔostRX.

**Genetic complementation**

ostR with 170 bp upstream of the coding sequence was amplified using primers OstR_Xbal_Fw and OstR_Kpn1_Rev (Table S4), which contain XbaI and Kpn1 sites, respectively. Digested PCR product was then cloned into the gentamicin-resistant pBBR1-MCS5 (38). The construct was transformed into E. coli TOP10 (Invitrogen), and plasmid was verified by sequencing. Tri-parental mating was performed to transfer plasmid harboring ostR to the B. thailandensis E264 ΔostR strain. Conjugation was performed by mixing overnight cultures of donor (E. coli TOP10 containing pBBR1-MCS5 plasmid harboring ostR, grown in the presence of 80 μg/ml gentamicin), recipient (ΔostR grown in the presence of 80 μg/ml tetracycline), and helper strain (HB101(pRK2013:Tn7)) in a ratio of 1:1:2. Residual antibiotics were removed by centrifuging the cells and washing pellets with 1.0 ml of LB four times. The pellets were then resuspended in 50 μl of LB and spotted on a preheated LB agar plate. After 12 h of incubation, cells were scraped off and resuspended in 1.0 ml of LB, and serial dilutions were spotted on LB-agar plates with 80 μg/ml tetracycline, 250 μg/ml gentamicin, and 8 μg/ml chloramphenicol. Individual colonies were screened to verify trans-conjugants by PCR with primers Con_pBBR_Xbal and Con_pBBR_Kpn1 (Table S4). The complemented strain is referred to as ΔostRc. Empty plasmid pBBR1-MCS5 without the ostR gene was similarly transferred to WT B. thailandensis, and the strain was referred to as WTe.

**Gene expression analysis**

WT and mutant strains were grown overnight, followed by a 1:100 dilution of each culture in fresh LB media. Cultures were grown until A600 of ~0.5, and cells were harvested by centrifugation and washed with ice-cold diethyl pyrocarbonate–treated water. To determine the effect of oxidants (H2O2 or CuCl2) and ZnCl2 on gene expression, cells were grown until A600 of ~0.5 and treated with 2 mM H2O2, 2 mM CuCl2, or 2 mM ZnCl2 for 30 min, followed by harvesting and washing with diethyl pyrocarbonate–treated water. The effect of tetracycline on gene expression in ΔostRX and ΔemrBX strains was determined after growing the cells overnight with tetracycline (5, 10, 20, 50, and 128 μg/ml). Cells were stored at ~80 °C. The Illustra RNAspin Mini Isolation kit (GE Healthcare) was used to isolate total RNA. Any genomic DNA contamination was removed by Turbo DNase (Ambion) treatment, and PCR was performed using fresh RNA samples to verify the absence of genomic DNA. NanoDrop (Thermo Scientific) was used to measure the concentration of RNA. cDNA was prepared using gene-specific primers (Table S4) and 1 μg of RNA with 1 mM MgCl2, 1 mM dNTP, 10 units of avian myeloblastosis virus reverse transcriptase (New England Biolabs) in 1× avian myeloblastosis virus reverse transcriptase buffer. The reaction mixture was incubated at 42 °C for 60 min. The qPCR was performed using a QuantStudio 6 Flex Real-Time PCR system and SYBR Green I (Sigma) to detect gene expression. Data were normalized to the reference gene glutamate synthase large subunit, which was amplified using primers GluSyn_qPCR_Fw and GluSyn_qPCR_Rv and expressed as 2−ΔΔCT. The comparative Ct method (2−ΔΔCT) was used to analyze the effect of antibiotics, oxidants, and ZnCl2 on gene expression. All expression analyses were performed in biological triplicates and are presented as mean ± S.D.

**Plate assay and MIC determination**

Sensitivity to antibiotics and oxidants was assessed by plate assays. Overnight cultures of WT and mutant strains were diluted 1:100 using LB medium and grown at 37 °C to A600 ~0.6. To assess the susceptibility to antibiotics, 5 μl of 10-fold serial dilutions were spotted on LB-agar plates containing the desired antibiotic (nalidixic acid, tetracycline, gentamicin, or trimethoprim). To determine the response to oxidants, 1 ml of each culture was incubated with 5 mM H2O2 or CuCl2 for 30 min at 37 °C, followed by spotting 5 μl of 10-fold serial dilutions on LB-agar plates. Plates were incubated at 37 °C for 18–22 h.

MICs were estimated by preparing 2-fold dilutions of antibiotics in LB in 96-well microtiter plates. Overnight cultures were freshly diluted 1:100 in LB and grown to exponential phase; an equal volume of culture was added to the microtiter wells at an A600 of ~0.6. Cell growth was determined visually after incubation at 37 °C for 24 h.

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**References**

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