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Identification and *In-vivo* Characterization of a Novel OhrR Transcriptional Regulator in *Burkholderia xenovorans* LB400

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Abstract

Transcriptional regulators (TRs) are an important and versatile group of proteins, yet very little progress has been achieved towards the discovery and annotation of their biological functions. We have characterized a previously unknown organic hydroperoxide resistance regulator from *Burkholderia xenovorans*LB400, Bxe_B2842, which is homologous to *E. coli's* OhrR. Bxe_B2842 regulates the expression of an organic hydroperoxide resistance protein (OsmC). We utilized frontal affinity chromatography coupled with mass spectrometry (FAC-MS) and electrophoretic mobility gel shift assays (EMSA) to identify and characterize the possible effectors of the regulation by Bxe_B2842. Without an effector, Bxe_B2842 binds a DNA operator sequence (DOS) upstream of *osmC*. FAC-MS results suggest that 2-aminophenol binds to the protein and is potentially an effector molecule. EMSA analysis shows that 2-aminophenol attenuates the Bxe_B2842's affinity for its DOS. EMSA analysis also shows that organic peroxides attenuate Bxe_B2842/DOS affinity, suggesting that binding of the TR to its DOS is regulated by the two-cysteine mechanism, common to TRs in this family. Bxe_B2842 is the first OhrR TR to have both oxidative and effector-binding mechanisms of regulation. This paper reveals further mechanistic diversity TR mediated gene regulation and provides insights into methods for function discovery of TRs.

Keywords: transcriptional regulator (TR), transcriptional regulator effector (TRE), fontal affinity chromatography coupled to mass spectrometry (FAC-MS), *Burkholderia xenovorans* LB400, organic hydroperoxide resistance regulator (OhrR), derepression, organic peroxides, Bxe_B2842 and 2-aminophenol

1. Introduction

Burkholderia xenovorans LB400 is the first nonpathogenic *Burkholderia* isolated and is one of the most important aerobic polychlorinated biphenyl degraders discovered thus far (Bedard et al., 1986; Chain et al., 2006; Goris et al., 2004; Maltseva, Tsoi, Quensen, Fukuda, & Tiedje, 1999; Seeger, Timmis, & Hofer, 1995; Seeger, Zielinski, Timmis, & Hofer, 1999). The *B. xenovorans* genome is 9.73 Mbp in size and contains approximately 9,000 coding sequences spanning three different chromosomes: 1, 2 and megaplasmid. The large chromosome (1) is considered the "core" chromosome because it carries the genes associated with core cellular function. The small chromosome (2) and megaplasmid carry genes associated with energy metabolism, secondary metabolism and inorganic ion and amino acid transport and metabolism. Genomic comparisons among the *Burknolderia* strains indicate that *B. xenovorans* LB400 is enriched in aromatic metabolic pathways that provide catabolic capacities for compounds such as biphenyl, 3-chlorocatechol, and 2-aminophenol (Chain et al., 2006). The genomiccontext of this strain suggests that genes of the central aromatic catabolic pathways are generally organized in operons with the genes encoding transcriptional retulators (TRs) adjacent to their regulated operons (Chain et al., 2006). In this paper, we investigate the Bxe_B2842, a TR from *B. xenovorans*, to understand its regulatory mechanism.

Sequence homology to known multiple antibiotic resistance regulator (MarR) TRs revealed that Bxe_B2842 is a member of the MarR family (Finn et al., 2010). This family of proteins comprises a wide range of TRs involved in regulating cellular processes such as metabolic pathways, stress responses, virulence and degradation or export of harmful chemicals, including phenolic compounds, antibiotics and common household detergents (Alekshun & Levy, 1999; Ariza, Cohen, Bachhawat, Levy, & Demple, 1994; Cohen, Hachler, & Levy, 1993;

Martin & Rosner, 1995; Sulavik, Gambino, & Miller, 1995). TRs in the MarR family typically have a winged helix-turn-helix (wHTH) DNA-binding motif and exist as dimmers (PDB#: 1JGS (Alekshun, Levy, Mealy, Seaton, & Head, 2001), 3VB2 and 3VOE). MarR family TRs act either to activate or repress transcription by binding to palindromic sequences within their target promoters, commonly known as DNA operator sequences (DOS) (Perera & Grove, 2010). MarR TRs can be characterized by the specific types of effectors they bind. MarR TRs that regulate metabolic pathways generally bind phenolic compounds; this binding event causes a conformational change in the TR that prevents it from binding to the DOS (Perera & Grove, 2010). Other MarR TRs regulate oxidative stress responses by interacting withreactive oxygen species (ROS) such as organic hydroperoxides. This interaction results in oxidation of cysteine residues on the TR, which causes a conformational change in the TR that attenuates its affinity for the DOS (Perera & Grove, 2010). ROS are commonly generated as by products of cellular processes. In bacteria, TRs such as OxyR, SoxR, PerR, and OhrR are responsive to elevated ROS concentrations (Fuangthong & Helmann, 2002; Helmann et al., 2003; Hidalgo, Leautaud, & Demple, 1998; Mongkolsuk & Helmann, 2002; Newberry, Fuangthong, Panmanee, Mongkolsuk, & Brennan, 2007; Zheng, Aslund, & Storz, 1998). Based on sequence homology, we postulate that the TR Bxe_B2842 is an OhrR homolog.

OhrR, the organic hydroperoxide (OHP) regulator, is a member of the MarR family of TRs and is widely found in many Gram-negative and Gram-positive bacteria. OhrR controls the expression of a gene (*ohr*) in *Bacillus subtilis*, *Xanthomonas campestris*, *Agrobacterium tumefaciens*, and *Streptomyces coelicolor* (Chuchue et al., 2006; Fuangthong, Atichartpongkul, Mongkolsuk, & Helmann, 2001; Oh, Shin, & Roe, 2007; Panmanee et al., 2002). The gene *ohr* encodes for a thiol peroxidase that reduces OHPs to their corresponding alcohols (Newberry et al., 2007). In Gram-negative bacteria, OhrR belongs to the two-cysteine OhrR family in which the conserved N-terminal cysteine residue is critical for its roles as a TR and OHP sensor (Atichartpongkul, Fuangthong, Vattanaviboon, & Mongkolsuk, 2010; Fuangthong & Helmann, 2002; Newberry et al., 2007; Panmanee, Vattanaviboon, Poole, & Mongkolsuk, 2006). OHP sensing by the two-cysteine OhrR family involves the initial oxidation by the OHP of the N-terminal cysteine to an unstable sulfenic acid intermediate, which then forms a disulfide bond with the conserved C-terminal cysteine (Panmanee et al., 2006). Structural evidence indicates that the oxidized OhrR undergoes a conformational change that results in dissociation from DOS (Newberry et al., 2007). Alternatively, for the one-cysteine family of OhrR, oxidation of the conserved cysteine results in the formation of a mixed disulfide bond with low molecular weight intracellular thiols (Fuangthong & Helmann, 2002; Lee, Soonsanga, & Helmann, 2007) followed by conformational change of the TR and release of the DOS.

Here, we report the characterization of *bxe_B2842*, an OhrR-typeTR found in a putative ROS-resistance operon in *B. xenovorans* composed of *osmC*, a predicted organic hydroperoxide resistance protein (*bxe_B2843*) and *phrB*, a predicted deoxyribodipyrimidine photo-lyase (*bxe_B2844*) in *Burkholderia xenovorans* (Figure 1). However, a recent publication by Cahoon and coworkers suggests that PhrB is a ROS-responsive protein involved in resistance to elevated cellular ROS and not a deoxyribodipyrimidine photo-lyase (Cahoon, Stohl, & Seifert, 2011). To date, all the reportedOhrRs have been responsive to hydroperoxides, but no studies have examined non-hydroperoxide ligands. We have utilized our recently developed frontal affinity chromatography coupled with mass spectrometry (FAC-MS) approach to TR discovery (Martí-Arbona et al., 2012) and electrophoretic mobility gel shift assays (EMSA) to identify and characterize the possible effectors of Bxe_B2842. Our investigation of Bxe_B2842 from *B. xenovorans* suggests that this TR regulates the expression of the putative ROS-resistance operon by binding to the phenolic ligand, 2-aminophenol, and that it is also responsive to OHPs.

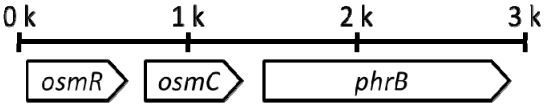


Figure 1. A putative ROS-resistance operon in *B. xenovorans* regulated by the TR *bxe_B2842* (*osmR*). The two ROS-resistance genes in the operon are *bxe_B2843* (*osmC*) and *bxe_B2844* (*phrB*)

2. Materials and Methods

2.1 Materials

Buffer components and effector molecules were purchased from Sigma Aldrich or Acros. The Exactive mass spectrometer was purchased from Thermo Scientific. DNA oligos were purchased from IDT or Invitrogen.

Restriction enzymes were purchased from New England Biolabs and polymerases from Invitrogen. Native and SDS gels were purchased from Invitrogen or Expedeon.

2.2 Cloning and Overexpression

2.2.1 GST-Tagged Protein

The gene *bxe_B2842* was cloned from the *B. xen*ovorans LB400 genomic DNA into the pGEX-KG expression vector. The *bxe_B2842* gene was amplified by conventional PCR methods (manufacturer's (Invitrogen) protocol for Platinum *Pfx* DNA polymerase) using two primers: forward 5'-GATCCATGGATGCAACGCAGCCTC-3' and reverse 5'-GATCGGATCCTCATGCCCGGACGCGG-3', containing *Nco*I and *BamH*I sites, respectively. The resulting PCR product was digested with the respective endonucleases and ligated into the pGEX-KG vector at the *Nco*I and *BamH*I sites. The recombinant plasmid was then transformed and the protein was expressed in Arctic Express cells. A single colony was used to inoculate a 50 mL overnight culture of LB medium containing 100 µg/mL ampicillin and 25 µg/mL gentimycin. Then, 5 mL of overnight culture was used to inoculate 1 L of LB media. The cells were grown at 30 ºC for approximately 5 hours or until the cell density reached ~0.5 OD at 600 nm, induced with 1.0 mM isopropylthiogalactoside and incubated overnight at 13 ºC.

2.2.2 His_8-Tagged Protein

The gene *bxe_B2842* was cloned from the *B. xen*ovorans LB400 genomic DNA into the pET42a(+) expression vector. The *bxe_B2842* gene was amplified by conventional PCR methods (manufacturer's (Invitrogen) protocol for Platinum *Pfx* DNA polymerase) using two primers: forward 5'-GGAATTCCATATGGACCCAGCGCCCCGCTTTGCCCTTCACGCTCGACG-3' and reverse 5'-CCGCTCGAGCCTGCGCGGCACACTCAACGATTACATGGACCGCTAG-3', containing *Nde*I and *Xho*I sites, respectively. The resulting PCR product was digested with the respective endonucleases and ligated into the pET42a(+) vector at the *Nde*I and *Xho*I sites. The C-terminal His_8-tagged Bxe_B2842 (Bxe_B2842-His_8) encoding recombinant plasmid was then transformed and the protein was expressed in Arctic Express cells. A single colony was used to inoculate a 50 mL overnight culture of LB medium containing 50 µg/mL kanamycin and 25 µg/mL gentimycin. Then, 5 mL of overnight culture was used to inoculate1 L of LB media. The cells were grown at 30 ºC for approximately 5 hours or until the cell density reached ~0.5 OD at 600 nm, induced with 1.0 mM isopropylthiogalactoside and incubated overnight at 13 ºC.

2.2.3 Protein Purification

After the overnight incubation, the cells were collected by centrifugation at 6,000 × *g* and the cell pellet was resuspended in a 20 mM Na_2PO_4, buffer containing 100 mM NaCl, 1mM dithiothretinol (DTT) and 1 mM ethylenediaminetethraacetate (EDTA) (buffer A). The resuspended cells were supplemented with 2 units/mL *DNase*I and 1 mg/mL phenylmethanesulfonyl fluoride, sonicated and centrifuged at 12,000 × *g* for 20 minutes. The cell lysate supernatant was filtered with a 0.45 µm filter and loaded onto an appropriate column (GSTrap or HisTrap column for GE Healthcare) pre-equilibrated with buffer A. The GST-Bxe_B2842 protein was eluted with a 0-50% gradient of 20 mM Tris at pH 8.0 containing 1 mM DTT) and 700 mM glutathione. The Bxe_B2842-His_8 protein was eluted with a 0-55% gradient of buffer A containing 700 mM imidazole. In each case, the protein was pooled, concentrated, and loaded onto a gel-filtration column pre-equilibrated with buffer A . The tagged-Bxe_B2842 protein was collected and verified by SDS-PAGE. The protein was stored in buffer A. The *GST*- Bxe_B2842 protein was utilized for the FAC-MS and PBM experiments described below, while the Bxe_B2842-His_8 protein was utilized for the EMSA experiments described below.

2.3 Frontal Affinity Chromatography - Mass Spectrometry

The frontal affinity chromatography coupled with mass spectrometry (FAC-MS) approach to TR discovery used here was similar to that described by Martí-Arbona et al. (Martí-Arbona et al., 2012). Briefly, GST-Bxe_B2842 protein (13 mg) was immobilized to a 1 ml GSTrap FF (GE Healthcare) affinity column, through which a mixture of effector-candidates was infused; the elution of these effector candidates was monitored with a mass spectrometer (Exactive Orbitrap, Thermo Scientific). The effector candidates were serine, cysteine, 2-aminophenol, homoserine, benzoic acid, *o*-phospho-L-serine, 2,5-dihydroxybenzoic acid, salicylic acid, ADP, adenine, adenosine, AMP, 2-deoxyguanosine, MTA, ATP, guanosine, hypoxanthine, xanthine, inosine, xanthosine, tryptophan, L-kynurenine, L-phenylalanine, anthranilic acid, 3-hydroxyanthranilic acid, 4-hydroxybenzoic acid, L-tyrosine, 3,4-dihydroxybenzoic acid, catechol, and chlorocatechol dissolved in the chromatography buffer (20 mM ammonium formate buffer at pH 7.3) to a final concentration of 10uM each. Compounds that were not bound by the Bxe_B2842 protein quickly eluted from the column while those for which Bxe_B2842 had affinity were retained in the column and eluted at a later time. The tighter a molecule was

bound to the protein, the later it eluted. Thus, the retention time of each compound was used to distinguish between binders and non-binders. The ion intensity for each compound was normalized to its highest and plotted (elution profile) using Sigma Plot 12.3.

2.4 Electrophoretic Mobility Shift Assay (EMSA)

2.4.1 Preparation of Bxe_B2842 to Identify Possible DOS

To investigate Bxe_B2842's binding to its DNA operator sequence, EMSAs were performed using the intergenomic DNA sequences upstream from the *bxe_B2842* gene. The 201 bases upstream sequence was cloned from *B. xen*ovorans LB400 genomic DNA by conventional PCR methods (manufacturer's (Invitrogen) protocol for Platinum *Pfx* DNA polymerase) with the following pairs of primers: 5'-Alexa488-CCTGCCGACCACACCGGCGGCCTGATTCC-3' /5'-GGGCAAAGCGGGGCGCTGGGTCATGG-3' and 5'-Alexa488-CATGGACCGCTAGCCCGCCGGCTCCAGAACG-3' /5'-GCTTTGTAGAGGATGTTCTAGATGCTGTTCCTTTGTCTG-3', respectively. The Alexa488-taggedPCR product was purified using the Qiagen Quick Purification kit and stored in TB buffer until EMSAs were performed.

2.4.2 Screening for the TR/DNA/Effector Interactions

Regulation by transcriptional regulators includes two binding events. In this case, the protein Bxe_B2842 binds its DOS (first binding event) and the binding of the effector molecule to the TR/DOS complex (second binding event) causes the collapse of the binary complex and the release of the DOS. The subsections below describe the characterization of the two binding events.

2.4.2.1 TR/DOS Binary Complex Formation

The TR/DOS binding reactions were performed with 20.25 nMDOS (in separate experiments) and various concentrations of the Bxe_B2842-His_8 in binding buffer containing 10 mM Tris, 200 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 5.0 mM DTT at pH 7.5. After incubating binding reactions at 37ºC for 1 hour, the samples were loaded onto a 7% polyacrylamide native gel for electrophoresis in a 34 mM HEPES and 43 mM imidazole running buffer that were pre-run at 150 V for 90 minutes. The electrophoretic mobility of the fluorescent Alex488-DOS was monitored with a Hitachi FMBio III fluoresence imager using a 100 micron resolution with an argon ion 488 nm laser and a 530 nm filter (25 nm bandpass, Semrock).

2.4.2.2 Effector Binding to the TR/DOS Binary Complex

Binding reactions in the presence of effectors were performed with 20.25 mM DOS, 500 nM Bxe_B2842-His_8 and variable concentrations of 2-aminophenol or anthranilic acid (5-20 mM), 0-20 μM hydroperoxides (hydrogen peroxide, t-butyl hydroperoxide or cumene hydroperoxide). After incubating binding reactions at 37ºC for 1 hour, the samples were loaded onto a 7% polyacrylamide native gel for electrophoresis in a 34 mM HEPES and 43 mM imidazole running buffer that were pre-run at 150 V for 90 minutes. The electrophoretic mobility of the fluorescent Alex488-DOS was monitored with a Hitachi FMBio III fluoresence imager using a 100 micron resolution with an argon ion 488 nm laser. Alexa-488 emission was detected by passage through a 530 nm filter (25 nm bandpass, Semrock).

3. Results and Discussion

3.1Bioinformatic Analysis

Bxe_B2842 is a TR belonging to the MarR family. With its 52% and 55% sequence identities (BLAST tool on NCBI website) to the characterized OHP resistance TRs (OhrR) of *B. subtilis* and *P. aeruginosa*, respectively, Bxe_B2842 mostly resembles an organic hydroperoxide resistance regulator (OhrR). Direct sequence comparisons (BLAST tool on NCI website) of Bxe_B2842 with its OhrR homologs indicate that Bxe_B2842 belongs to a two-cysteine family of OhrRs, having two mechanistically important conserved cysteines at residues 16 and 21 (Chuchue et al., 2006; Fuangthong, Atichartpongkul, Mongkolsuk, & Helmann, 2001; Oh, Shin, & Roe, 2007; Panmanee et al., 2002). According to the current model (Panmanee *et al*., 2006), oxidation of the reactive Cys-16 would result in disulfide formation with Cys-121 and subsequent attenuation of DNA-binding. This allows RNA polymerase to bind to the promoter and initiate expression of regulated genes, in this case b*xe_B2843* and b*xe_B2844*.

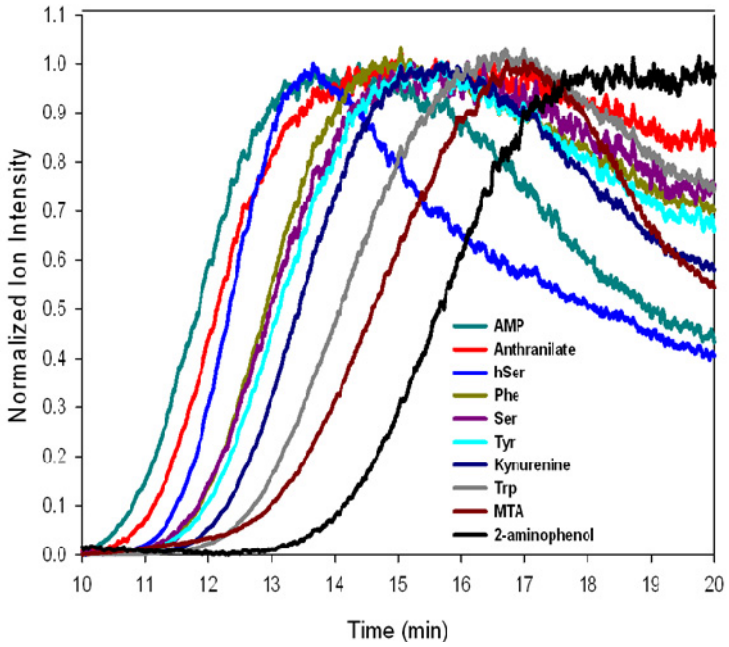


Figure 2. FAC-MS screening for possible effectors binding to GST-Bxe_B2842. The color-coded elution profiles for the individual candidate effectors (10uM dissolved in 20 mM ammonium formate, pH 7.3) are keyed in the figure itself. Compounds that did not bound to the immobilized GST-Bxe_B2842 protein quickly eluted from the column while those with higher affinity to GST-Bxe_B2842 were retained in the column and eluted at a later time. 2-Aminophenol showed the highest binding affinity of all the compounds tested

The genomic context of *bxe_B2842* shows that it is located immediately upstream of *bxe_B2843* and *bxe_B2844*. We suggest the annotation of *bxe-B2843* gene as *osmC* based on its 42% and 43% shared sequence identity with the *ohrA* and *ohrB* genes (thiol-dependent peroxidases), respectively, in *B. subtilis* and its 69% identity to the *ohr* gene in *P. aeruginosa*. Immediately downstream of the *bxe_B2843* (osmC) gene is the *bxe_B2844* gene (*phrB*), which is annotated in the NCBI database as a putative deoxyribodipyrimidine photo-lyase. The *phrB* (*bxe_B2844*) has 40% shared sequence identity with the *phrB* gene in *P. aeruginosa* PA7 and 41% with the *phrB* gene in *E. coli* K12. A recent publication by Cahoon and coworkers suggests that PhrB is a ROS-responsive protein involved in resistance to elevated cellular ROS and not a deoxyribodipyrimidine photo-lyase (Cahoon, Stohl, & Seifert, 2011). Given the genomic context of these genes, it is highly probable that the gene *bxe_B2842* is involved in the regulation of the expression of the *osmC* and *phrB* genes and is involved in the modulation of *Burkholderia*'s resistance to OHPs. Our experimental work was designed to investigate this possible function of Bxe_B2842 protein.

3.2 Discovery of Effectors by Frontal Affinity Chromatography - Mass Spectrometry (FAC-MS)

OhrR TRs bind various phenolic compounds. Structural studies of these homologs indicate a conservation of a hydrophobic effector site (Hong, Fuangthong, Helmann, & Brennan, 2005). Close examination of the genomic context of the *bxe_B2842* gene and its closest homologs in other bacteria identified a conserved cluster of annotated genes that included AMP nucleosidase, deoxyribodipyrimidine photo-lyase and homoserine kinase. A library of effector candidates was constructed that included substrates and products of these enzymes and their analogs as well as a set of commercially available phenolic compounds and organic acids. The results of the FAC-MS assay (Figure 2) showed that the immobilized GST-Bxe_B2842 was capable of binding to phenolic ligands, with 2-aminophenol being the most tightly bound of the compounds tested. The other metabolites were either not bound or weakly bound by the TR.

3.3 Identification of DNA-Binding Motif

Electrophoretic mobility shift assays (EMSA) using the intergenomic sequences of *bxe_B2842* and *osmC*, a near genomic neighbor of *bxe_B2842*, provide evidence that this TR binds to the promoter regions of these two genes in a concentration-dependent manner. We analyzed the upstream regions of *bxe_B2842* and its nine closest homologs from different *Burkholderia* species for similarity to uncover a common DOS, with a general sequence

for 5'-A(A\T)T(C\A)ATTTG(C\T)(A\G)(T\C)(G/A)CAAAT(G/T)A(A\T)T-3' (palindromic operator sequence, Figure 3). Moreover, when we performed the same search for *osmC* (*bxe_B2843*) and its nine closest homologs, the same palindromic sequence was readily identified. A highly symmetrical and large consensus sequence was discovered that is present in all 20 sequences. This level of conservation and large palindromic symmetry is highly consistent withthe possibility that there is a common DNA operator sequence that is used to regulate both the *ohrR* TR gene and the downstream *osmC* gene and very likely the other member of the ROS-responsive operon *phrB*.

In the absence of an effector molecule, the EMSA result (Figure 4) demonstrated that the TR binds to the DOS sequencein a concentration-dependent manner creating a TR/DOS binary complex. This suggests that the TR acts as a repressor; when there is no effector present, the TR binds to the DOS and prevents transcription of the putative ROS-responsive operon and the expression of the OsmC protein. The common DOS suggests that Bxe_B2842 controls its own expression and that of OsmC and very likely the expression of the PhrB protein.

3.4 Characterization of the Effector and Its Effects on the TR/DOS Binary Complex

EMSA results suggested that micromolar concentrations OHPs and millimolar concentrations of 2-aminophenol are capable of disrupting the TR/DOS binary complex and of releasing the DOS. In the presence of 2-aminophenol, the protein is released from the DOS (Figure 4). Anthranilic acid was used a control because it had been identified by the FAC-MS effector screen as not being bound by the TR. As could be expected from that result, anthranilic acid did not disrupt the TR/DOS binary complex (Figure 5). This suggests that 2-aminophenol is an active effector of Bxe_B2842. Therefore, Bxe_B2842 is the first OhrR shown to have non-hydroperoxide effector-regulated activity. It is well known that 2- or 4-aminophenols are oxidized by O_2 producing ROS, which could lead to the formation of organic peroxides (Prati, Rossi, & Ravasio, 1992). It makes sense that the expression of genes involved in the detoxification of ROS (i.e., *bxe_B2843* and *bxe_B2844*) are controlled by a TR that is activated by either the presence of ROS or 2-aminophenol.

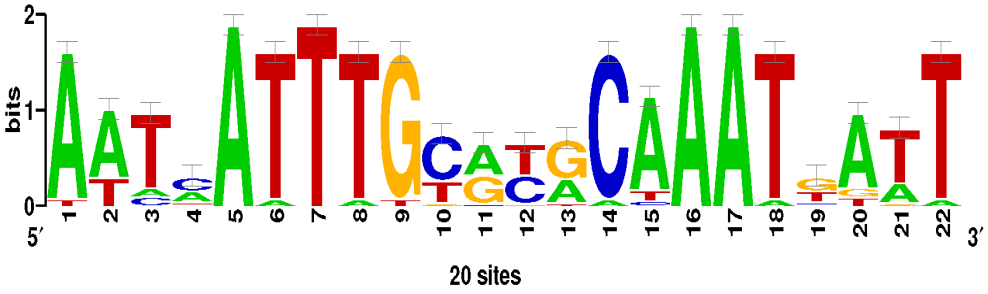


Figure 3. Consensus DNA sequence conserved in the promoter regions of *bxe_B2842* and its nine closest homologues from different *Burkholderia* species

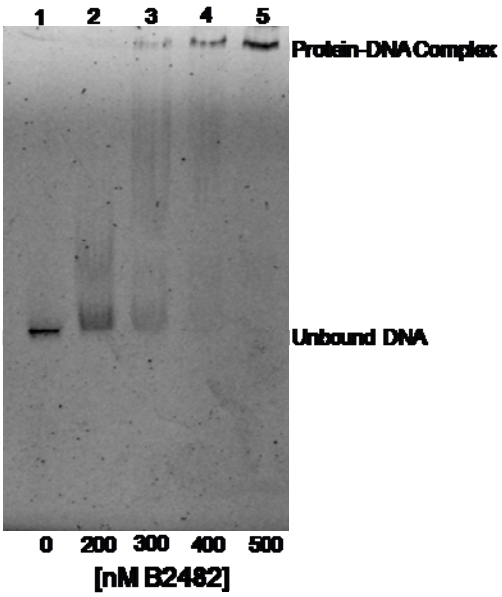


Figure 4. EMSA test for Bxe_B2842 binding to its own promoter (201 bp upstream, DOS). The concentration dependency of the DNA binding was tested by the incremental titration of the Bxe_B2842-His_8

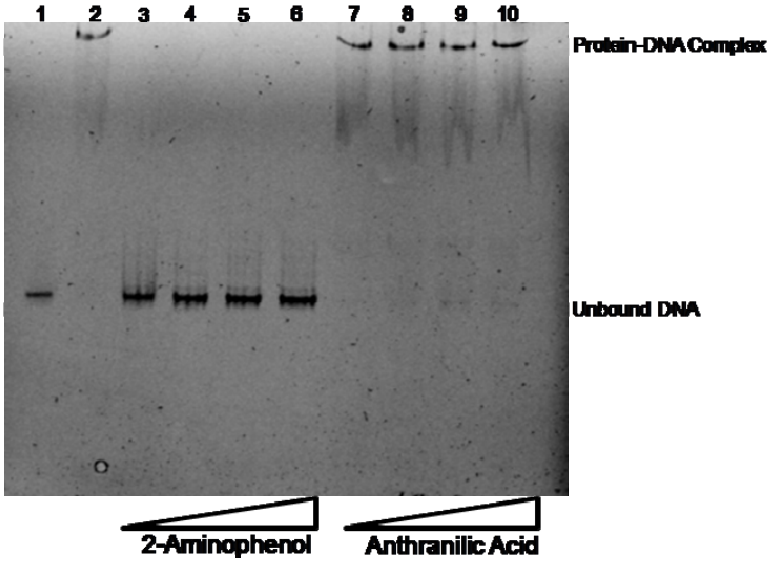


Figure 5. EMSA test for Bxe_B2842-His_8 (500 nM)/DOS (20.25 nM) with two metabolites from the FAC-MS library: anthranilate (a non-binder in the FAC-MS) and 2-aminophenol (the best binder in the FAC-MS). Lane 1 contains labeled DOS, no protein and effector. Lane 2 contains Bxe_B2842-His_8/DOS but no effector. Lanes 3 to 6 contain Bxe_B2842-His_8/DOS with incremental concentrations of 2-aminophenol ranging from 5 to 20 mM. Lanes 7 to 10 contain Bxe_B2842-His_8/DOS with incremental concentrations of 2-aminophenol ranging from 5 to 20 mM

Also consistent with this TR having non-hydroperoxide and hydroperoxide effectors are the results from EMSA with three hydroperoxides (Figure 6). H_2O_2, t-butyl hydroperoxide and cumene hydroperoxide disrupted the TR/DOS binary complex, suggesting the oxidation of the two conserved cysteines in Bxe_B2842. At low concentrations of OHP, only the TR/DOS binary complex can be detected on the gel; as the concentrations of the OHPs increase, the TR/DOS binary complex is disrupted and the free DOS can be observed. This suggests that TR oxidation causes the release of the DOS. EMSA results also indicate that the presence of hydroperoxides in the absence of protein has no effect on the DNA's electrophoretic mobility. These results are consistent with

previous studies on homologous OhrRs from *B. subtilis* and *X. campestris* (Chuchue et al., 2006; Fuangthong, Atichartpongkul, Mongkolsuk, & Helmann, 2001; Oh, Shin, & Roe, 2007; Panmanee et al., 2002) and suggest that Bxe_B2842 is the regulatory proteinOhrRthat governs the transcription of the ROS-responsive operon composed of OsmC and PhrB in *B. xenovorans*. More experiments are needed to confirm the *in-vivo* regulatory effect of 2-aminophenol on *bxe*_B2842 in *B. xenovorans*.

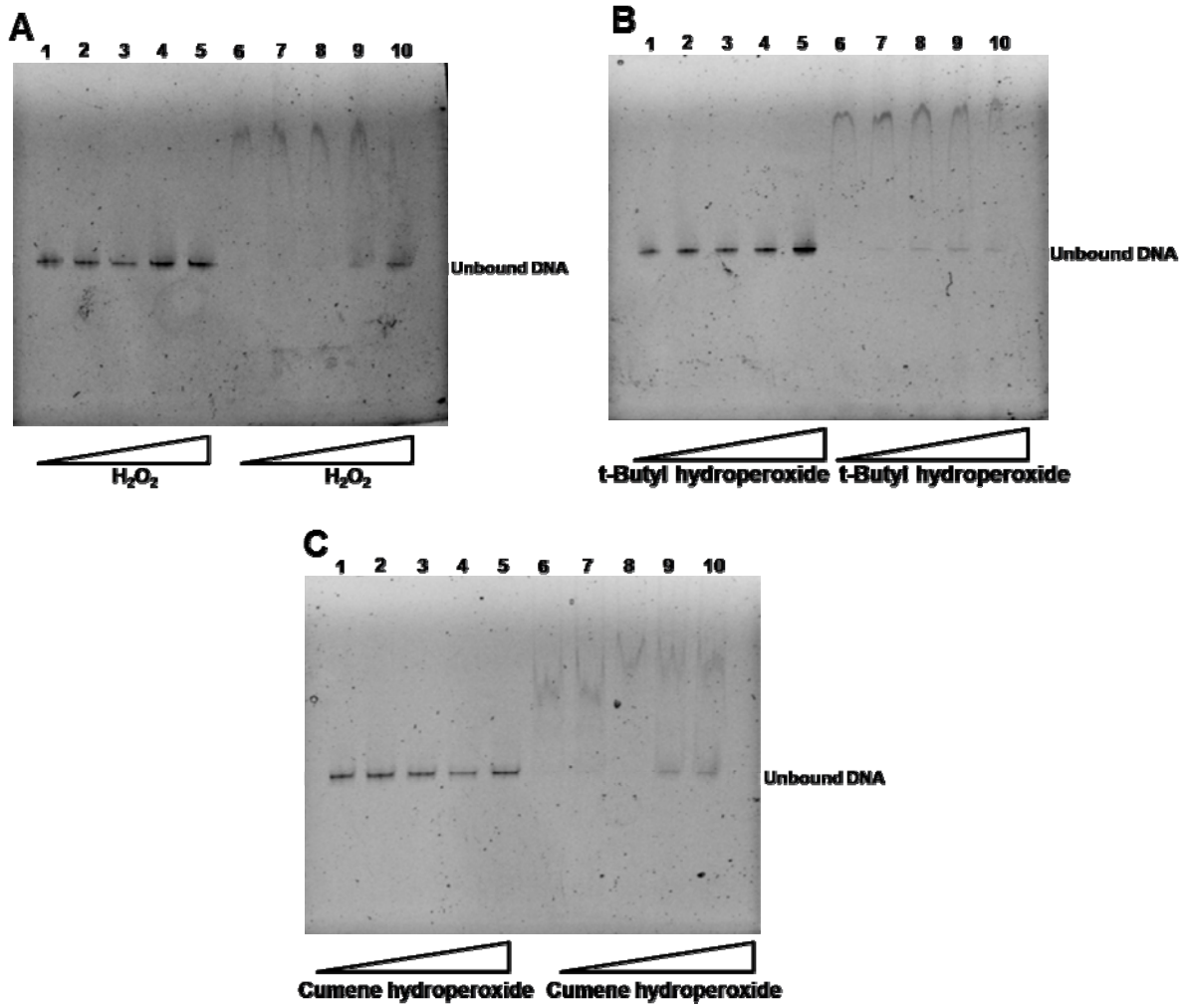


Figure 6. EMSA test for Bxe_B2842-*His*$_8$ (500 nM)/DOS (20.25 nM) in the presence of different hydroperoxides (A- hydrogen peroxide, B- t-butyl hydroperoxide, C- cumene hydroperoxide) at concentrations ranging from 0 - 20 µM. In all three gels (A-C), lanes 1 to 5 correspond to reactions containing the DOS and the hydroperoxide without the TR. Lanes 6 to 10 correspond to reactions containing Bxe_B2842-*His*$_8$/DOS and incremental concentrations of the hydroperoxides

3.5 Conclusion

Here, we identified the first organic hydroperoxide responsive TR (OhrR, Bxe_B2842) that is responsive to either hydroperoxides and the expected chemical modification or the binding of a small molecule effector that cannot modify the TR. Organic hydroperoxides are known to act by chemically oxidizing the two conserved cysteines on the C terminal of the OhrR. Oxidation of the cysteines is very unlikely for 2-aminophenol, which lacks oxidizing properties. The 2-aminophenol can thus be expected to act as a typical small molecule effector whose binding induces a conformational change and the release of the DOS. As an effector, 2-aminophenol plays a role in *B. xenovorans'* response to ROS because 2-aminophenol is an intermediate in tryptophan or nitrobenzene metabolism and can be metabolized to generate ROS. Many bacterial TRs bind an effector molecule, but the finding of two functionally related but structurally distinct types of molecules to which this TR can respond has not been previously reported.

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References

Alekshun, M. N., & Levy, S. B. (1999). Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli marRAB* locus, by multiple chemicals in vitro. *Journal of Bacteriology., 181*(15), 4669-4672.

Alekshun, M. N., Levy, S. B., Mealy, T. R., Seaton, B. A., & Head, J. F. (2001). The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. *Nature Structural Biology, 8*(8), 710-714. http://dx.doi.org/10.1038/90429

Ariza, R. R., Cohen, S. P., Bachhawat, N., Levy, S. B., & Demple, B. (1994). Repressor mutations in the *marRAB* operon that activate oxidative stress genes and multiple antibiotic resistance in *Escherichia coli*. *Journal of Bacteriology, 176*(1), 143-148.

Atichartpongkul, S., Fuangthong, M., Vattanaviboon, P., & Mongkolsuk, S. (2010). Analyses of the regulatory mechanism and physiological roles of Pseudomonas aeruginosa OhrR, a transcription regulator and a sensor of organic hydroperoxides. *Journal of Bacteriology, 192*(8), 2093-2101. http://dx.doi.org/10.1128/JB.01510-09

Bedard, D. L., Unterman, R., Bopp, L. H., Brennan, M. J., Haberl, M. L., & Johnson, C. (1986). Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Applied and Environmental Microbiology, 51*(4), 761-768.

Chain, P. S., Denef, V. J., Konstantinidis, K. T., Vergez, L. M., Agullo, L., Reyes, V. L., … Tiedje, J. M. (2006). *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. *Proceedings of the National Academy of Sciences of the United States of America, 103*(42), 15280-15287. http://dx.doi.org/10.1073/pnas.0606924103

Chuchue, T., Tanboon, W., Prapagdee, B., Dubbs, J. M., Vattanaviboon, P., & Mongkolsuk, S. (2006). OhrR and ohr are the primary sensor/regulator and protective genes against organic hydroperoxide stress in Agrobacterium tumefaciens. *Journal of Bacteriology, 188*(3), 842-851. http://dx.doi.org/10.1128/JB.188.3.842-851.2006

Cohen, S. P., Hachler, H., & Levy, S. B. (1993). Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. *Journal of bacteriology, 175*(5), 1484-1492.

Finn, R. D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J. E., … Bateman, A. (2010). The Pfam protein families database. *Nucleic Acids Research, 38*(Database issue), D211-222. http://dx.doi.org/10.1093/nar/gkp985

Fuangthong, M., Atichartpongkul, S., Mongkolsuk, S., & Helmann, J. D. (2001). OhrR is a repressor of ohrA, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. *Journal of Bacteriology, 183*(14), 4134-4141. http://dx.doi.org/10.1128/JB.183.14.4134-4141.2001

Fuangthong, M., & Helmann, J. D. (2002). The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proceedings of the National Academy of Sciences of the United States of America, 99*(10), 6690-6695. http://dx.doi.org/10.1073/pnas.102483199

Goris, J., De Vos, P., Caballero-Mellado, J., Park, J., Falsen, E., Quensen, J. F., … Vandamme, P. (2004). Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400T and relatives as *Burkholderia xenovorans* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 54*(Part 5), 1677-1681. http://dx.doi.org/10.1099/ijs.0.63101-0

Helmann, J. D., Wu, M. F., Gaballa, A., Kobel, P. A., Morshedi, M. M., Fawcett, P., & Paddon, C. (2003). The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. *Journal of Bacteriology, 185*(1), 243-253.

Hidalgo, E., Leautaud, V., & Demple, B. (1998). The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator. *The EMBO Journal, 17*(9), 2629-2636. http://dx.doi.org/10.1093/emboj/17.9.2629

Lee, J. W., Soonsanga, S., & Helmann, J. D. (2007). A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proceedings of the National Academy of Sciences of the United States of America, 104*(21), 8743-8748. http://dx.doi.org/10.1073/pnas.0702081104

Maltseva, O. V., Tsoi, T. V., Quensen, J. F., 3rd, Fukuda, M., & Tiedje, J. M. (1999). Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. *Biodegradation, 10*(5), 363-371.

Martí-Arbona, R., Teshima, M., Anderson, P. S., Nowak-Lovato, K. L., Hong-Geller, E., Unkefer, C. J., & Unkefer, P. J. (2012). Identification of new ligands for the methionine biosynthesis transcriptional regulator (MetJ) by FAC-MS. Jo*urnal of Molecular Microbiology and Biotechnology, 22*(4), 205-214. http://dx.doi.org/10.1159/000339717

Martin, R. G., & Rosner, J. L. (1995). Binding of purified multiple antibiotic-resistance repressor protein (MarR) to mar operator sequences. *Proceedings of the National Academy of Sciences of the United States of America, 92*(12), 5456-5460.

Mongkolsuk, S., & Helmann, J. D. (2002). Regulation of inducible peroxide stress responses. *Molecular Microbiology, 45*(1), 9-15.

Newberry, K. J., Fuangthong, M., Panmanee, W., Mongkolsuk, S., & Brennan, R. G. (2007). Structural mechanism of organic hydroperoxide induction of the transcription regulator OhrR. *Molecular Cell, 28*(4), 652-664. http://dx.doi.org/10.1016/j.molcel.2007.09.016

Oh, S. Y., Shin, J. H., & Roe, J. H. (2007). Dual role of OhrR as a repressor and an activator in response to organic hydroperoxides in Streptomyces coelicolor. *Journal of Bacteriology, 189*(17), 6284-6292. http://dx.doi.org/10.1128/JB.00632-07

Panmanee, W., Vattanaviboon, P., Eiamphungporn, W., Whangsuk, W., Sallabhan, R., & Mongkolsuk, S. (2002). OhrR, a transcription repressor that senses and responds to changes in organic peroxide levels in *Xanthomonas campestris* pv. phaseoli. *Molecular Microbiology, 45*(6), 1647-1654.

Panmanee, W., Vattanaviboon, P., Poole, L. B., & Mongkolsuk, S. (2006). Novel organic hydroperoxide-sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress. *Journal of Bacteriology, 188*(4), 1389-1395. http://dx.doi.org/10.1128/JB.188.4.1389-1395.2006

Perera, I. C., & Grove, A. (2010). Molecular mechanisms of ligand-mediated attenuation of DNA binding by MarR family transcriptional regulators. *Journal of Molecular Cell Biology, 2*(5), 243-254. http://dx.doi.org/10.1093/jmcb/mjq021

Seeger, M., Timmis, K. N., & Hofer, B. (1995). Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the bph locus of *Pseudomonas* sp. strain LB400. *Applied and Environmental Microbiology, 61*(7), 2654-2658.

Seeger, M., Zielinski, M., Timmis, K. N., & Hofer, B. (1999). Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the bph-encoded catabolic pathway of *Burkholderia* sp. strain LB400. *Applied and Environmental Microbiology, 65*(8), 3614-3621.

Sulavik, M. C., Gambino, L. F., & Miller, P. F. (1995). The MarR repressor of the multiple antibiotic resistance (mar) operon in *Escherichia coli*: prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. *Molecular Medicine, 1*(4), 436-446.

Zheng, M., Aslund, F., & Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science, 279*(5357), 1718-1721.