Redox-sensitive MarR homolog BifR from *Burkholderia thailandensis* regulates biofilm formation

Ashish Gupta, Stanley M. Fuentes, Anne Grove

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

Content:

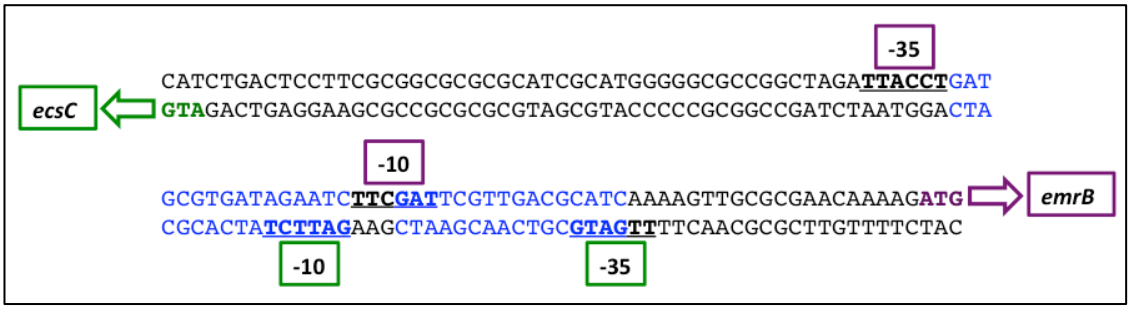

Figure S1. Predicted promoter elements for divergent genes overlap with the identified palindromes. The palindromes are shown in blue. Annotated translational start codons for *ecsC* and *emrB* are shown in green and magenta, respectively, with corresponding -10 and -35 promoter elements bolded and underlined. Promoter elements were predicted using phiSITE.[1]

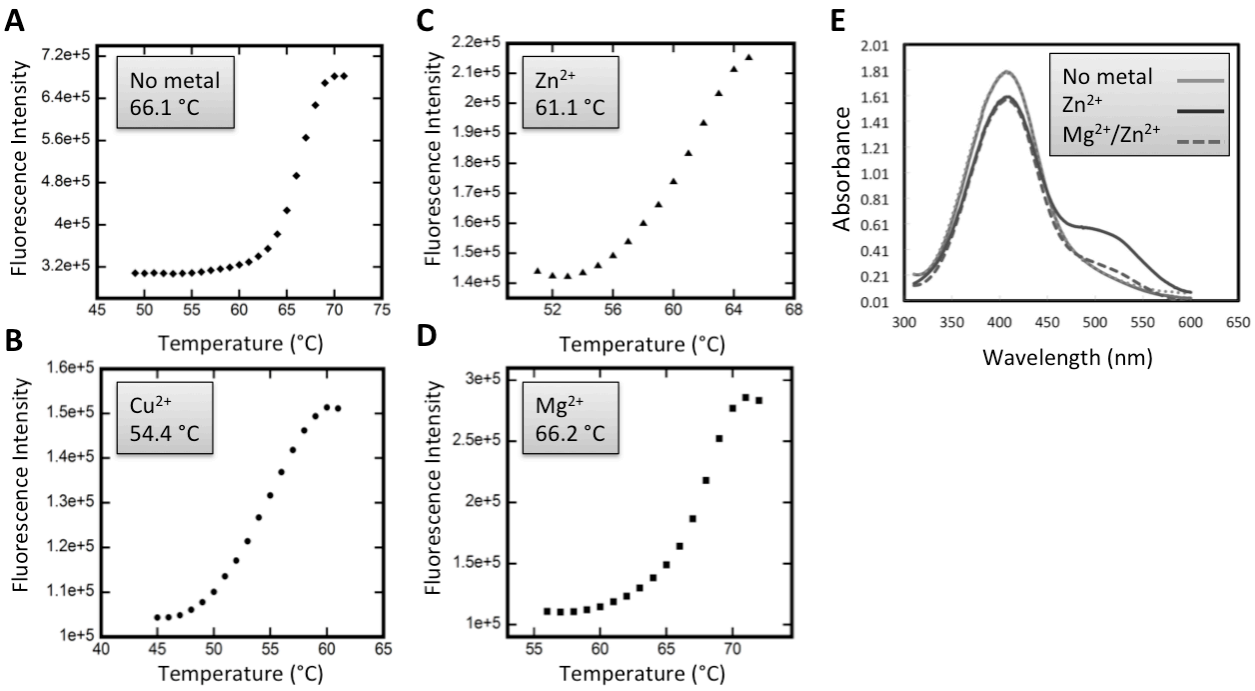

Figure S2. Metal binding by BifR. (A)-(D) Thermal denaturation of BifR determined by differential scanning fluorometry. (A) BifR only (10 μM). (B) BifR incubated with 1 mM $CuCl_2$ followed by dialysis. (C) BifR incubated with 2 mM $ZnCl_2$. (D) BifR incubated with 2 mM $MgCl_2$, followed by addition of 2 mM $ZnCl_2$. (E) Release of zinc from BifR observed by absorbance of the $(PAR)_2Zn^{2+}$ complex. Absorbance spectrum of buffer containing PAR (• dotted line; peak absorbance at 408 nm), native protein (grey line), BifR incubated with $ZnCl_2$ followed by dialysis to remove unbound metal (dark grey line), BifR incubated with $MgCl_2$, followed by $ZnCl_2$ and dialysis to remove unbound metal (dashed dark grey line).

Reduced BifR was quite stable, unfolding in a one-step melting transition with Tm = 66.1 °C. Copper(II)-oxidized BifR had a significantly lower melting temperature (Tm = 54.4 °C, Panel B). Other oxidants (tBHP, CHP, H_2O_2) had little effect on protein stability, perhaps in part reflecting the more efficient oxidation by copper(II) compared to the other oxidants. That BifR does bind metal was verified based on the reduced stability of zinc(II)-bound protein (Tm = 61.1 °C, Panel C). In contrast, addition of magnesium(II) to BifR had no affect on protein stability (Tm = 66.2 °C, Panel D).

To confirm metal-binding to BifR, we used a metal chelator 4-(2-pyridylazo) resorcinol (PAR) that forms complex with various divalent metals resulting in a diagnostic absorbance of the metal-PAR complex.[1] BifR was incubated with metal ions followed by dialysis to remove unbound metal. Subsequent denaturation of BifR to release bound metals was performed in presence of PAR, and the absorbance of metal-PAR complex was monitored (Panel E). BifR previously incubated with $ZnCl_2$ (dark continuous line) showed a significant increase in absorbance at ~500 nm compared to native BifR (grey line), reflecting release of BifR-bound zinc(II). Since the extinction coefficient for the Mg^{2+}-PAR complex is very low resulting in Mg^{2+} being effectively undetectable by this assay, Mg^{2+}-bound protein was incubated with $ZnCl_2$ and dialyzed. No change in the wavelength of PAR absorbance was observed when protein was incubated with magnesium(II) prior to addition of zinc(II) (dashed grey line), suggesting that Mg^{2+} competed with Zn^{2+} for binding to BifR. This is consistent with the measurement of thermal stability showing that zinc(II)-bound BifR has lower Tm than bipyridyl-treated BifR, whereas magnesium(II)-bound BifR or BifR incubated successively with $MgCl_2$ and $ZnCl_2$ exhibited the same thermal stability as bipyridyl-treated BifR (Table 1).

Metal binding to BifR-C104A was assessed by the thermal stability assay. A modest increase in thermal stability of BifR-C104A (Tm = 68.9 °C) was observed compared to wild-type BifR. Zinc(II)-binding modestly stabilized BifR-C104A (Tm = 71.4 °C), whereas incubation with $CuCl_2$ (Tm = 67.9 °C) had no

effect on protein stability. These data are consistent with Cys oxidation by $CuCl_2$ in wild-type BifR and they further suggest that the Cys to Ala substitution alters, but does not abolish metal (zinc) binding. That BifR has a metal-binding site other than that involving Cys104 is also suggested by the observation that incubation with Mg^{2+} or Zn^{2+} did not attenuate BifR oxidation by Cu^{2+} (disulfide bond formation; data not shown).

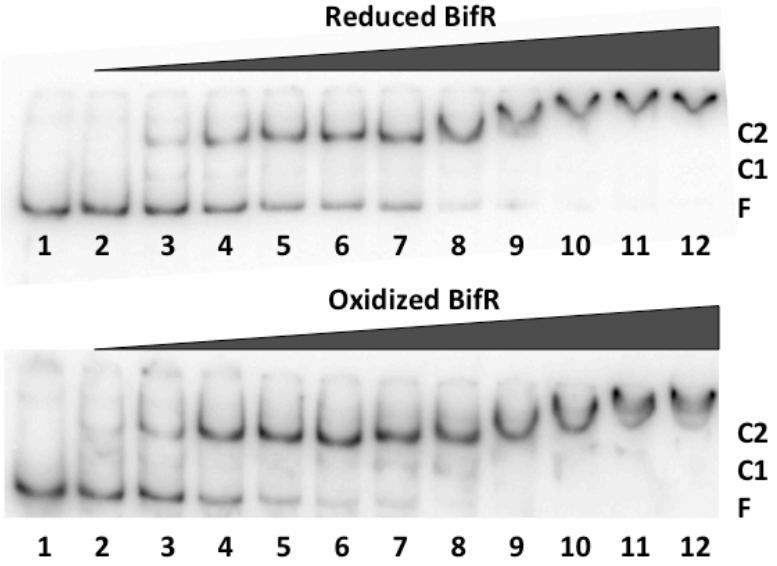

Figure S3. Oxidation by $CuCl_2$ does not significantly alter BifR binding. EMSA showing 355 bp operator DNA (0.8 nM) titrated with increasing concentration of reduced (top panel) or Cu^{2+}-oxidized (bottom panel) BifR (0.5 nM; lane 2, 1 nM; lane 3, 2.5 nM; lane 4, 5 nM; lane 5, 8 nM; lane 6, 10 nM; lane 7, 25 nM; lane 8, 50 nM; lane 9, 100 nM; lane 10, 150 nM; lane 11, 250 nM; lane 12); reactions in lanes 1 contains DNA only. Free DNA is identified as F and complexes are identified as C1 and C2 at the right.

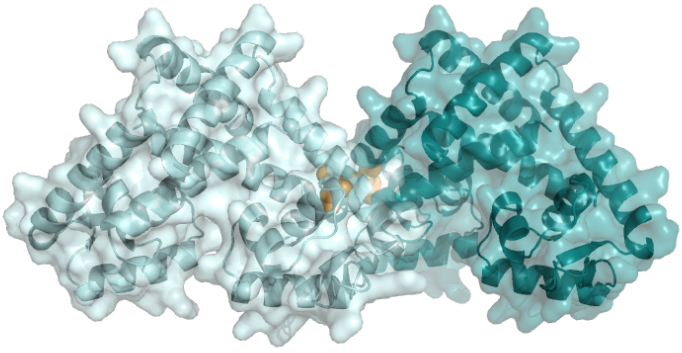

Figure S4. Representation of BifR dimer-of-dimers. The two dimers are assembled by rigid-body docking and shown in light and dark teal, respectively, with the darker dimer behind the lighter dimer. Cysteine residues involved in disulfide bond formation are shown as yellow spheres. Figure generated with PyMol.

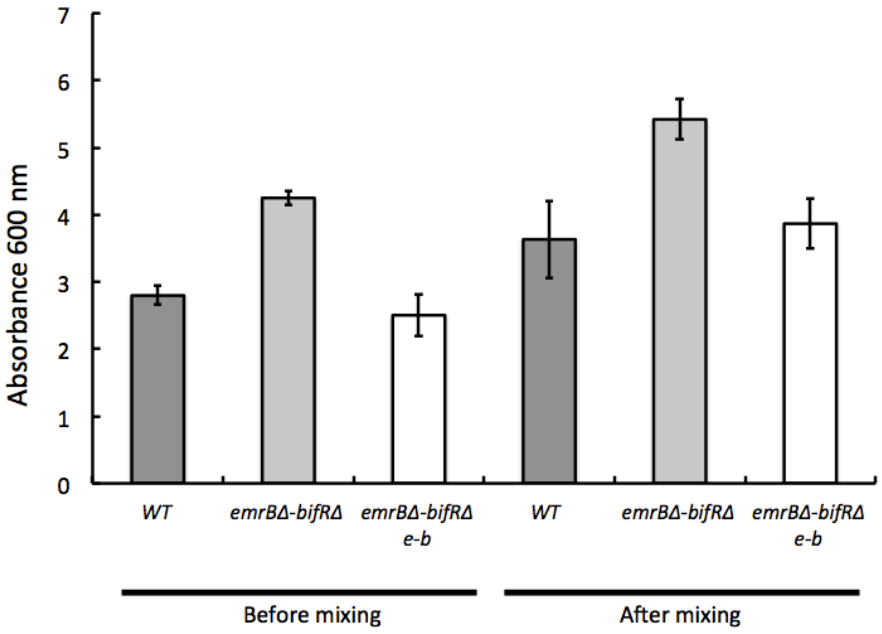

Figure S5. Absorbance of WT, *emrBΔ-bifRΔ*, and *emrBΔ-bifRΔ e-b*. Biofilm was formed in static culture and culture absorbance was measured before/after mixing the biofilm. Error bars represent the standard deviation of 3 replicates.

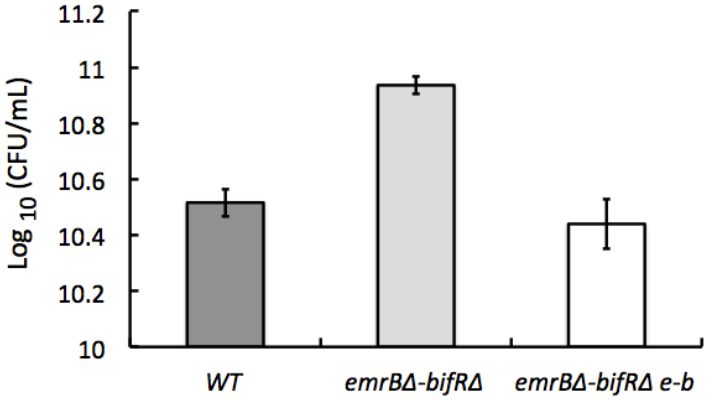

Figure S6. Log $_{10}$(CFU/mL) of WT, *emrBΔ-bifRΔ*, and *emrBΔ-bifRΔ e-b* after mixing the biofilm. Cultures were serially diluted to 10^6 and 20 µL of each diluted culture was spreaded on LB agar plates. Error bars represent the standard deviation of 5 replicates.

Table S1. Primers used

I541_Mut_fw	AAAGATGGCAGTCCATACCG
I541_Mut_Rev	AACATGTCGAACGGCAGAAT
LacZ_148	GGGTAACGCCAGGGTTTTCC

BifR_clon_Fw	CAGTCAGCATATGGAAGAACAGG
BifR_clon_Rev	CATCCTTGAATTCGCGCTCAC
C104A_Fw	GCGTTCGAGGCG**GCG**CTGCCGCGCCGCAACGCGTTC
C104A_Rev	GCGGCAG**CGC**CGCCTCGAACGCCGCGC
542 Top	GCTAGATTACCTGATGCGTGATAGAATCTTCGATTCGTTGACGCATCAAAAGTTGCG
542 Bot	CGCAACTTTTGATGCGTCAACGAATCGAAGATTCTATCACGCATCAGGTAATCTAGC
BifR_355_fw	CTTGCCGATCATTTTCTCGAC
BifR_355_Rev	GAGCATCGTGACGAAGGAGAT
Complementation	
EmrB_Xba_pBBR_Fw	GTTCCA**TCTAGA**TCCTTCGCGGCGCGCGCATCGC
EmrB_KpnI_pBBR_Rev	CTGCGGCTCC**GGTACC**CGTGAATGATGTGGCG
EmrB_BifR_kpnI_pBBR_Rev	CGCG**GGTACC**TTCACTTCGCGCTCACTTCGC
Veri_pBBR_XbaI fw	GTAATACGACTCACTATAGGGC
Veri_pBBR_KpnI_Rev	GCAATTAACCCTCACTAAAGG
qRT-PCR	
EmrB_qpCR_Fw	CGTTGACGCATCAAAAGTTG
EmrB_qpCR_Rev	AGCATCGTGACGAAGGAGAT
BifR_qPCR_Fw	GCGTGACGAACGTGAGACT
BifR_qPCR_Rev	GGACAGCGCACTGAGCAC
Glusynlg_qPCR_Fw	GCAAGAAGAGCCACGAAATC
Glusynlg_qPCR_Rev	CCATCTCCTCGCGATAGAAC
Nudix_qPCR_Fw	AGTACGAAGGCAAAATCCTGCT
Nudix_qPCR_Rev	GTTCTTGCGGATGAATTCGTAG

EcsC_qPCR_Fw	CGACATCGGCTATTTCGTGCTG
EcsC_qPCR_Rev	GTCGATGAAGAGCGTGTTGACCAT
PhzF_qPCR_Fw	ACGTTCGAGGTCCGCTGCCTGATGAC
PhzF_qPCR_Rev	ATCCAGATCTTGCCGGCGTCGTCGTAAT

Table S2. C_T values for qRT-PCR reference gene (GlusynIg)

Treatment/type of cells	C_T value
WT	26.5 ± 0.2
H_2O_2 treated WT	26.8 ± 0.1
Zn^{2+} treated WT	26.4 ± 0.2
Cu^{2+} treated WT	26.6 ± 0.1
Mg^{2+} treated WT	26.5 ± 0.3
bifRΔ	26.5 ± 0.1
bifRΔ e-b	26.4 ± 0.3
Cu^{2+} treated *bifRΔ*	26.6 ± 0.1
emrBΔ-bifRΔ	26.5 ± 0.2

Mean and standard deviation from three experiments.

Experimental

Thermal stability assay

Protein was diluted in thermal stability buffer (100 mM Tris (pH 8.0) and 200 mM NaCl) containing 5X SYPRO Orange dye (Invitrogen). To measure the effect of oxidants (tBHP, CHP and hydrogen peroxide) and metal ($CuCl_2$, $MgCl_2$, $ZnCl_2$), protein was mixed with oxidants/metals in a ratio of 1:100. Protein previously dialyzed against 50 mM bipyridyl to remove bound metals and protein incubated with metal, followed by dialysis to remove unbound metal, were also analyzed. An Applied Biosystems 7500 real time PCR system was used with increasing temperature from 5 °C to 94 °C in 1 degree increments. The Sigma Plot 9 four–parameter equation was used to fit the sigmoidal part of the melting curve. The data represents the mean of three replicates.

Metal binding by BifR

The metal binding assay was performed as described.[2] In brief, protein was incubated with 50 mM bipyridyl (metal chelator) at 4 °C. Bipyridyl-treated protein was dialyzed overnight against buffer (50 mM Tris-Cl (pH 7.0), 300 mM NaCl, 10% glycerol, and 2 mM DTT). The protein was then incubated with 2 mM $ZnCl_2$ or with 2 mM $MgCl_2$ followed by addition of 2 mM $ZnCl_2$. After every treatment, protein was incubated for 15 minutes. To remove excess unbound metal, the protein was subjected to overnight dialysis. The protein was denatured by addition of 1 % SDS and heated at 94 °C for 10 minutes, followed by the addition of 125 µM 4-(2-pyridylazo) resorcinol (PAR). The absorbance was recorded from 300 nm to 630 nm using an Agilent 8453 spectrophotometer. All experiments were performed in triplicate.

References

1. Klucar, L., Stano, M. and Hajduk, M. (2010) phiSITE: database of gene regulation in bacteriophages, Nucleic Acids Res. 38 (database issue), D366-D370.
2. Grove, A., Kushwaha, A. K. and Nguyen, K. H. (2015) Determining the role of metal binding in protein cage assembly, Methods Mol. Biol. 1252, 91-100.