Supplemental information

Inhibition of *Pseudomonas aeruginosa* ExsA DNA-binding activity by *N*-hydroxybenzimidazoles

Marsden et. al

Table S1. Bacterial strains and plasmids used in this study.

Bacterial strains	Relevant characteristics	Reference
Pseudomonas aeruginosa		
PA103	Wild-type parental strain	(1)
PA103 *exsA*::Ω	Chromosomal interposon insertion in *exsA*	(2)
PA103 Δ*exsA*	In frame deletion of *exsA*	(3)
PA103 Δ*exoU exoT*::Tc	In frame deletion of *exoU*, Tc cassette insertion in *exoT*	(4)
Escherichia coli		
DH5α	*recA* cloning strain	(5)
BL21 (DE3) Tuner	Protein expression and purification	Novagen
GS162	Wild-type strain carrying Δ*lac*U169	(6)

Reporter fusions	Integration plasmid	Experiment	Primer or primer pair	Reference
$P_{exsC\text{-}lacZ}$	mini-CTX-$P_{exsC\text{-}lacZ}$ (-186 to +17)	Fig. S4A	NA	(7)
$P_{exoT\text{-}lacZ}$	mini-CTX-$P_{exoT\text{-}lacZ}$ (-179 to +17)	Fig. S4B, C	NA	(8)
$P_{exoS\text{-}gfp}$	pJNE05	Fig. 3, S2	NA	(9)

Expression plasmids	Relevant characteristics	Experiment	Primer or primer pair(s)	Reference
pJN105	Arabinose-inducible expression vector	Fig. S4A-C	NA	(10)
pEB124	Wild-type *exsA* expression vector	Fig. S4A-C	NA	(8)
pET16b	IPTG-inducible protein expression vector with an N-terminal His10 tag		NA	Novagen
pET16b *exsA*	Source of purified $ExsA_{His}$	Fig. 2A-C; 5B; 7A; S5	NA	(8)
pET16b *lcrF*	Source of purified $LcrF_{His}$	Fig. 5C, 7C	NA	(11)
pET16b *ascA*	Source of purified $AscA_{His}$	Fig. 5D	124682246,124682249	This study
pET16b $exsA_{Vp}$	Source of purified $ExsA_{Vp\text{-}His}$	Fig. 5E	124682248,124682251	This study
pET16b *exsA*-CTD	Source of purified ExsA-CTD_{His}	Fig. 2D	124727245,117236112	This study
pET16b $exsA_{Vp}$-CTD	Source of purified $ExsA_{Vp}$-CTD_{His}	Fig. 6A	124727247,124682251	This study
pET16b *vfr*	Source of purified Vfr_{His}	Fig. 6B	NA	(12)

Table S1. (continued) Bacterial strains and plasmids used in this study.

Expression plasmids	Relevant characteristics	Experiment	Primer or primer pair(s)	Reference
pAM196	Source of purified ExsA(S207T)$_{His}$	Fig. 7B, S5	117236111,126035215; 117236112,126035214	This study
pAM202	Source of purified ExsA(M177E)$_{His}$		117236111,127030665; 117236112,127030664	This study
pAM203	Source of purified ExsA(H180E)$_{His}$	Fig. S6A	117236111,127030667; 117236112,127030666	This study
pAM204	Source of purified ExsA(Y181E)$_{His}$		117236111,127030669; 117236112,127030668	This study
pAM205	Source of purified ExsA(N183E)$_{His}$	Fig. S6B	117236111,127030671; 117236112, 127030670	This study
pAM206	Source of purified ExsA(W185E)$_{His}$		117236111,127030675; 117236112,127030674	This study
pAM207	Source of purified ExsA(I217E)$_{His}$	Fig. S6C	117236111,126904065; 117236112,126904064	This study
pAM208	Source of purified ExsA(R221E)$_{His}$		117236111,126904067; 117236112,126904066	This study
pAM209	Source of purified ExsA(F245E)$_{His}$		117236111,126904069; 117236112,126904068	This study
pAM210	Source of purified ExsA(S247E)$_{His}$		117236111,126904071; 117236112,126904070	This study
pAM211	Source of purified ExsA(Y250E)$_{His}$		117236111,126904073; 117236112,126904072	This study
pAM212	Source of purified ExsA(E184A)$_{His}$	Fig. S6D	117236111,126825499; 117236112,126825498	This study
pAM213	Source of purified ExsA(K186A)$_{His}$	Fig. S6E	117236111,130973192; 117236112,130973191	This study
pAM214	Source of purified ExsA(P213A)$_{His}$	Fig. S6F	117236111,130973194; 117236112,130973193	This study
pAM215	Source of purified ExsA(R214A)$_{His}$	Fig. S6G	117236111,130973196; 117236112,130973195	This study
pAM216	Source of purified ExsA(M241A)$_{His}$	Fig. S6H	117236111,130973198; 117236112,130973197	This study
pAM217	Source of purified ExsA(S246A)$_{His}$	Fig. S6I	117236111,130973200; 117236112,130973199	This study

Table S1. (continued) Bacterial strains and plasmids used in this study.

Expression plasmids	Relevant characteristics	Experiment	Primer or primer pair(s)	Reference
pAM231	Source of purified LcrF(T203S)$_{His}$	Fig. 7D	132425570,132342334 124818301,132342333	This study

Table S2. Primers used in this study.

Primer ID	Name	Primer Sequence
22963127	Pc5'Hind	5'-ACGCAAGCTTATGAAGGACGTCCTGCAGCTCATCC
49188917	Pc3'EcoRI	5'-TGATGAATTCGCCTCCTAAAGCTCAGCGCATGC
85333731	algD5_nonsp	5'-CAGGGGTGTCGGAGGGACGAACGGTA
85333730	algD3_nonsp	5'-ACGGCTATTACTTCAGCGCCGAGCAATC
124727245	exsA CTD Gibson	5'-TATCGAAGGTCGTCATATGGAGAATCTTTATTTTCAGGGCAACCGGCATGTCGAG CGTCTGCAGC
117236111	exsA Gibson pET16b 1	5'-CATATCGAAGGTCGTCATATGCAAGGAGCCAAATCTC
117236112	exsA Gibson pET16b 2	5'-GCTTTGTTAGCAGCCGGATCCTCAGTTATTTTTAGCCCGG
124727249	ascA Gibson	5'-TATCGAAGGTCGTCATATGGAGAATCTTTATTTTCAGGGCAACCGCCAGGTTGAA CGATTACAGC
124682249	AxsA3'BampET16b	5'-GCTTTGTTAGCAGCCGGATCCTTAATCTTTGCCATGTCTGGC
124727247	vxsA CTD Gibson	5'-TATCGAAGGTCGTCATATGGAGAATCTTTATTTTCAGGGCAACCGAACCTCAGAC CGCTTACGCC
124682251	VxsA3'BampET16b	5'-GCTTTGTTAGCAGCCGGATCCTCAATTAGCGATGGCGACTTG
124727246	lcrF CTD Gibson	5'-TATCGAAGGTCGTCATATGGAGAATCTTTATTTTCAGGGCAACCGCCCAGAAGA ACGGTTGCAAA
124818301	LcrF3'pET16b Gibson	5'-GCTTTGTTAGCAGCCGTTAGCCTGTGGTTGCTATTTTAG
124682246	AxsA5'NdepET16b	5'-CATATCGAAGGTCGTCATATGAAAGGCATTACAACCA
124682248	VxsA5'NdepET16b	5'-CATATCGAAGGTCGTCATATGGATGTGTCAGGCCAAC
126035214	ExsAS207TGibsonfor	5'-AAGGAGCTGTTCGGCACCGTCTACGGGGTTTCG
126035215	ExsAS207TGibsonrev	5'-CGAAACCCCGTAGACGGTGCCGAACAGCTCCTT
127030664	ExsAM177EGibsonfor	5'-CTGCAGCTATTCGAGGAGAAGCACTAC
127030665	ExsAM177EGibsonrev	5'-GTAGTGCTTCTCCTCGAATAGCTGCAG
127030666	ExsAH180EGibsonfor	5'-TTCATGGAGAAGGAGTACCTCAACGAG
127030667	ExsAH180EGibsonrev	5'-CTCGTTGAGGTACTCCTTCTCCATGAA
127030668	ExsAY181EGibsonfor	5'-ATGGAGAAGCACGAGCTCAACGAGTGG
127030669	ExsAY181EGibsonrev	5'-CCACTCGTTGAGCTCGTGCTTCTCCAT
127030670	ExsAN183EGibsonfor	5'-AAGCACTACCTCGAGGAGTGGAAGCTG
127030671	ExsAN183EGibsonrev	5'-CAGCTTCCACTCCTCGAGGTAGTGCTT
127030674	ExsAW185EGibsonfor	5'-TACCTCAACGAGGAGAAGCTGTCCGAC
127030675	ExsAW185EGibsonrev	5'-GTCGGACAGCTTCTCCTCGGTGAGGTA
126904064	ExsAI217EGibsonfor	5'-CCGCGCGCCTGGGAGAGCGAGCGGAGA
126904065	ExsAI217EGibsonrev	5'-TCTCCGCTCGCTCTCCCAGGCGCGCGG
126904066	ExsAR221EGibsonfor	5'-ATCAGCGAGCGGGAGATCCTCTATGCC

Table S2. (continued) Primers used in this study.

Primer ID	Name	Primer Sequence
126904067	ExsAR221EGibsonrev	5'-GGCATAGAGGATCTCCCGCTCGCTGAT
126904068	ExsAF245EGibsonfor	5'-ATGGAGGCGGGCGAGTCCAGCCAGTCC
126904069	ExsAF245EGibsonrev	5'-GGACTGGCTGGACTCGCCCGCCTCCAT
126904070	ExsAS247EGibsonfor	5'-GCGGGCTTTTCCGAGCAGTCCTATTTC
126904071	ExsAS247EGibsonrev	5'-GAAATAGGACTGCTCGGAAAAGCCCGC
126904072	ExsAY250EGibsonfor	5'-TCCAGCCAGTCCGAGTTCACCCAGAG
126904073	ExsAY250EGibsonrev	5'-CTCTGGGTGAACTCGGACTGGCTGGA
33075941	2UY27A	5'-TATGAATCCCATGCCGAACCGGTAGCC
33075940	2UY27B	5'-GGGGGCAGGTAGGGGATCCGCTCGGCAATCGG
126825498	ExsAE184AGibsonfor	5'-CACTACCTCAACGCCTGGAAGCTGTCC
126825499	ExsAE184AGibsonrev	5'-GGACAGCTTCCAGGCGTTGAGGTAGTG
130973191	ExsAK186AGibsonfor	5'-CTCAACGAGTGGGCCCTGTCCGACTTC
130973192	ExsAK186AGibsonrev	5'-GAAGTCGAACAGGGCCCACTCGTTGAG
130973193	ExsAP213AGibsonfor	5'-TACGGGGTTTCGGCCCGCGCCTGGATC
130973194	ExsAP213AGibsonrev	5'-GATCCAGCCGCGGGCCGAAACCCCGTA
130973195	ExsAR214AGibsonfor	5'-GGGGTTTCGCCGGCCGCCTGGATCAGC
130973196	ExsAR214AGibsonrev	5'-GCTGATCCAGGCGGCCGGCGAAACCCC
130973197	ExsAM241AGibsonfor	5'-GTCGACATCGCCGCCGAGGCGGGCTTT
130973198	ExsAM241AGibsonrev	5'-AAAGCCCGCCTCGGCGGCGATGTCGAC
130973199	ExsAS246AGibsonfor	5'-GAGGCGGGCTTTGCCAGCCAGTCCTAT
130973200	ExsAS246AGibsonrev	5'-ATAGGACTGGCTGGCAAAGCCCGCCTC
132342333	LcrF T203S for	5'-GAACTGTTTGGTAGCGTTTATGGCATT
132342334	LcrF T203S rev	5'-AATGCCATAAACGCTACCAAACAGTTC
132425570	LcrF5'pET16bGibson	5'-CATATCGAAGGTCGTCATATGGCATCACTAGAGATTATT

REFERENCES

1. **Liu PV.** 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. 3. Identity of the lethal toxins produced in vitro and in vivo. J. Infect. Dis. **116:**481-489.
2. **Frank DW, Nair G, Schweizer HP.** 1994. Construction and characterization of chromosomal insertional mutations of the Pseudomonas aeruginosa exoenzyme S trans-regulatory locus. Infect. Immun. **62:**554-563.
3. **Brutinel ED, Vakulskas CA, Yahr TL.** 2010. ExsD inhibits expression of the Pseudomonas aeruginosa type III secretion system by disrupting ExsA self-association and DNA binding activity. J. Bacteriol. **192:**1479-1486.
4. **Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, Frank DW.** 1998. ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system. Proc. Natl. Acad. Sci. U. S. A. **95:**13899-13904.
5. **Hanahan D.** 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. **166:**557-580.
6. **Stauffer GV, Plamann MD, Stauffer LT.** 1981. Construction and expression of hybrid plasmids containing the Escherichia coli glyA genes. Gene **14:**63-72.
7. **McCaw ML, Lykken GL, Singh PK, Yahr TL.** 2002. ExsD is a negative regulator of the Pseudomonas aeruginosa type III secretion regulon. Mol. Microbiol. **46:**1123-1133.
8. **Brutinel ED, Vakulskas CA, Brady KM, Yahr TL.** 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the Pseudomonas aeruginosa type III secretion system. Mol. Microbiol. **68:**657-671.
9. **Urbanowski ML, Brutinel ED, Yahr TL.** 2007. Translocation of ExsE into Chinese hamster ovary cells is required for transcriptional induction of the Pseudomonas aeruginosa type III secretion system. Infect. Immun. **75:**4432-4439.
10. **Newman JR, Fuqua C.** 1999. Broad-host-range expression vectors that carry the L-arabinose-inducible Escherichia coli araBAD promoter and the araC regulator. Gene **227:**197-203.
11. **King JM, Schesser Bartra S, Plano G, Yahr TL.** 2013. ExsA and LcrF recognize similar consensus binding sites, but differences in their oligomeric state influence interactions with promoter DNA. J. Bacteriol. **195:**5639-5650.
12. **Fuchs EL, Brutinel ED, Klem ER, Fehr AR, Yahr TL, Wolfgang MC.** 2010. In vitro and in vivo characterization of the Pseudomonas aeruginosa cyclic AMP (cAMP) phosphodiesterase CpdA, required for cAMP homeostasis and virulence factor regulation. J. Bacteriol. **192:**2779-2790.

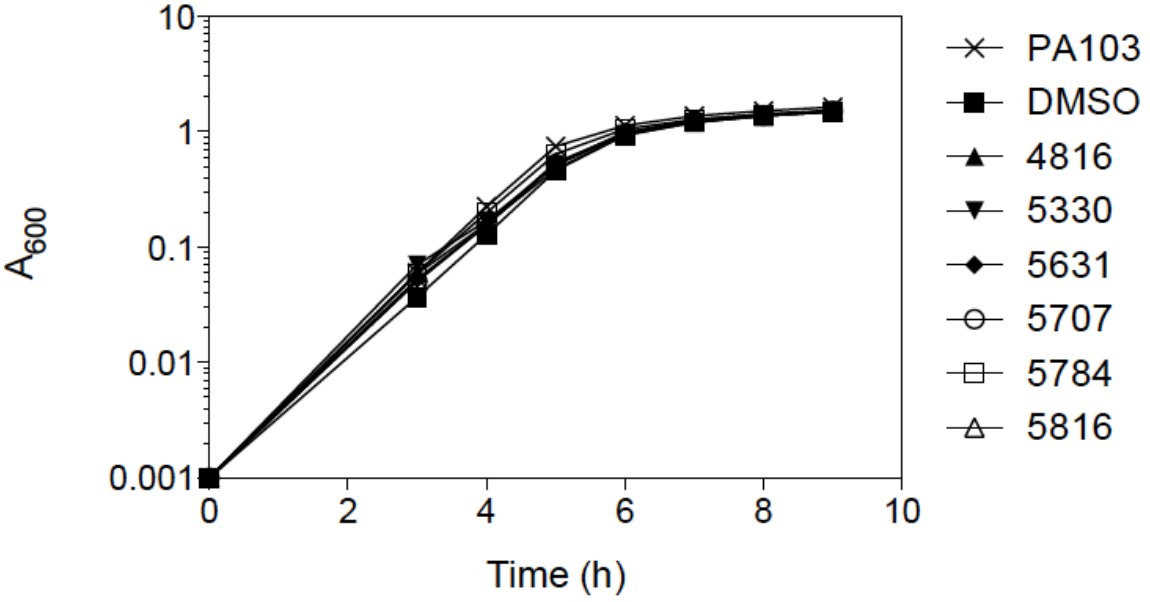

FIG S1. Growth curve for PA103 in the presence of *N*-hydroxybenzimidazoles. Wild-type *P. aeruginosa* (PA103) was grown overnight on VBM agar then suspended at an A_{600} of 0.001 with each *N*-hydroxybenzimidazole (125 μM) or DMSO (2.5%) and grown at 37°C with shaking.

Figure S1

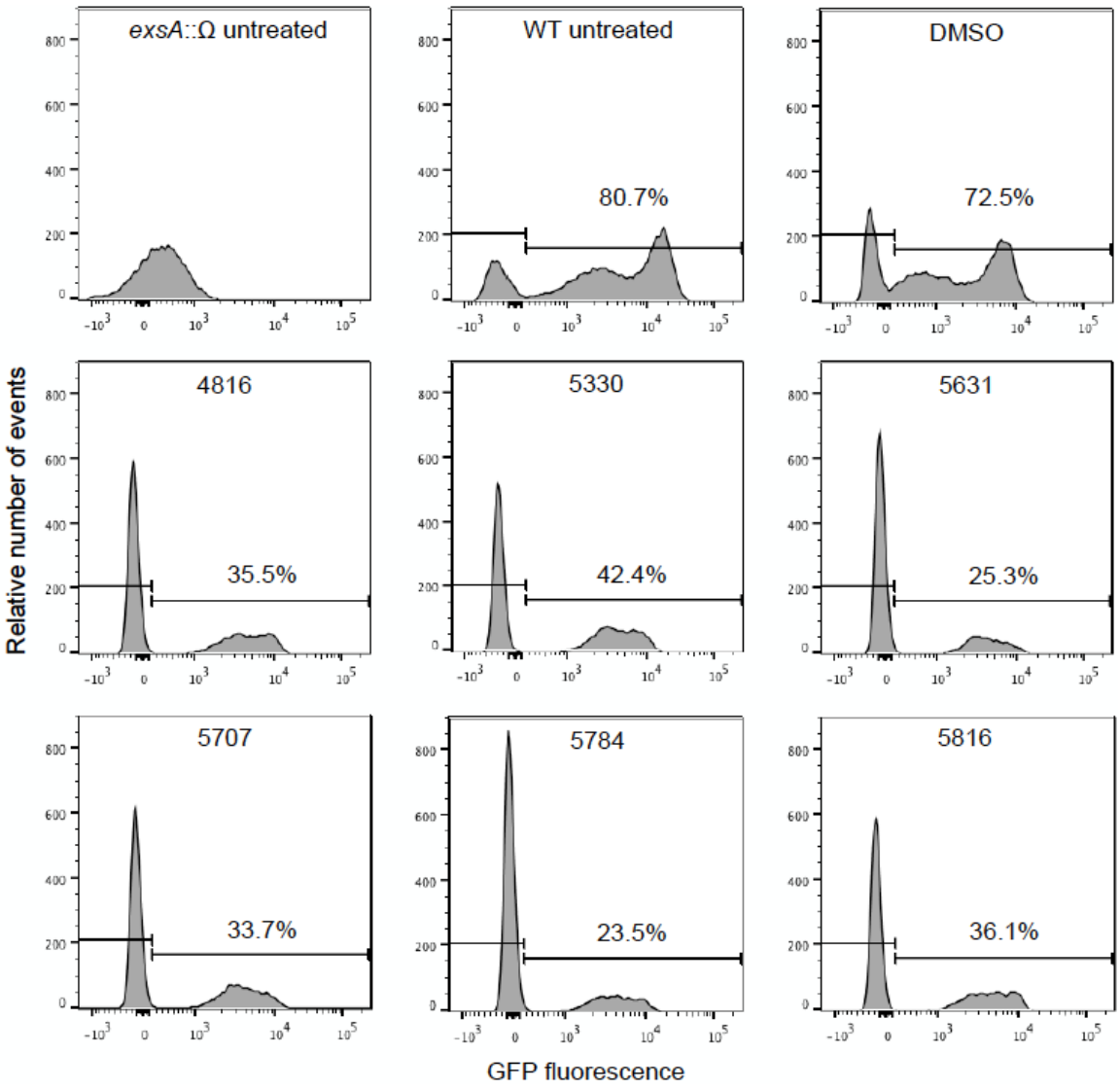

FIG S2. *N*-hydroxybenzimidazoles inhibit ExsA-dependent gene expression. Representative data from an *exoU*, *exoT* mutant carrying a GFP transcriptional reporter (PA103 Δ*exoU exoT*::Tc $P_{exoS\text{-}gfp}$) incubated with DMSO (2.5%) or the indicated *N*-hydroxybenzimidazole (125 μM) for 15 min prior to incubation with CHO cells for 4 hr at 37°C. Reporter activity was measured by flow cytometry.

Figure S2

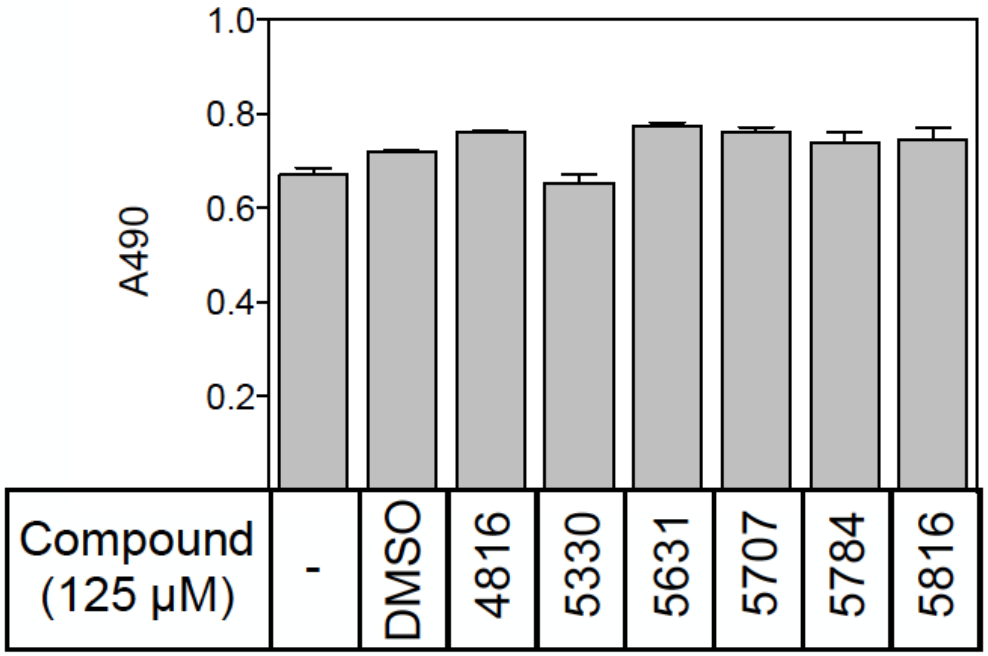

FIG S3. *N*-hydroxybenzimidazoles do not inhibit purified lactate dehydrogenase activity. Purified LDH enzyme was incubated with DMSO (2.5%) or the indicated *N*-hydroxybenzimidazole (125 μM) for 15 min at 25°C and activity was assayed.

Figure S3

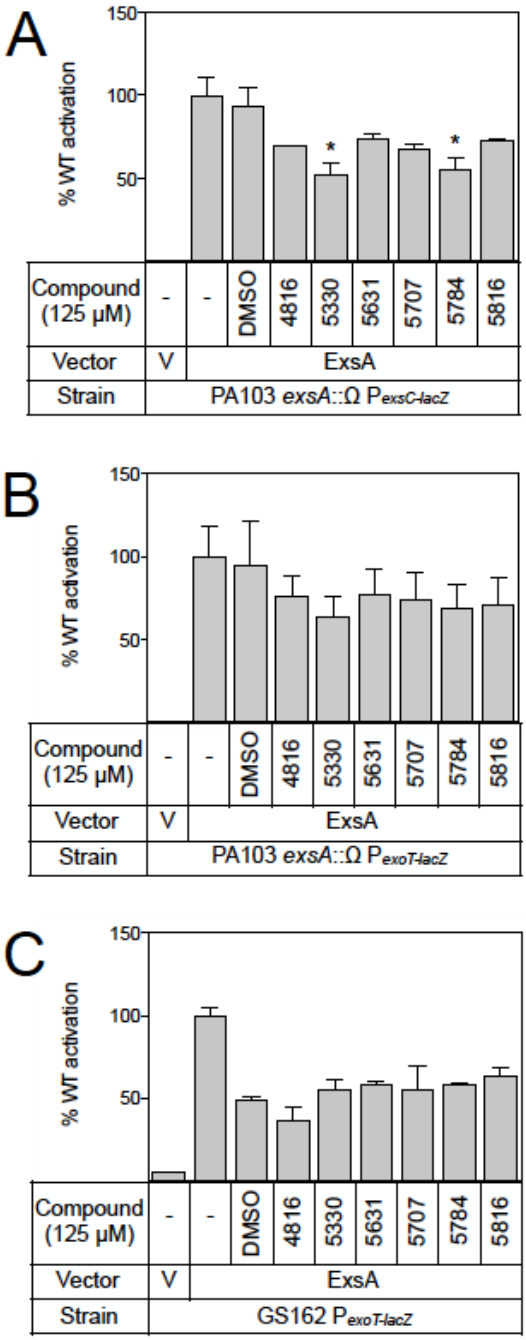

FIG S4. *N*-hydroxybenzimidazoles do not significantly inhibit ExsA-dependent promoter activity in broth cultures. (*A*-*B*) *P. aeruginosa exsA* mutants or (*C*) *E. coli* GS162 carrying $P_{exsC\text{-}lacZ}$ (*A*) or $P_{exoT\text{-}lacZ}$ (*B* and *C*) transcriptional reporters and either a vector control (V) or an arabinose-inducible *exsA* expression vector (ExsA) were grown with 0.1% arabinose to an A_{600} of 1.0 in the presence of each *N*-hydroxybenzimidazole (125 μM) at 30°C. β-galactosidase activity was assayed and expressed as a percentage of the activity measured in untreated samples expressing *exsA*. Statistical differences were determined by comparison to DMSO treatment. *, $P < 0.01$.

Figure S4

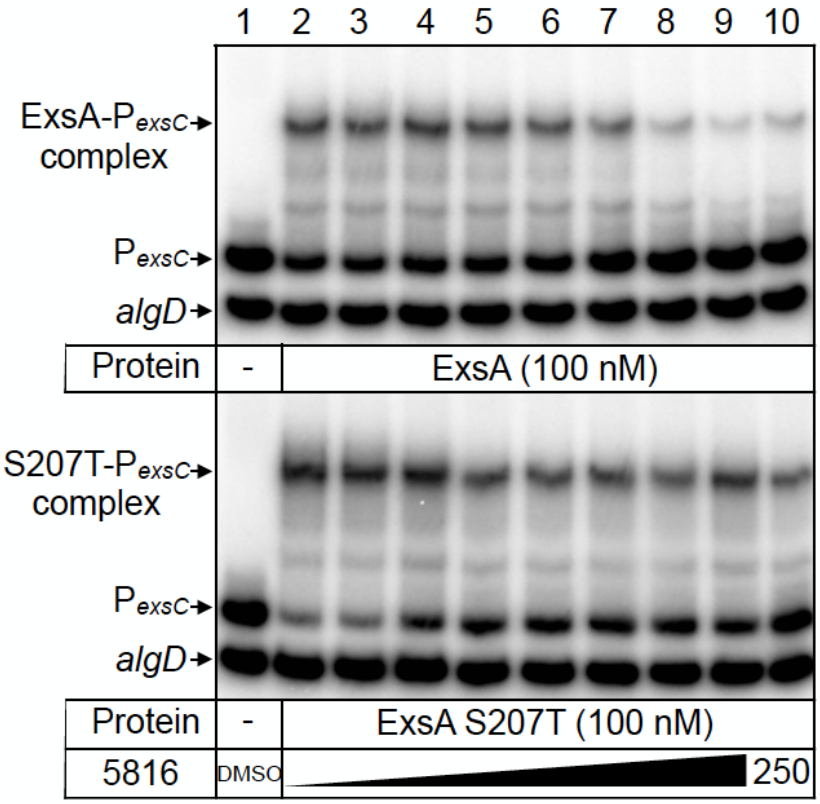

FIG S5. Amino acid substitution S207T in recognition helix 1 alters ExsA-DNA binding in the presence of *N*-hydroxybenzimidazole 5816. ExsA or $ExsA_{S207T}$ (100 nM) were incubated with the indicated concentrations of compound 5816 (0-250 nM) to determine the half maximal inhibitory concentration (IC_{50}) for inhibition of ExsA-P_{exsC} complex formation.

Figure S5

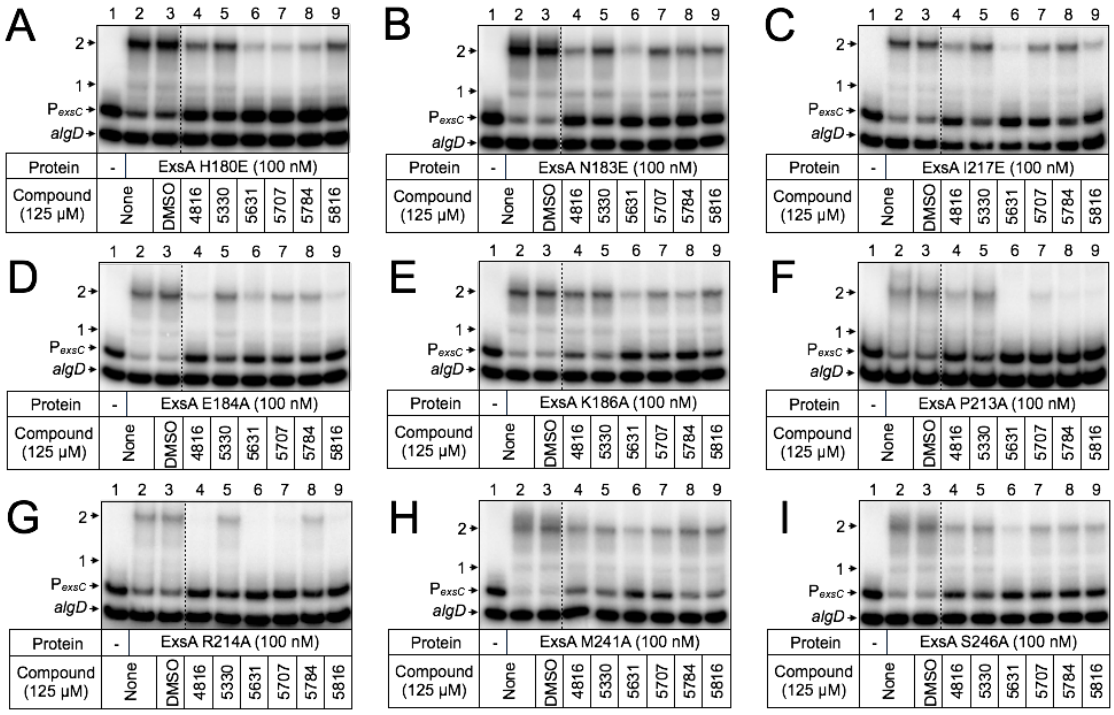

FIG S6. Glutamic acid and alanine substitutions in the putative inhibitor-binding pocket affect ExsA-DNA binding in the presence of *N*-hydroxybenzimidazoles. $ExsA_{H180E}$ *(A),* $ExsA_{N183E}$ (*B*), $ExsA_{I217E}$ (*C*), $ExsA_{E184A}$ (*D*), $ExsA_{K186A}$ (*E*), $ExsA_{P213A}$ (*F*), $ExsA_{R214A}$ (*G*), $ExsA_{M241A}$ (*H*), and $ExsA_{S246A}$ (*I*) (100 nM) were treated with DMSO (2.5%) or each *N*-hydroxybenzimidazole (125 μM) for 5 min before incubation with specific (P_{exsC}) and nonspecific (*algD*) radiolabeled probes (0.05 nM each) for 15 min at 25°C. Native polyacrylamide gel electrophoresis and phosphorimaging was performed to examine binding reactions. Protein-DNA complexes and unshifted specific and nonspecific probes are indicated.

Figure S6

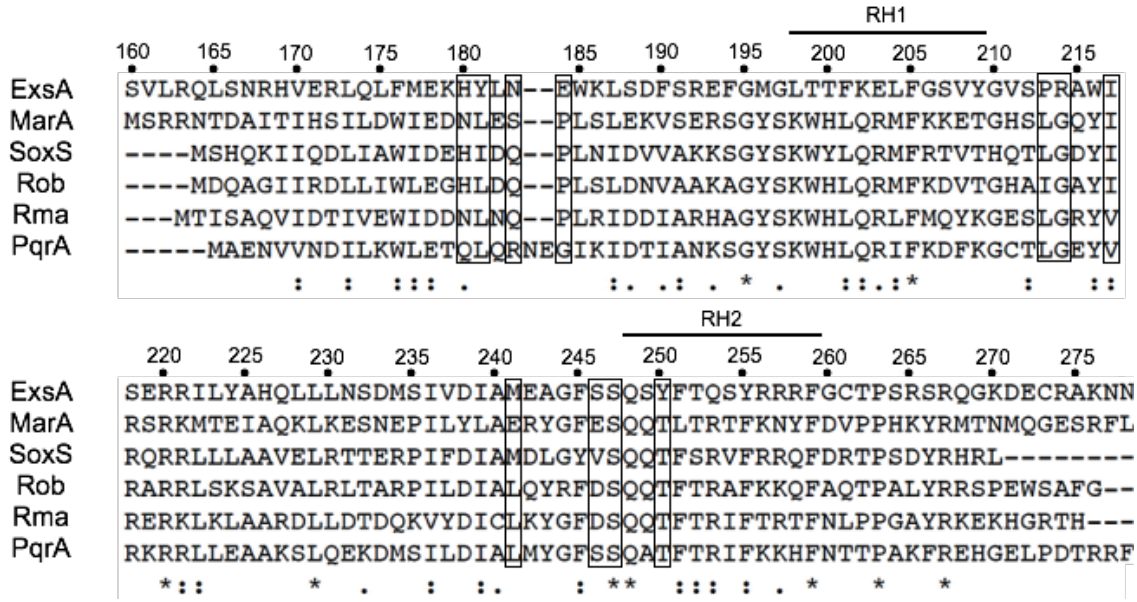

FIG S7. Amino acid alignment of AraC family protein DNA-binding domains. ExsA amino acids are numbered, and recognition helices (RH1 and RH2) are indicated with a bold line. Boxes outline amino acids that were mutagenized in ExsA.

Figure S7