Supplemental material for:

A Bacterial Stress Response Regulates Expression of Respiratory Protein Complexes to Control Envelope Stress Adaptation

Randi L. Guest, Junshu Wang, Julia L. Wong, and Tracy L. Raivio*

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

*Corresponding author:

Phone: +1 (780) 492-3491

Fax: +1 (780) 492-9234

Email: traivio@ualberta.ca

Supplementary Materials and Methods

Growth curve. Bacteria were grown overnight in LB-Lennox broth at 37°C with shaking at 225 RPM. After standardizing the OD_{600} of the overnight cultures, the cultures were diluted by a factor of 1:100 into 200µL fresh LB-Lennox broth in a 96 well plate. Cells were grown at 37°C with aeration by shaking at 225 RPM. The OD_{600} was measured using a plate reader (Perkin Elmer) every hour post-subculture for nine hours, and again after 24 hours.

Sensitivity assays. To determine the sensitivity to increasing concentrations of amikacin, overnight cultures were standardized to an OD_{600} of 1 and serially diluted. 10µL of each dilution was plated on LB-Lennox agar, or LB-Lennox agar supplemented with 1.5, 3, 6, or 12 µg/mL amikacin. Cells were grown overnight at 37°C. Susceptibility to carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma) was determined using gradient agar plates (1). 25mL of LB-Lennox agar containing DMSO (Sigma) was poured into a nine centimeter squared petri plate. One side of the plate was elevated to allow the agar to solidify on a slant. Next, 25mL of LB-Lennox agar containing 50µM CCCP (from a 50mM CCCP stock dissolved in DMSO) was added on top of the DMSO layer, leveled, and allowed to solidify for five hours. Overnight cultures of wildtype and Δ*cpxRA* mutant EPEC were diluted by a factor of 1:100 into fresh LB-Lennox broth, and grown to early log phase. 20µL of this culture was spread across the surface of the CCCP gradient agar. The plates were incubated at 37°C overnight.

β-galactosidase assay for activation by CCCP. Overnight cultures of MC4100 containing a *cpxP-lacZ* transcriptional reporter were diluted by a factor of 1:100 into 2mL LB-Lennox broth in a 13x100mm glass test tube and grown to mid-log phase at 37°C with aeration at 225 RPM. 100µM CCCP or an equivalent volume of DMSO was then added and cultures were grown for an additional thirty minutes. *cpxP-lacZ* expression was determined as described in the Materials and Methods section of the main text.

Western blot. Overnight cultures of E2348/69 were diluted by a factor of 1:100 into 10mL LB-Lennox broth in a 125mL Erlenmeyer flask and grown to an OD_{600} of approximately 0.5 at 37°C with shaking at 225 RPM. 100μM CCCP or an equivalent volume of DMSO was then added and cultures were grown for an additional thirty minutes. 1mL of culture was pelleted and resuspended in 50μL 2x SDS sample buffer (Sigma). Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). 20μg of total protein was loaded onto a SDS-polyacrylamide gel and proteins were separated by electrophoresis. Western blotting was performed as previously described (2). Primary MBP-CpxR, MBP-CpxA, and PhoA antibodies were used at a 1:10000, 1:50000, and 1:25000 dilution, respectively. Alkaline-phosphatase anti-rabbit secondary antibodies (Sigma) were used at a 1:25000 dilution. Proteins were detected using the Immun-Star alkaline phosphatase chemiluminescence kit (Bio-Rad) and the Bio-Rad ChemiDoc MP imaging system.

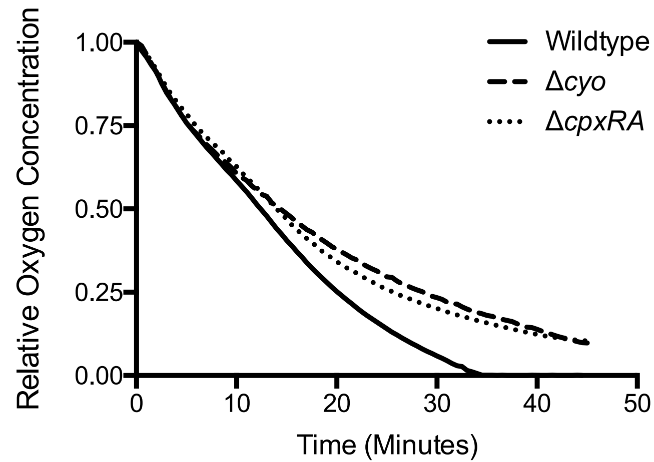

Figure S1. Rate of Oxygen consumption of the Δ*cpxRA* mutant is similar to the Δ*cyo* mutant. Oxygen concentration was measured every thirty seconds for 45 minutes in a closed system containing wildtype EPEC, or EPEC lacking the Cpx response (Δ*cpxRA*) or cytochrome bo_3 (Δ*cyo*). The oxygen concentration at each time point was standardized to the oxygen concentration at zero seconds. Data are representative of two independent experiments.

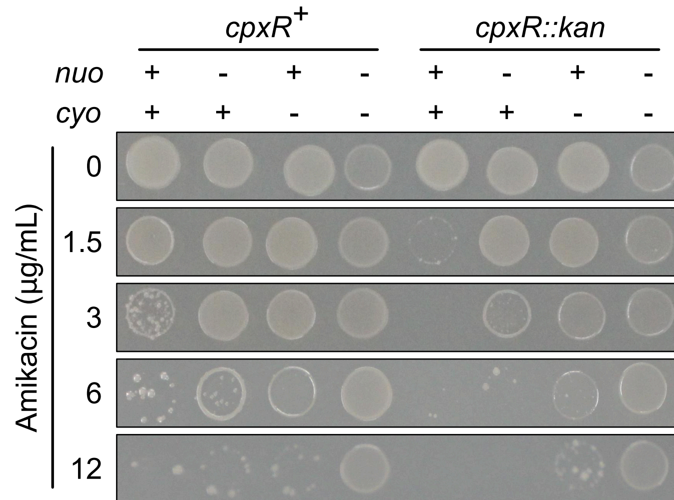

Figure S2. Deletion of both NDH-I and cytochrome *bo₃* increases resistance to amikacin. Growth of EPEC strains on LB or LB supplemented with 1.5µg/mL, 3µg/mL, 6µg/mL, or 12µg/mL amikacin. Displayed in this figure is the growth of a 10^{-3} dilution of an overnight culture at each concentration of amikacin. The strains shown are wildtype EPEC, Δ*nuo,* Δ*cyo*, and *cpxR::kan* single mutants, Δ*nuo* Δ*cyo,* Δ*nuo cpxR::kan,* and Δ*cyo cpxR::kan* double mutants, and the Δ*nuo* Δ*cyo cpxR::kan* triple mutant. +, presence of an operon; -, deletion of an operon. Data are representative of two independent experiments.

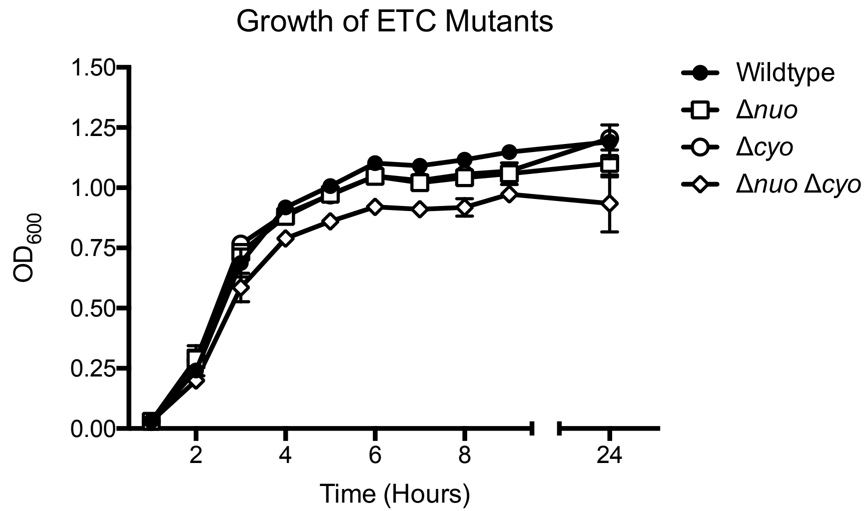

Figure S3. Growth of electron transport chain mutants. Growth curve of wildtype, Δ*nuo,* Δ*cyo,* and Δ*nuo* Δ*cyo* EPEC in LB-lennox broth. OD_{600} was read every hour for nine hours, and again after 24 hours post-subculture. Data correspond to the means and standard deviations of five replicate cultures.

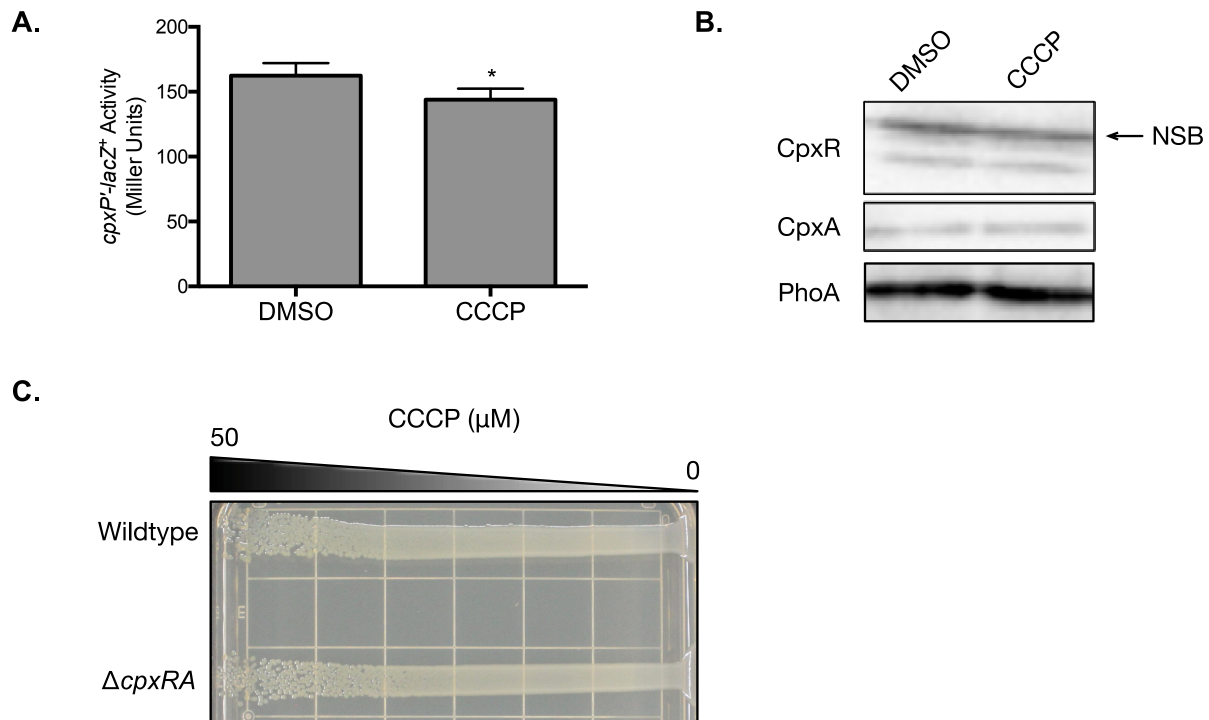

Figure S4. The *E. coli* Cpx response is not strongly affected by CCCP. (A) *cpxP-lacZ* expression in wildtype MC4100 treated with DMSO or 100µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Data correspond to the means and standard deviations of three replicate cultures. (B) CpxR and CpxA protein levels in wildtype EPEC exposed to DMSO or 100µM CCCP. PhoA protein levels were determined and used as a loading control. (C) Growth of wildtype and Δ*cpxRA* mutant EPEC in the presence of increasing concentrations of CCCP. The asterisk (*) indicates a statistically significant difference from the DMSO control (*P* = 0.0124, unpaired t-test). NSB, nonspecific band.

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

Strain or plasmid	Description	Source or reference
Bacterial strains		
E2348/69	Prototypical EPEC O127:H6 laboratory strain	(3)
ALN195	E2348/69 *cpxA24*	(4)
ALN88	E2348/69 *cpxR::kan*; Kan^R	(5)
RG139	E2348/69 Δ*cyoABCDE*	This study
RG141	ALN88 Δ*cyoABCDE*	This study
RG148	E2348/69 Δ*nuoABCDEFGHIJKLMN*	This study
RG149	E2348/69 Δ*cyoABCDE* Δ*nuoABCDEFGHIJKLMN*	This study
RG149	ALN88 Δ*cyoABCDE* Δ*nuoABCDEFGHIJKLMN*	This study
RG157	ALN88 Δ*nuoABCDEFGHIJKLMN*	This study
RG222	E2348/69 Δ*cpxRA*	This study
TR50	MC4100 λRS88[*cpxP'-lacZ*$^+$]	(6)
Plasmids		
pCA-24N	Vector control for ASKA library containing $P_{T5\text{-}lac}$ IPTG-inducible promoter; Cam^R	(7)
pCA-*nlpE*	IPTG-inducible *nlpE* overexpression vector from ASKA library; Cam^R	(7)
pCA-*cyoA*	IPTG-inducible *cyoA* overexpression vector from ASKA library; Cam^R	(7)
pCA-*cyoC*	IPTG-inducible *cyoC* overexpression vector from ASKA library; Cam^R	(7)
pJW25	pJW15 luminescence reporter plasmid containing	(4)

	cpxP promoter; Kan^R	
pJW15-P*nuo*	pJW15 luminescence reporter plasmid containing *nuo* operon promoter from E2348/69; Kan^R	This study
pJW15-Pnuo_{SUB}	pJW15-P*nuo* with base pairs –194 to –208 upstream of the *nuoA* coding region mutated to 5’-CAGTATCAGTCAGTA-3’	This study
pJW15-P*cyo*	pJW15 luminescence reporter plasmid containing *cyo* operon promoter from E2348/69; Kan^R	This study
pRE112	Suicide vector for allelic exchange; Cam^R	(8)

Table S2: Oligonucleotide primers used in this study

Primer name	Sequence*
PnuoAFwdCln	5'-TTTTGAATTCCACAACGGACACGATTCAAC-3'
PnuoARevCln	5'-TTTTGGATCCACCGCCTACCAGCATCAG-3'
Pnuolux-CpxRScrFwd	5'-CAAATGTTGTTGTCATTGTCAGTATCAGTCAGTA-3'
Pnuolux-CpxRScrRev	5'-TCAACTTTAACAAAGGTTTCTACTGACTGATACTG-3'
PcyoAClnFwd	5'-TTTTCGATCGGCTGTAGTAATCATCCGCCG-3'
PcyoAClnRev	5'-TTTTGGATCCTTACAGCCACTGAGCAATAC-3'
cpxRADelUpF	5'-TTTTGGTACCGAGTGATTATCGTCGGCAGG-3'
cpxRADelUpR	5'-CGGAGTTTAACTCCGTTTATTCATTGTTTAAATAC-3'
cpxRADelDnF	5'-AAACAATGAATAAACGGAGTTAAACTCCGCATTTG-3'
cpxRADelDnR	5'-TTTTGAGCTCCTTACCTCTATCTGGTCACG-3'
nuoDelUpF	5'-TTTTGGTACCTCTACCTTTAAGGCATTGAACCTGCG-3'
nuoDelUpR	5'-TGTTTACATCAGCGGTGACATACTCATTGCTTACT-3'
nuoDelDnF	5'-GCAATGAGTATGTCACCGCTGATGTAAACAGTCAG-3'
nuoDelDnR	5'-TTTTGGTACCGCGCTGGAGATGATTGGTTATTACG-3'
cyoDelUpF	5'-TTTTTCTAGATACTGGCGACCATTGTTGGCGCATTGTACG-3'
cyoDelUpR	5'-TTGTGTTACCACACAGCCCTGAGTCTCATTTAACGAC-3'
cyoDelDnF	5'-TAAATGAGACTCAGGGCTGTGTGGTAACACAACCT-3'
cyoDelDnR	5'-TTTTGAGCTCCTGGCGATTCTTTACTGATAAGTGAAGGC-3'

* Underlined sequences denote restriction endonuclease cut sites (*Eco*RI: GAATTC, *Bam*HI: GGATCC, *Pvu*I: CGATCG, *Kpn*I: GGTACC, *Sac*I: GAGCTC, *Xba*I: TCTAGA).

REFERENCES

1. **Bina XR**, **Provenzano D**, **Nguyen N**, **Bina JE**. 2008. Vibrio cholerae RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. Infection and Immunity **76**:3595–3605.

2. **Raivio TL**, **Popkin DL**, **Silhavy TJ**. 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263–5272.

3. **Levine MM**, **Bergquist EJ**, **Nalin DR**, **Waterman DH**, **Hornick RB**, **Young CR**, **Sotman S**. 1978. Escherichia coli strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet **1**:1119–1122.

4. **MacRitchie DM**, **Ward JD**, **Nevesinjac AZ**, **Raivio TL**. 2008. Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic Escherichia coli. Infection and Immunity **76**:1465–1475.

5. **Nevesinjac AZ**, **Raivio TL**. 2005. The Cpx Envelope Stress Response Affects Expression of the Type IV Bundle-Forming Pili of Enteropathogenic Escherichia coli. J Bacteriol **187**:672–686.

6. **Raivio TL**, **Silhavy TJ**. 1997. Transduction of envelope stress in Escherichia coli by the Cpx two-component system. J Bacteriol **179**:7724–7733.

7. **Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H**. 2005 Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA Res* **12**:291–299.

8. **Edwards RA**, **Keller LH**, **Schifferli DM**. 1998. Improved allelic exchange vectors and their

use to analyze 987P fimbria gene expression. Gene **207**:149–157.