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The *Escherichia coli* Cpx Envelope Stress Response Regulates Genes of Diverse Function That Impact Antibiotic Resistance and Membrane Integrity

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The Cpx envelope stress response mediates adaptation to stresses that cause envelope protein misfolding. Adaptation is partly conferred through increased expression of protein folding and degradation factors. The Cpx response also plays a conserved role in the regulation of virulence determinant expression and impacts antibiotic resistance. We sought to identify adaptive mechanisms that may be involved in these important functions by characterizing changes in the transcriptome of two different *Escherichia coli* strains when the Cpx response is induced. We show that, while there is considerable strain- and condition-specific variability in the Cpx response, the regulon is enriched for proteins and functions that are inner membrane associated under all conditions. Genes that were changed by Cpx pathway induction under all conditions were involved in a number of cellular functions and included several intergenic regions, suggesting that posttranscriptional regulation is important during Cpx-mediated adaptation. Some Cpx-regulated genes are centrally involved in energetics and play a role in antibiotic resistance. We show that a number of small, uncharacterized envelope proteins are Cpx regulated and at least two of these affect phenotypes associated with membrane integrity. Altogether, our work suggests new mechanisms of Cpx-mediated envelope stress adaptation and antibiotic resistance.

All cells possess protective mechanisms against stresses that lead to the misfolding of proteins that are secreted across the cytoplasmic membrane. Gram-negative bacteria employ two major envelope stress responses to respond to misfolded, secreted proteins (2, 5). The transcription factor σ^E is activated upon the initiation of a proteolytic cascade by misfolded outer membrane proteins (OMPs), leading to the destruction of the membrane-bound anti-σ factor RseA and the release of σ^E (3). σ^E then associates with RNA polymerase (RNAP) and guides it to the promoters of genes encoding chaperones, proteases, and outer membrane biogenesis factors (4). The σ^E response plays an important homeostatic role in sensing and mediating adjustments to changes in the biogenesis of secreted proteins, specifically OMPs.

A second envelope stress response in Gram-negative bacteria is the Cpx envelope stress response (5). This response is activated by a set of inducing signals that are distinct from those that turn on the σ^E response. These are diverse and include alkaline pH, chloride ions, copper, mutations that impact protein folding in the periplasm, the overexpression of periplasmic proteins that misfold and aggregate at the inner membrane, and adherence to abiotic surfaces (for a review, see reference 5 and references within). It has long been thought that the Cpx-inducing cue involves misfolded periplasmic proteins since all of the activating signals are expected to generate these and the first identified genes of the Cpx regulon encode envelope protein folding and degrading factors, such as the protease/chaperone DegP, the peptidyl-prolyl-isomerase PpiA, the disfulfide oxidase DsbA, and the chaperones Spy and CpxP (7–11). In addition, it has been demonstrated that Cpx-mediated adaptation also involves the regulation of genes involved in phospholipid metabolism, general outer membrane porins, and other stress responses (12–14). The Cpx response also leads to downregulated production of envelope-spanning protein complexes, including pili, secretion systems, and conjugation machinery, in a number of enteric pathogens (1, 15–21). Cumulatively, these Cpx signals and adaptations have been demonstrated to play roles in adherence, pathogenesis, biofilm formation, and horizontal gene transfer (1, 15, 16, 19, 20, 22–27).

The Cpx response is regulated by the two-component system consisting of the transmembrane sensor kinase CpxA in conjunction with the cytoplasmic transcription factor CpxR. Although the molecular signature of the cues that activate the response is unknown, all envelope-associated inducers require the kinase CpxA for detection and, more specifically, its periplasmic sensing domain (28, 29). Additionally, two auxiliary regulators are involved in signal detection in the envelope. The outer membrane lipoprotein NlpE is required for efficient adhesion to abiotic surfaces, and it also signals this event via CpxA to induce the Cpx response and facilitate this process (27). CpxA activity is induced upon titration of a chaperone protein, CpxP (8, 11, 30–32), in the presence of a variety of conditions that cause proteins to misfold in the periplasm (28, 33–35). Activation of CpxA by any of these mechanisms ultimately leads to its autophosphorylation, followed by phosphorylation of CpxR (29), which then facilitates changes in the expression of adaptive genes, as described above.

One microarray study of the Cpx regulon in *Escherichia coli* has been published; however, that study analyzed changes in gene expression in strains in which the Cpx response either was lacking or was induced by overexpression of the unphosphorylated regulator

Received 31 January 2013 **Accepted** 2 April 2013
Published ahead of print 5 April 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00105-13.
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doi:10.1128/JB.00105-13

CpxR, and it failed to identify several well-characterized Cpx regulon members (36). We wished to more fully characterize the genes that are changed in expression immediately after induction of the Cpx response by an envelope stress cue in order to gain insight into the molecular mechanisms by which this signaling pathway impacts the numerous fundamental biological processes it has been linked to. Our results suggest that the Cpx response mediates adaptation primarily at the inner membrane and that it impacts several cellular functions not previously associated with this pathway. Among these are a number of known and predicted regulatory small RNAs (sRNAs), genes involved in respiration that impact antibiotic resistance, and small envelope proteins that alter membrane integrity.

MATERIALS AND METHODS

Growth conditions. *E. coli* K-12 and enteropathogenic *E. coli* (EPEC) strains MC4100 and E2348/69 were grown on LB agar plates or with shaking at 37°C or 30°C in LB broth or Dulbecco's modified Eagle's medium-F12 tissue culture medium (DMEM/F12; Invitrogen) supplemented with the appropriate antibiotics, as indicated. Bacterial strains for which secretion assays were performed were statically grown in DMEM/F12 in 5% CO_2 at 37°C. Antibiotics were used at the following concentrations: amikacin (3 μg/ml), kanamycin (30 μg/ml) (*E. coli* K-12 strains), kanamycin (50 μg/ml) (EPEC strains), chloramphenicol (25 μg/ml), and streptomycin (50 μg/ml).

Bacterial strains and plasmids. All strains and plasmids used in this study are listed inTable 1. Knockout mutants were generated using P1 transduction to move the desired mutant alleles from the Keio collection (37) into wild-type MC4100 as previously described (38). The inducible pCA24N-based plasmids used in this study were obtained from the ASKA collection (39). Promoter-*lacZ* transcriptional reporter genes were made as described previously using the transcriptional fusion vector pRS415 and the λRS88 phage for the transfer of a single-copy fusion to the chromosome (40). Restriction enzyme-tagged primers (see Table S1 in the supplemental material) were used to amplify each promoter region, including the predicted CpxR binding site, if present. The PCR product was purified (MP Biomedicals GeneClean III kit), restriction digested with EcoRI and BamHI (Invitrogen), and cloned into EcoRI-BamHI-digested pRS415 vector, which contains a promoterless *lacZ* gene. The λRS88 phage was used to move the promoter fusion onto the chromosome of MC4100 at the λ*att* site in single copy as described previously (40).

Microarray analysis of gene expression. For microarray analysis of genes with altered expression in the presence of NlpE overproduction, two subcultures each (MC4100 [pCA-*nlpE*] or EPEC E2348/69 [pCA-*nlpE*] strains) were grown in LB broth and DMEM/F12 (Invitrogen), with shaking, at 37°C, to an absorbance at 600 nm (A_{600}) of 0.35. At this point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to one culture for each strain and condition at a final concentration of 1 mM to induce the overproduction of NlpE from the plasmid pCA-*nlpE* (39). At least two experimental replicates were carried out. The cultures were grown for another 25 min after IPTG addition before two 10-ml samples were harvested per strain and condition and RNA was isolated using a MasterPure RNA purification kit as described by the manufacturer (Epicentre). Isolated RNA was resuspended in 100 μl of nuclease-free water per ml of cultured sample. Quantification of RNA samples was performed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). To assay for the quality of the RNA, a sample from each RNA isolation was standardized to 250 ng/μl and 1 μl was run on an Agilent 2100 BioAnalyzer using an Agilent Prokaryotic Total RNA 6000 Nano kit per the protocol of the manufacturer (Agilent Technologies, Inc.). A 10-ng volume of isolated RNA was mixed with 10 μl of 300 ng/μl random primers (Invitrogen), 3 μl of 10 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen), and nuclease-free water to a final volume of 30 μl. The random primers and RNA were allowed to anneal (70°C for 10 min, 25°C for 10 min). After the annealing step, the RNA/primer hybridization mix was added to 30 μl of SuperScript II Master Mix (12 μl of 5× first-strand buffer, 6 μl of 100 mM dithiothreitol [DTT], 7.5 μl of SuperScript II, and 4.5 μl of nuclease-free H_2O) (Invitrogen), bringing the final volume to 60 μl, and incubated for cDNA synthesis (25°C for 10 min, 37°C for 1 h, 42°C for 1 h, and 70°C for 10 min). The cDNA was purified using a QIAquick PCR purification kit per the instructions of the manufacturer (Qiagen) and eluted in 20 to 50 μl of nuclease-free water. Each cDNA sample was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). All microarrays performed utilized an Affymetrix GeneChip *E. coli* Genome 2.0 Array (Affymetrix, Inc.). cDNA fragmentation, labeling, and hybridization to the microarray chip were performed per the protocols of the manufacturer (Affymetrix, Inc.). To ensure that NlpE overproduction had induced the Cpx response in our experiments, a 100-ng aliquot of each cDNA sample was set aside and used as a template for quantitative PCR (Q-PCR)

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
E. coli strains		
E2348/69	Prototype O127:H7 EPEC strain	90
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150* Str^r *relA1 flbB5301 deoC1 ptsF25 rbsR*	91
TR20	MC4100 *cpxA101 zii*::Tn*10*	29
TR1195	MC4100 *cyoA*::Kn	This study
TR1198	MC4100 *efeO*::Kn	This study
TR1199	MC4100 *nuoA*::Kn	This study
TR1200	MC4100 *sdhC*::Kn	This study
TR1214	MC4100 *ydiY*::Kn	This study
SL195	MC4100 λRS88 [*yjfN-lacZ*]	This study
SL196	MC4100 λRS88 [*yqaE-lacZ*]	This study
SL197	MC4100 λRS88 [*yncJ-lacZ*]	This study
SL198	MC4100 λRS88 [*ynfD-lacZ*]	This study
SL195 *cpxA24*	MC4100 λRS88 [*yjfN-lacZ*] *cpxA24*	This study
SL196 *cpxA24*	MC4100 λRS88 [*yqaE-lacZ*] *cpxA24*	This study
SL197 *cpxA24*	MC4100 λRS88 [*yncJ-lacZ*] *cpxA24*	This study
SL198 *cpxA24*	MC4100 λRS88 [*ynfD-lacZ*] *cpxA24*	This study
BT1	SL195 (pCA-*nlpE*)	This study
BT2	SL195 (pCA24N)	This study
BT3	SL196 (pCA-*nlpE*)	This study
BT4	SL196 (pCA24N)	This study
BT5	SL197 (pCA-*nlpE*)	This study
BT6	SL197 (pCA24N)	This study
BT7	SL198 (pCA-*nlpE*)	This study
BT8	SL198 (pCA24N)	This study
BT17	BT1 *cpxR*::*spc*	This study
BT18	BT2 *cpxR*::*spc*	This study
BT19	BT3 *cpxR*::*spc*	This study
BT20	BT4 *cpxR*::*spc*	This study
BT21	BT5 *cpxR*::*spc*	This study
BT22	BT6 *cpxR*::*spc*	This study
BT23	BT7 *cpxR*::*spc*	This study
BT24	BT8 *cpxR*::*spc*	This study
Plasmids		
pCA vectors	Gene of interest cloned downstream of the IPTG-inducible promoter on pCA24N	39
pCA24N	Vector control for the ASKA library, containing $P_{T5\text{-}lac}$ IPTG-inducible promoter	39
pCA-*nlpE*	$P_{T5\text{-}lac}$-*nlpE*	39
pRS415	*bla-T1*$_4$-EcoRI-SmaI-BamHI-*lacZ*$^+$	40

[a] Str, streptomycin.

analysis of *degP*, *cpxP*, and *rpoD* (control gene). Primers were designed using the Primer Express program (version 3; Applied Biosciences). The primers are listed in Table S1 in the supplemental material. Q-PCRs and analyses were carried out as outlined previously (14).

Statistical analysis of microarray data. Principal component analysis (PCA) was performed to examine the clustering of experimental replicates. This analysis revealed that, in general, the replicates (two biological replicates, each with two experimental replicates) of MC4100 in LB, MC4100 in DMEM, and EPEC in LB were tightly clustered, indicating a high degree of reproducibility. However, EPEC grown in DMEM showed a high degree of variability between samples despite our performing three biological replicates, each of which contained two experimental replicates. At present, we have no explanation for this finding. All microarray chips (using both strains and under all medium conditions) were analyzed together using the robust multichip average (RMA) algorithm (41, 42). Normalization of the data sets was performed through interquartile range (IQR) filtering, which removed transcripts from the analysis that showed little variance across all samples (including both strains and media). The remaining IQR-filtered transcripts were utilized for comparisons between stimulated and control samples to identify genes showing significant expression differences in each strain (MC4100 or EPEC) and under each set of medium conditions (LB or DMEM/F12) using a modern Bayesian *t* test with a probability of false discovery rate (FDR) of lower than 0.05.

Functional cluster analysis of genes identified as significantly changed by NlpE overexpression. To identify functional relationships among genes identified by microarray analysis as displaying 2-fold or higher changes in expression after transient NlpE overexpression, we used the functional annotation clustering algorithm that is part of the DAVID (Database for Annotation, Visualization, and Integrated Discovery) (43, 44) suite of bioinformatics resources (http://david.abcc.ncifcrf.gov/). This tool analyzes a submitted gene list based on a user-selected suite of annotation terms and groups together genes with similar annotation terms. An enrichment score is calculated for each functional cluster based on a modified Fisher's exact *P* value for each term in the cluster that indicates the probability that a particular term in the cluster would be identified by chance alone (i.e., that the results were not enriched for that term). In our analysis, we submitted separate gene lists for each strain and condition and also divided the genes into categories according to whether expression was positively or negatively influenced by NlpE overproduction. The annotation categories used in our analysis were SP PIR KEYWORDS, GOTERM BP FAT, and KEGG PATHWAY. These were chosen because they resulted in the inclusion of the largest numbers of genes in the analysis from our submitted lists. We considered functional clusters having an enrichment score of greater than 2 and individual Fisher's exact *P* values of less than 0.05 for each term in the cluster and involving greater than 5% of the genes on our submitted list.

Antibiotic sensitivity assays. An isolated colony of each strain was inoculated into 5 ml of LB broth containing appropriate antibiotics and grown overnight at 30°C, with shaking (250 rpm). The next day, serial 10-fold dilutions of each strain were made in LB broth, in a 96-well microtiter plate, and 5 μl of each dilution was spotted onto an LB plate carrying the indicated concentrations of antibiotics. The plate was incubated at 37°C overnight, and pictures were taken the next morning to record the results.

β-Galactosidase assays. β-Galactosidase assays were based on a 96-well plate assay described previously (45). Briefly, 2-ml overnight cultures grown in LB plus appropriate antibiotics were subcultured (1/50) into fresh medium of the same kind and grown with the following treatments. For NlpE induction from the pCA-*nlpE* plasmid, cultures were grown to the mid-log phase and then induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside; Invitrogen) for 1 h. For pH 8.0 induction, cultures were grown to the early log phase in LB at pH ~7.0, spun down for 10 min at 4,000 rpm, resuspended in LB at pH 5.8 (shutoff) or pH 8.0 (induction), and then grown for an additional 2 h. For *cpxA24* induction, cultures were grown with shaking at 30°C. For all conditions, the final optical density at 600 nm (OD_{600}) was ~0.6 to 0.7. After growth under the appropriate conditions, cultures were centrifuged and cells were resuspended in 2 ml of freshly prepared 1× Z-buffer (10 ml 10× Z-buffer [600 mM $Na_2HPO_4 \cdot 7H_2O$, 400 mM $NaH_2PO_4 \cdot H_2O$, 100 mM KCl, 10 mM $MgSO_4 \cdot 7H_2O$], 90 ml double-distilled water [dH_2O], 270 μl β-mercaptoethanol). The OD_{600} was read in 96-well polystyrene plates using a PerkinElmer Wallac Victor^2 1420 plate reader. Cells were lysed using chloroform and 0.1% SDS, and the cellular debris were removed by centrifugation. The lysed cell mixture was diluted in 1× Z-buffer in 96-well plates (50 μl lysed cell mixture, 150 μl 1× Z-buffer), and 50 μl 10 mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (Sigma) was added. The absorbance at 420 nm (A_{420}) was read 20 times over approximately 30 min in the plate reader, and Miller units were calculated. Experiments were done in triplicate. Fold changes were measured as the Miller units determined for the induced condition (plus IPTG, [pH 8.0], or *cpxA24*) divided by the Miller units determined for the uninduced condition (no IPTG, [pH 5.8], or wild-type *cpxA*).

Secretion assays. Overnight cultures were diluted 1:100 in 2 ml of prewarmed DMEM/F12 (catalog no. 11330-032; Invitrogen) containing the appropriate antibiotics in a 24-well tissue culture plate. Cultures were statically incubated in 5% CO_2 at 37°C. For strains carrying plasmids that included genes controlled by IPTG-inducible promoters, after 2 h of growth IPTG was added to reach a final concentration of 0.1 mM and the cultures were incubated for an additional 3 to 5 h to an A_{600} of 0.6 to 0.8. A 1-ml volume of sample was taken from the culture, and cells were pelleted using a tabletop centrifuge. The supernatant was transferred to a fresh tube containing 10% tricarboxylic acid (TCA) and put on ice for at least 1 h. The cell pellet was resuspended in 2× sample buffer (46) and stored at −20°C. The precipitated, secreted proteins were pelleted at 14,000 rpm for 15 min at 4°C. The supernatant was removed, and the protein was resuspended in 2× sample buffer. Secreted protein was subjected to SDS-PAGE with Coomassie blue (10% Coomassie blue dye, 10% methanol:H_2O [1:1], 10% acetic acid) staining.

LIVE/DEAD BacLight assay. A LIVE/DEAD BacLight kit (Invitrogen) was used according to the manufacturer's directions. Briefly, overnight cultures were subcultured (1:50) into 30 ml of fresh LB medium and grown with shaking at 37°C to the late log phase (OD_{600}, ~0.8). A 25-ml volume of culture was transferred to a conical tube, centrifuged for 10 min at 10,000 × *g*, and resuspended in 2 ml 0.85% NaCl. Half of the concentrated culture was added to 20 ml of either 0.85% NaCl (for live bacteria) or 70% isopropanol (for dead bacteria) and incubated at room temperature for 1 h. Cells were pelleted (10,000 × *g*, 10 min) and resuspended in 20 ml of 0.85% NaCl. The OD_{670} was standardized to ~0.12, and then mixtures of five different proportions (0:100, 10:90, 50:50, 90:10, and 100:0) of live and dead bacteria in 2-ml total volumes were prepared. A 100-μl volume of each mixture was divided into aliquots in triplicate into a 96-well plate, a 100-μl volume of 2× stain solution (prepared according to the LIVE/DEAD BacLight kit instructions; Invitrogen) was added to each well, and the plate was allowed to stand for 15 min before the absorbances at ~530 nm and ~630 nm were measured in a PerkinElmer Wallac Victor^2 plate reader. The ratio of green fluorescence to red fluorescence (live bacteria/dead bacteria) was plotted against the known percentage of live cells in the suspension. Experiments were done in triplicate.

Phenotype MicroArray study. Strain MC4100, together with strains containing in-frame deletions of *yqaE*, *yncJ*, *yjfN*, and *ynfD* in which the gene was replaced with a kanamycin cassette, was sent to Biolog, Inc. (Hayward, CA), for Phenotype MicroArray (PM) analyses. All five strains were run on the full array of panels (20 panels) in the Microbial PM collection.

RESULTS AND DISCUSSION

Transient NlpE overexpression is a reliable tool for the identification of the Cpx regulon. In order to identify new Cpx-regulated genes, we analyzed changes in the *E. coli* transcriptome shortly after overexpression of the lipoprotein NlpE, a well-characterized

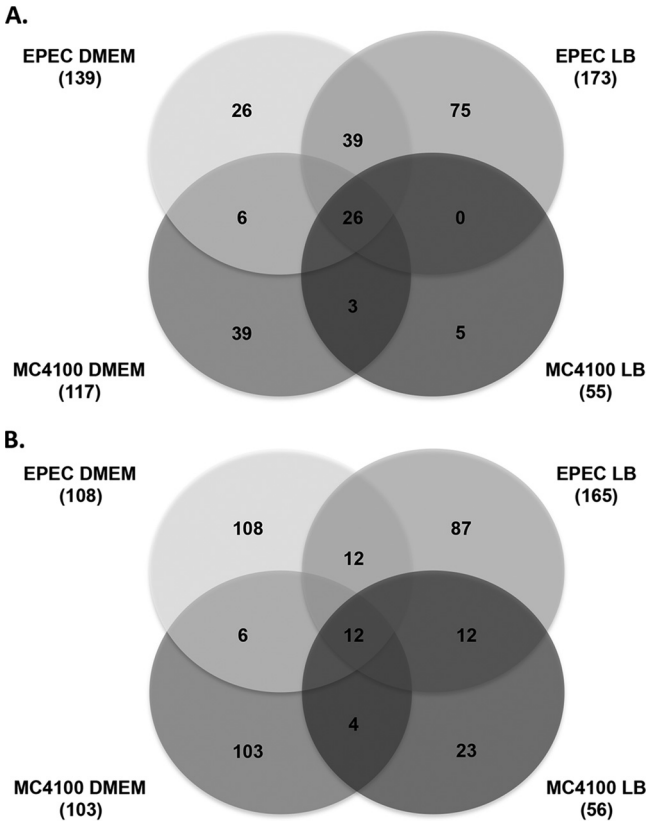

FIG 1 NlpE-induced changes in gene expression are strain and medium dependent. (A) Venn diagram showing comparison of genes with a minimal 2-fold increase in expression after NlpE overexpression in MC4100 or E2348/69 grown in LB broth or DMEM/F12 media. (B) Venn diagram showing comparison of genes with a minimal 2-fold decrease in expression after NlpE overexpression in MC4100 or E2348/69 grown in LB broth or DMEM/F12 media. Comparison lists were generated using the online Venn Diagram Generator at http://www.pangloss.com/seidel/Protocols/venn.cgi.

Cpx-inducing signal (47). We performed these experiments in the *E. coli* K-12 laboratory isolate MC4100, which has been extensively used to study the Cpx response, together with the enteropathogenic *E. coli* (EPEC) type strain E2348/69, where we have shown that inhibition of virulence determinant production is part of the adaptive mechanism mediated by the Cpx response (1, 20, 48). In addition, we analyzed gene expression changes in these strains when grown in two different types of media, LB, in which most studies of the Cpx response have been carried out, and the defined tissue culture medium Dulbecco's modified Eagle's medium (DMEM), which stimulates virulence determinant production in EPEC (49).

After statistical filtering, our data indicated that transient NlpE overexpression led to greater-than-2-fold changes in the expression of several hundred genes in a strain- and medium-dependent manner (probability of false discovery < 0.05) (Fig. 1; see also Table S2 in the supplemental material). Altogether, 247 genes were changed in EPEC grown in DMEM, 338 in EPEC grown in LB, 220 in MC4100 grown in DMEM, and 111 in MC4100 grown in LB (Fig. 1). Of these, there was an almost even split between genes that were upregulated after NlpE overexpression and those that were downregulated (Fig. 1). The fold changes in expression detected for known Cpx regulon members were generally in strong agreement with published observations (14), with the exception of the *rpoE rseABC* operon, which was previously observed to be inhibited by the Cpx response and here appears to have been induced by NlpE overexpression (Table 2). Our previous examination of *rpoE rseABC* regulation by the Cpx response utilized transcriptional reporters, while microarray analyses also reveal the impact of some posttranscriptional events. Thus, one possibility is that the combined impact of transcriptional and posttranscriptional inputs leads to an accumulation of the *rpoE rseABC* mRNA under Cpx-inducing signals. Alternatively, it is possible that the *rpoE rseABC* operon was induced in our study as a result of NlpE-dependent effects that do not require the CpxAR two-component system. Although the genes that we analyzed by Q-PCR mostly appeared to require CpxR for NlpE-dependent expression changes (see Table 5), we cannot rule out the possibility that some of the gene expression changes we observed via microarray occurred independently of the Cpx response. These hypotheses await further testing. Several genes (i.e., *ppiD*, *ompC*, and *ung*) previously shown to be weakly regulated by the Cpx response (14) were not detected in our study (Table 2). The lack of detection of these transcripts under the conditions used in our microarray experiments precluded the possibility of making conclusions about their Cpx regulation based on this study. Overall, however, our observations suggest that the transient NlpE overexpression used here to discover new Cpx-regulated genes was very effective at inducing the known Cpx envelope stress response and, further,

TABLE 2 Comparison of microarray data to previously published data

Gene(s)	Fold change in gene expression					
	lux luminescence[a]		Cpx microarray[b]			
	*cpxA**	NlpE OXP	MC LB	MC DM	EP LB	EP DM
cpxP	113	25.5	5.354	6.325	2.210	4.636
ycfS	93.3	45.4	4.152	4.094	1.903	8.744
yebE	84.2	17.7	11.563	32.656	6.554	36.058
ftnB (*yecI*)	100.4	8.3	4.435	11.249	1.546	7.816
dsbA	97.9	5.0	2.136	2.642	2.009	3.620
yccA	30.0	7.5	2.490	2.331	2.018	3.637
yqjA	24.2	4.5	2.858	3.513	1.812	4.132
degP (*htrA*)	105.9	2.1	6.843	22.231	10.095	15.762
psd	32.5	2.3	1.614	2.203	2.696	3.433
spy	24.2	4.5	9.022	27.191	6.255	32.298
ppiA	2.3	2.1	1.832	NF	1.698	1.738
cpxRA	2.0	2.6	2.600	2.948	1.704	2.579
ompF	0.017	0.23	0.196	0.101	0.263	0.163
rpoE rseABC	0.06	0.32	NF	2.332	2.909	2.097[f]
efeU (*ycdN*)	0.062	0.22	0.327	0.282	0.415	0.049
aroK	0.15	0.43	NF	NF	NF	NF
ybaJ	24.2	1.2	2.089	2.501	1.856	1.860
ydeH	16.8	0.83	8.932	34.578	2.255	8.456
csgDEFG	0.13	0.63	NF	NF	NF	NF
aroG	0.16	0.83	NF	0.475	0.648	0.265
acrD	4.9	0.53	NF	NF	NF	NF
mdtABCD	1.0	0.040	NF	NF	NF	NF
ung	2.2	0.67	NF	NF	NF	NF
ompC	1.4	0.71	NF	4.231	NF	NF
ppiD	1.4	1.2	NF	NF	NF	NF

[a] Data are from Price and Raivio (14).
[b] MC, K-12 MC4100 strain; EP, EPEC E2348/69 strain; LB, LB broth; DM, DMEM; NF, not found.

TABLE 3 Functional cluster analysis of genes changed more than 2-fold upon NlpE overexpression[a]

Function or structure associated with gene cluster	Enrichment score (% genes in cluster)							
	EPEC DMEM+	EPEC LB+	MC4100 DMEM+	MC4100 LB+	EPEC DMEM−	EPEC LB−	MC4100 DMEM−	MC4100 LB−
Membrane	6.5 (19)	4.1 (15.4)	5.9 (19.2)	3.4 (22.6)	30.6 (46)	38.8 (37.5)	27.7 (51.3)	13.6 (36.1)
Transporter(s)	−	−	−	−	6.2 (21)	2.6 (10.8)	2.3 (9.7)	3.5 (11.1)
e^- trpt/TCA cycle	−	−	−	−	3.6 (10.9)	3.3 (6.3)	3.4 (11.5)	−
ox-phos	−	−	−	−	27.4 (23)	25.4 (18.3)	23.4 (26.5)	−
Fe/metal binding	−	−	−	−	5.1 (12.3)	−	2.4 (8)	−
Glu/Asp metab	−	−	−	−	3.5 (6.5)	−	−	−
Ion tpt & siderophore biosyn	−	−	−	−	−	4.5 (9.2)	−	−
S metab	−	−	6.7 (13.7)	−	−	−	−	−
Aromatic aa metab	−	−	−	−	−	−	4.9 (24.8)	−

[a] Functional clusters of genes identified are listed at the left; strains and conditions are listed at the top. e^- trpt, electron transport; ox-phos, oxidative phosphorylation; biosyn, biosynthesis; S metab, sulfur metabolism; −, negatively influenced by NlpE overexpression; +, positively influenced by NlpE overexpression. Data represent the enrichment scores for a given gene list and functional cluster, as calculated by DAVID (43, 44), and (in parentheses) the percentages of genes in that category that are accounted for in the specified functional cluster.

that the genes we identified have a high probability of being genuinely associated with the Cpx envelope stress response.

The Cpx regulon is enriched for inner membrane-associated proteins and functions. Interestingly, there was a high degree of variability in the genes that were changed by NlpE overexpression that was dependent on the strain and medium (Fig. 1). We wondered if this variability might reflect different functions of the Cpx response in different strains and under different environmental conditions. To address this question, we used DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://david.abcc.ncifcrf.gov/) (43, 44) to determine what functional classes of genes, if any, were enriched within our data sets. We separated the lists of genes changed in expression more than 2-fold by transient NlpE overexpression according to strain, medium, and whether they were affected positively or negatively. All gene sets were enriched for genes known or predicted to encode membrane-localized proteins, and most of these were inner membrane proteins (Table 3 and data not shown; see also Table S2 in the supplemental material). Among downregulated genes, those predicted or known to encode inner membrane transport proteins were enriched (Table 3). Similarly, under all conditions except for MC4100 grown in LB, genes encoding proteins involved in electron transport, the TCA cycle, and oxidative phosphorylation were enriched for among downregulated genes (Table 3). Genes encoding products involved in functions involving iron and metals were enriched specifically among genes that were downregulated in DMEM (Table 3). Gene products involved in glutamate/aspartate metabolism, iron transport and siderophore synthesis, sulfur metabolism, and aromatic amino acid metabolism were enriched for among genes that were changed by NlpE overexpression in only one data set (Table 3).

Our data suggest an important role for the Cpx response in the physiology of the inner membrane under all conditions but also argue that environmental and strain parameters impact the types of adaptive functions that are influenced. This makes sense in light of the fact that different conditions result in the expression of unique transcriptomes and that the Cpx response has been demonstrated to positively regulate the transcription of some genes only in the presence of other activators (50). Further, a transcript could not be negatively regulated by the Cpx response unless specific conditions were present that led to the expression of the mRNA. Accordingly, our data reflect the activity of the Cpx response in the context of other transcription factors and repressors that are active under the conditions being examined and we expect these data to vary in a manner dependent on condition. The dramatic difference in the numbers of genes that exhibit expression changes upon NlpE overproduction in E2348/69 versus MC4100 suggests to us that EPEC possesses a more complex response to changes in its envelope. This might be expected given that the E2348/69 envelope itself is quite different from that of MC4100. Some notable differences in the EPEC envelope include the capacity to express a number of multiprotein complexes necessary for infection (51), motility, and the production of an O antigen. In addition, E2348/69 was more recently isolated from a natural environment in which a wide array of envelope stresses are expected to occur (52, 53).

Genes that are uniformly regulated by Cpx are involved in multiple cellular functions. Despite the variability in the genes with changed expression upon transient NlpE overproduction, a common set of 38 genes was affected independently of the strain or medium (Fig. 2, Table 4). This set of genes consisted of 26 that were positively regulated by NlpE overexpression and 12 that ex-

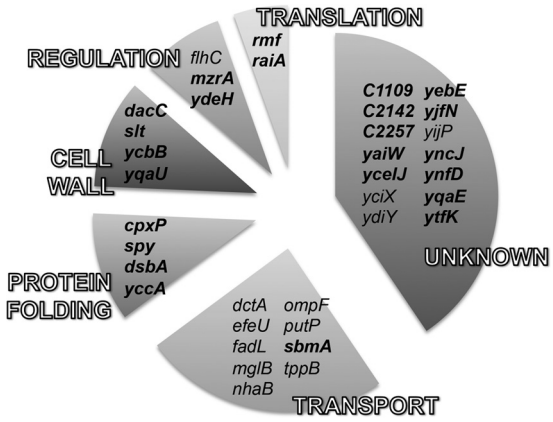

FIG 2 Genes of a core group are changed by NlpE overexpression independently of conditions. The pie chart shows genes that demonstrate a 2-fold or greater change in expression upon NlpE overexpression regardless of strain or medium. Upregulated genes are shown in bold.

TABLE 4 Annotated functions, cellular locations, and presence of predicted or proven CpxR binding site of genes that exhibit greater than 2-fold expression changes when NlpE is overexpressed independently of strain or media

Gene(s) and function category	Transcription regulation category[a]	Function	Cellular location[b]	CpxR binding site[c]
Unknown				
c1109	+		?	−
c2142	+		?	−
c2257	+		?	−
yaiW	+		OM	+P
yceI	+		PP	−
yceJ	+		IM	−
yciX-c1744	−		C	−
ydiY	−		OM	−
yebE	+		IM	+E
yjfN	+		PP	+P
yijP	−		IM	−
yncJ	+		PP	−
ynfD	+		PP	−
yqaE	+		IM	+P
ytfK	+		C	−
Transport/solute diffusion				
dctA	−	C4 dicarboxylate transporter	IM	−
efeU	−	Ferrous iron transporter	IM	+E
fadL	−	Long-chain fatty acid uptake	IM	−
mglB	−	Galactose periplasmic binding protein	PP	−
nhaB	−	Sodium/proton antiporter	IM	−
ompF	−	Porin	OM	+E
putP	−	Sodium/proline symporter	IM	−
sbmA	+	Putative peptide importer	IM	+P
tppB	−	Proton-dependent peptide importer	IM	−
Protein folding				
cpxP	+	Putative periplasmic chaperone	PP	+E
dsbA	+	Disfulfide oxidase	PP	+E
spy	+	Chaperone	PP	+E
yccA	+	Regulator of FtsH proteolysis	IM	+E
Cell wall				
dacC	+	D-Alanyl-D-alanine carboxypeptidase, PBP6	IM	−
slt	+	Lytic murein transglycosylase	OM	+P
ycbB	+	L,D-Transpeptidase	IM	−
ygaU	+	LysM cell wall degradation motif	IM	+P
Regulation				
flhC	−	Transcriptional regulator of flagella	C	−
mzrA	+	Modulator of EnvZ/OmpR	IM	+E
ydeH	+	Diguanylate cyclase regulator of motility and biofilm	C	+E
Translation				
rmf	+	Ribosome modulation factor	C	−
raiA	+	Ribosome-associated inhibitor	C	−

[a] +, upregulated transcription in the presence of NlpE overexpression; −, downregulation in the presence of NlpE overexpression.
[b] C, cytoplasm; IM, inner membrane; OM, outer membrane: PP, periplasm.
[c] +, presence of a predicted (P) or experimentally demonstrated (E) CpxR binding site; −, absence of site. Where no CpxR binding site has been demonstrated, the presence of a CpxR binding site within 500 bp upstream of the translational start codon was predicted using the program Virtual Footprint (http://prodoric.tu-bs.de/vfp/). References (in parentheses) for experimentally established DNA binding sites were as follows: *yebE* (92); *efeU* (92); *ompF* (12); *cpxP* (10, 92, 93); *dsbA* (10, 92); *spy* (94, 95); *yccA* (92); *mzrA* (92); *ydeH* (92).

hibited reduced expression. The majority (32/38) of these gene products are known or predicted to be localized to the envelope (Table 4). Interestingly, almost all of the genes that were negatively impacted by NlpE overexpression encode inner membrane transporters (Fig. 2, Table 4). Among the genes positively regulated by NlpE, the largest (12/26) class consisted of "*y*" genes of unknown function (Fig. 2, Table 4). A second class of positively regulated genes was made up of previously identified Cpx regulon members *cpxP*, *spy*, *yccA*, and *dsbA*—all known or predicted to be involved in protein folding or proteolysis in the envelope (30, 54–56). In-

TABLE 5 Microarray and Q-PCR changes in gene expression after NlpE overexpression

Gene(s) and function category	Fold change in gene expression					
	Microarray[a]				Q-PCR[b]	
	EPEC DMEM	EPEC LB	MC4100 DMEM	MC4100 LB	MC4100	MC4100 *cpxR::spc*
Unknown						
c1109	3.3	6.2	2.5	2.6	ND[c]	ND
c2142	13.7	6.2	3.7	3.1	ND	ND
c2257	14.9	7.8	4.9	6.1	ND	ND
yaiW	3.9	8.2	3.2	2.8	3.5	1.5
yceI	3.4	3.8	5.3	3.5	5.7	1.1
yceJ	3	3.1	2.8	2	4.6	0.7
yciX-c1744	0.34	0.29	0.3	0.4	ND	ND
ydiY	0.5	0.17	0.17	0.29	0.6	0.9
yebE	36.1	6.6	32.7	11.6	39.7	1.0
yjfN	49	6	34.5	2.7	3.0	1.0
yijP	0.44	0.33	0.44	0.4	0.7	1.1
yncJ	40.1	5.9	29.5	17.3	51.4	1.5
ynfD	3.9	2.2	4	2.4	3.4	0.9
yqaE	4.3	3	4	3	6.3	0.9
ytfK	9.2	9.3	4.9	9.2	8.6	1.2
Transport						
dctA	0.35	0.26	0.24	0.5	0.6	0.6
efeU	0.16	0.41	0.28	0.33	0.4	3.7
fadL	0.44	0.27	0.33	0.44	0.9	0.8
mglB	0.35	0.09	0.31	0.36	0.5	0.5
nhaB	0.28	0.49	0.43	0.37	0.3	1
ompF	0.16	0.26	0.1	0.2	0.05	0.3
putP	0.38	0.47	0.4	0.39	0.5	0.5
sbmA	10.5	15.5	6.9	4.2	8.6	2.1
tppB	0.2	0.28	0.3	0.35	0.2	1.5
Protein folding						
cpxP	4.6	2.2	6.3	5.4	20.8	1.8
dsbA	3.6	2	2.6	2.1	4.5	1.2
spy	32.3	6.3	27.2	2.5	15.4	1.2
yccA	3.6	2	2.3	2.5	4.8	1.0
Cell wall						
dacC	4.6	2.2	5.9	4.2	5.5	1.0
slt	2.5	2.8	6.9	2.5	4.1	0.9
ycbB	5.2	3.5	5.5	2.9	2.4	0.9
ygaU	2.5	2.5	3.1	2.4	3.3	0.8
Regulation						
flhC	0.42	0.22	0.31	0.38	0.5	0.7
mzrA	4.3	2.1	4.3	2.8	4.4	1.0
ydeH	8.5	2.3	34.6	8.9	36.4	1.6
Translation						
rmf	2.1	4	2.8	4.1	2.6	0.9
raiA	4	2.4	3	3.7	5.3	0.7

[a] Average fold change in gene expression after 25 min of IPTG induction of NlpE overexpression as observed in microarray analysis.
[b] Fold change in gene expression after 25 min of IPTG induction of NlpE overexpression as observed by Q-PCR analysis. Q-PCR analysis was carried out on cDNA derived from mRNA isolated from cultures grown in LB. Each Q-PCR was repeated three times in each experiment. Numbers represent the averages of the mean changes observed in two experiments (six replicates).
[c] ND, not determined.

terestingly, another class of four genes that were upregulated in response to induction of NlpE expression encodes known or predicted cell wall modification enzymes: DacC, Slt, YcbB, and YgaU (Table 4) (57–62). Finally, three genes are involved in regulation of motility and biofilm formation (*ydeH* [upregulation] and *flhC* [downregulation]) or EnvZ/OmpR-mediated osmoregulation (*mzrA*) (63–68), and two upregulated gene products, Rmf and RaiA, affect translation (Table 4) (69–75). Expression changes upon NlpE overproduction were also detected under all conditions for three putative open reading frames annotated in the uro-

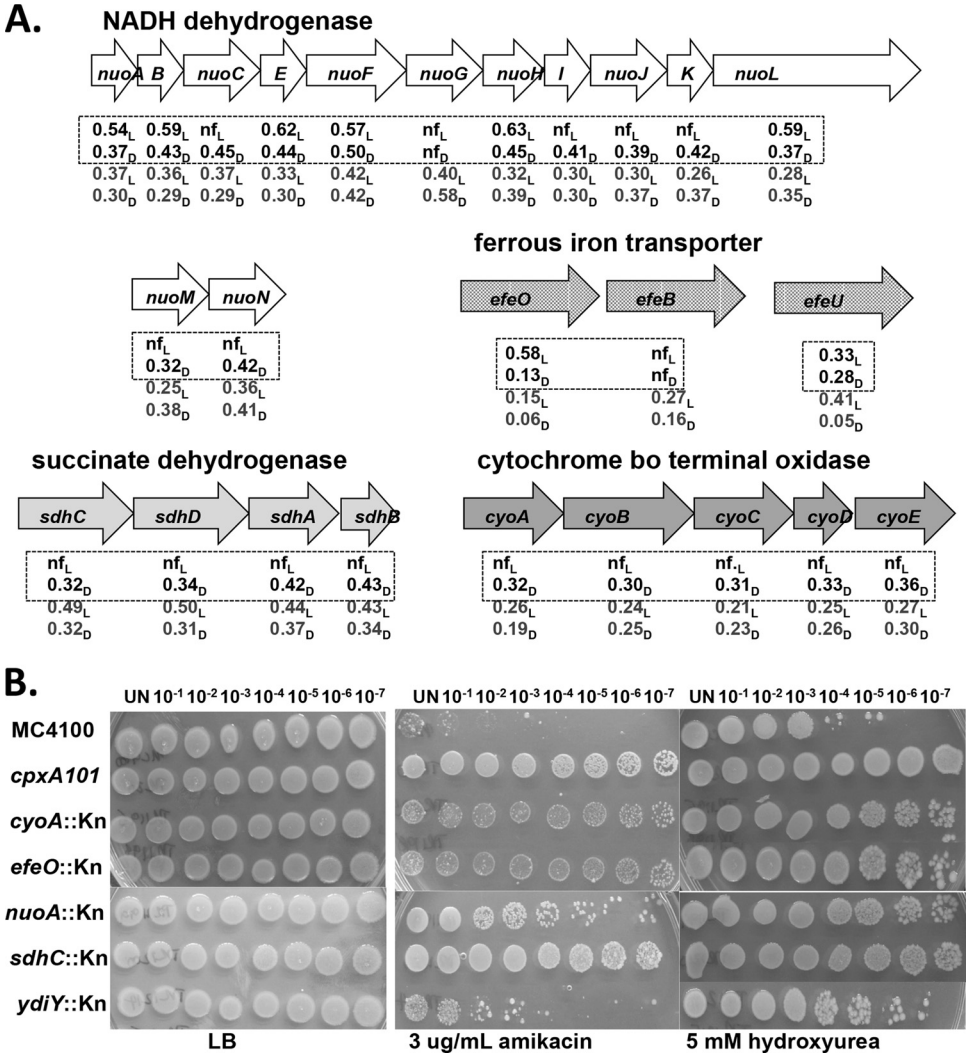

FIG 3 The Cpx response downregulates genes involved in respiration that impact antibiotic resistance. (A) Diagrammatic representation of gene clusters encoding NADH dehydrogenase (*nuo*), succinate dehydrogenase (*sdh*), cytochrome bo terminal oxidase (*cyo*), and the EfeUOB ferrous iron transporter (*efe*). Listed underneath each gene are the fold changes in transcript accumulation observed in strains MC4100 (boxed numbers) and E2348/69 in LB (L) and DMEM (D) after transient NlpE overexpression. (B) Strains MC4100, TR20 (contains *cpxA101* allele which constitutively activates the Cpx response), TR1195 (*cyoA*::Kn), TR1198 (*efeO*::Kn), TR1199 (*nuoA*::Kn), TR1200 (*sdhC*::Kn), and TR1214 (*ydiY*::Kn) were grown overnight in LB containing 30 μg/ml kanamycin (TR1195, TR1198, TR1199, TR1200, TR1214) or amikacin (TR20) at 30°C with shaking, and then 5 μl of serial 10-fold dilutions in LB was plated on LB agar (left), LB agar containing 3 μg/ml amikacin (middle), and LB agar containing 5 mM hydroxyurea (right). The plates were incubated overnight at 37°C, and then photographs were taken to obtain the images shown. UN, undiluted.

pathogenic *E. coli* (UPEC) genome but not in those of E2348/69 or MC4100. These putative open reading frames occur in genomic positions that overlap and are oriented in the opposite direction from the Cpx-regulated genes *spy*, *yccA*, and *yebE* (Fig. 1, Table 4). The fact that we detected these transcripts in two disparate strains and under very different growth conditions argues strongly that transcription from these genomic regions does occur, although at present it is unknown if these RNAs encode proteins.

Q-PCR analysis was performed to confirm the observed gene expression changes observed by microarray analysis (Table 5). Most genes exhibited very similar fold changes in expression upon NlpE overexpression (Table 5). Of the 26 genes that were upregulated by NlpE overexpression and confirmed by Q-PCR analysis, 22 required CpxR for induction (Table 5). The *yaiW sbmA* operon was still induced by NlpE overexpression in a *cpxR* mutant strain, albeit to a lesser extent than in a wild-type strain (1.5- to 2.1-fold compared to 3.5- to 8.6-fold in a wild-type strain background; Table 5). Among the genes that were negatively impacted by NlpE overproduction independently of conditions, most exhibited diminished fold reductions in the absence of CpxR, while 3 (*dctA*, *mglB*, and *putP*) were downregulated by NlpE overexpression regardless of the presence of an intact *cpxR* gene (Table 5). Our data thus suggest the exciting possibility that, in addition to inducing the Cpx envelope stress response, NlpE overexpression also leads to changes in the activity of other, unidentified signaling pathway(s).

The common set of Cpx-induced genes suggests that the mechanisms involved in Cpx-mediated adaptation to envelope stress are more diverse than previously thought. Specifically, it appears that the Cpx response, akin to the eukaryotic unfolded protein

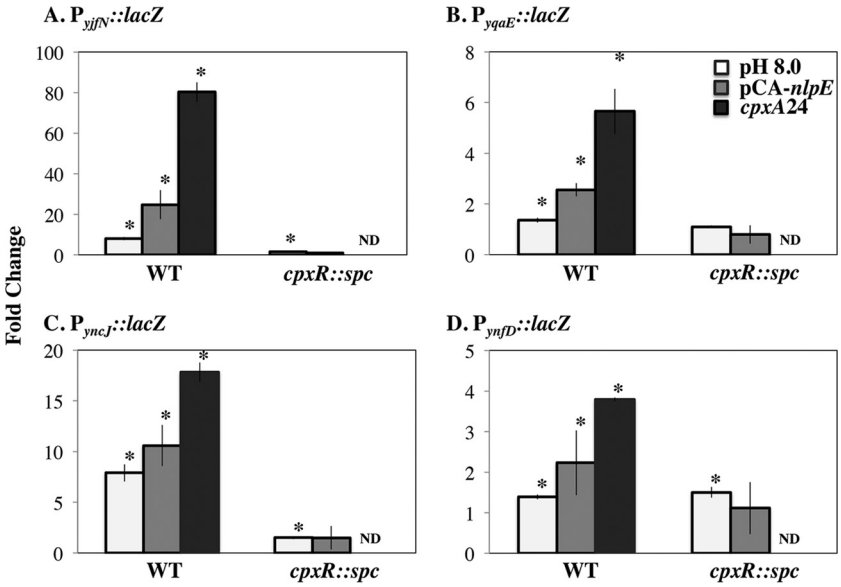

FIG 4 Genes encoding small proteins of unknown function are transcriptionally regulated by the Cpx envelope stress response. Strains carrying single-copy, transcriptional, chromosomal *lacZ* reporters for each of the four small protein genes (*yjfN* [A], *yqaE* [B], *yncJ* [C], and *ynfD* [D]) were subcultured into fresh LB medium after overnight growth in LB medium and grown with shaking at 37°C to a final OD_{600} of ~0.6 to 0.7, with the exception of the *cpxA24* strains, which were grown at 30°C. For NlpE induction from the pCA-*nlpE* plasmid, cultures were grown to the mid-log phase and then induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside; Invitrogen) for 1 h; for pH 8.0 induction, cultures were grown to the early log phase in LB at pH ~7.0, spun down for 10 min at 4,000 rpm, resuspended in LB at pH 5.8 (shutoff) or pH 8.0 (induction), and then grown for an additional 2 h. Cells were lysed using chloroform and SDS, and the β-galactosidase levels were measured using a PerkinElmer Wallac Victor^2 1420 plate reader after addition of ONPG in a 96-well plate. Fold changes between induced (plus IPTG [pH 8.0] or *cpxA24*) and uninduced (no IPTG [pH 5.8] or wild-type [WT] *cpxA*) conditions for triplicate cultures within at least two separate experiments are shown. ND, not determined. Asterisks indicate that the data determined for the induced sample are significantly different from those of the uninduced control ($P < 0.05$).

response (UPR), adapts the envelope to protein misfolding stresses not only by upregulating the chaperone and protease content of the periplasm but also by downregulating secreted proteins (specifically those localized to the inner membrane), altering translation, and coordinating with other regulators that control envelope-related functions (76).

The Cpx response regulates adaptive gene expression at the transcriptional and posttranscriptional levels. Among the 38 genes that showed NlpE-dependent expression changes, CpxR has been demonstrated to bind to the promoters of 9 (*yebE*, *efeU*, *ompF*, *cpxP*, *spy*, *dsbA*, *yccA*, *mzrA*, and *ydeH*) (Table 4). We used the program Virtual Footprint (http://prodoric.tu-bs.de/vfp/) to search for putative CpxR binding sites in the remaining 29. This analysis revealed an additional six genes or operons (*sbmA yaiW*, *yqaE*, *yjfN*, *slt*, and *ygaU*) that contained CpxR consensus binding motifs within 500 bp upstream of the translational start site of the gene (Table 4). The remaining 23 genes that exhibited expression changes upon NlpE overexpression were not predicted to contain a CpxR consensus binding motif within 500 bp upstream of the translational start site (Table 4), suggesting that Cpx-regulated intermediary factors are involved in many of the observed changes in gene expression.

Related to this conclusion, we observed that the expression of several known small RNAs (sRNAs) is changed by NlpE overexpression by 2-fold or more under at least one microarray condition (Table S2 in the supplemental material). These sRNAs included CyaR, MicF, OmrA, OmrB, RprA, and RybB. In agreement with our findings, recent studies have shown that the Cpx response leads to upregulated production of the *omrAB* gene cluster through its direct regulation of *mzrA*, a gene encoding a small membrane protein capable of activating the EnvZ-OmpR two-component system, which in turn regulates *omrAB* transcription (68, 77). Further, in addition to inhibiting *ompF* expression, MicF negatively regulates the transcription factor Lrp, which in turn is responsible for the regulation of genes involved in a number of functions that we also identified in our microarray data, including amino acid metabolism and transport (78). These findings suggest that the Cpx-mediated upregulation of *micF* expression that we observed functions as part of a feed-forward loop to ensure efficient inhibition of some targets during envelope stress. Interestingly, MicF has also been shown to inhibit *cpxR* expression, thus forming a negative-feedback loop (79). It appears that the Cpx-mediated downregulation of *cyaR* transcription that we observed may be important in a regulatory circuit with the *yqaE* gene, which is positively regulated by NlpE overexpression (Fig. 1, Table 5). CyaR is known to inhibit *yqaE* expression (80), and so its downregulation may serve to enhance the elevated expression of *yqaE* under conditions in which the Cpx response is induced. We conclude that the regulation of sRNA expression, and the consequent posttranscriptional effects on gene expression, are likely to be an important part of the complex regulatory circuitry that dictates adaptation to envelope stress that is conferred by the Cpx response.

In addition to known sRNAs, 35 intergenic (IG) regions exhibited at least a 2-fold change in expression upon NlpE overexpression under at least one microarray condition (see Table S2 in the supplemental material). A total of 23 IG regions were changed in expression under only 1 condition, 8 exhibited altered expression

under 2 or 3 conditions, and 4 exhibited changes in transcript levels under all 4 conditions examined. Although it may turn out that not all of these IG regions encode sRNAs, our data suggest that additional, uncharacterized sRNAs may be part of the Cpx-mediated response to envelope stress.

Newly identified Cpx-regulated genes affect antibiotic resistance. Collins and coworkers have proposed that all bactericidal antibiotics act through a similar mechanism involving a burst of NADH levels leading to elevated electron transport, the generation of superoxide, and the subsequent production of reactive oxygen species via the Fenton reaction (81). A number of recent studies support this hypothesis (82, 83), and it has been shown that mutations in *cpxA* lead to antibiotic resistance and the diminished production of reactive oxygen species (84). Recent findings indicate that antibiotic resistance is conferred through activation of the Cpx response (85), which occurs in a *cpxA* null strain through unregulated phosphorylation of CpxR by low-molecular-weight phosphodonors (8). In light of this fact, we were struck by the enrichment of genes encoding proteins involved in aerobic respiration, metal binding, and ion transport among genes that were downregulated by NlpE overexpression (Fig. 2 and 3A, Table 4). We observed significant downregulated expression of genes encoding succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase, and the EfeUOB ferrous iron transporter under multiple conditions in our array experiments (Fig. 3A). To test whether Cpx-mediated downregulation of these genes could be involved in antibiotic resistance, we examined the impact of deleting these genes on resistance to the aminoglycoside amikacin, to which hyperactivated CpxA* mutants are known to be resistant (86), and the drug hydroxyurea (HU) (resistance to HU is also impacted by mutations to *cpxA*) (84). In all cases, elimination of these genes led to an increase in survival in the presence of either amikacin or HU, similar to what is seen in the presence of a *cpxA** mutation that constitutively activates the Cpx response, without any apparent overall change in viability (Fig. 3B). Interestingly, the randomly selected control gene *ydiY* also caused a slight increase in resistance to amikacin and HU (Fig. 3B), although other strains carrying kanamycin insertion cassette mutations did not (data not shown). These data strongly suggest that activation of the Cpx response can confer antibiotic resistance and that this effect is at least partly due to the downregulated expression of genes involved in electron transport and iron import.

Small, Cpx-regulated envelope proteins are associated with membrane integrity and function. We were curious about the functions of the unknown "*y*" genes that are induced by the Cpx response under all our experimental conditions (Fig. 2, Table 4). Several of these are predicted to encode small periplasmic (YnfD, 101 amino acids [aa]; YjfN, 91 aa; YncJ, 76 aa) or inner membrane (YqaE, 52 aa) proteins. Initially, we confirmed the Cpx regulation of these genes using transcriptional *lacZ* reporter genes. The DNA upstream of each gene was cloned upstream of a promoterless *lacZ* gene, and the resulting constructs were integrated into the chromosome on a λ phage, as previously described (40). β-Galactosidase activity was then examined in wild-type and *cpxR* mutant strains in the presence of either the constitutively active *cpxA24* allele, alkaline pH, or NlpE overexpression. In all cases, induction of the Cpx response by either cue led to elevated expression of each reporter gene, and induction by NlpE and alkaline pH was dependent on the presence of an intact CpxAR signaling pathway

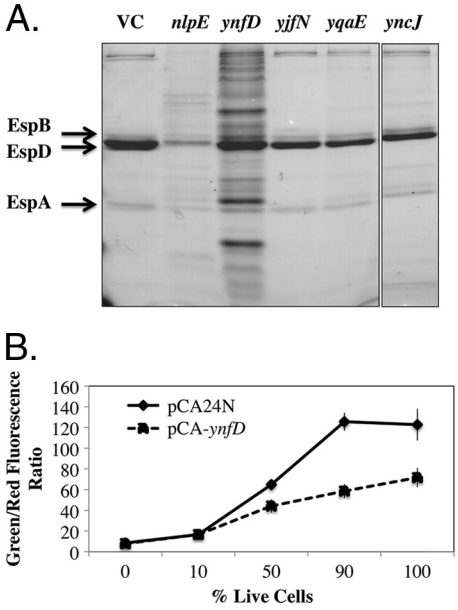

FIG 5 Overexpression of YnfD causes a leaky-membrane phenotype. (A) Coomassie blue-stained SDS-PAGE gel from a T3S assay on EPEC E2348/69 containing one of the pCA24N, pCA-*nlpE*, pCA-*ynfD*, pCA-*yjfN*, pCA-*yqaE*, and pCA-*yncJ* plasmids induced with 0.1 mM IPTG. T3S samples were collected from samples statically grown in 24-well polystyrene plates in 2 ml of DMEM with 5% CO_2 at 37°C to an OD_{600} of ~0.7. (B) A LIVE/DEAD BacLight kit (Invitrogen) was used on late-log-phase cultures of EPEC E2348/69 (pCA24N) and E2348/69 (pCA-*ynfD*) with OD_{670} values standardized to ~0.12; half of the collected cells were killed with isopropanol, and then mixtures of five different proportions (0:100, 10:90, 50:50, 90:10, and 100:0) of live/dead bacteria were made, divided into aliquots, placed in a 96-well plate, and stained with SYTO 9 and propidium iodide, and the absorbances at ~530 nm and ~630 nm were measured in a PerkinElmer Wallac Victor^2 plate reader. The ratio of green/red fluorescence (live/dead bacteria) was plotted against the known percentage of live cells in the suspension. Experiments were done in triplicate.

(Fig. 4). These results, together with the microarray and Q-PCR validation experiment data (Table 5), demonstrate that the transcription of *ynfD*, *yjfN*, *yncJ*, and *yqaE* is controlled by the Cpx envelope stress response.

When the Cpx pathway is activated in EPEC, it shuts down the production of the T3SS (type III secretion system) and the BFP (bundle-forming pili) and motility (1, 20, 48). Further, our microarray data suggest that a number of inner membrane proteins, primarily transporters, are also expressed at lower levels when the Cpx response is induced (Fig. 2, Table 4). Recent studies suggest that stress-regulated small envelope proteins may play roles in regulating inner membrane protein function (87–89). To test the possibility that YnfD, YjfN, YncJ, and/or YqaE is involved in the shutdown of envelope processes during Cpx activation, we examined the effects of upregulating these four genes on the T3SS in EPEC strain E2348/69. There appeared to be only minor effects on the T3S of the translocators EspD, EspB, and EspA upon overexpression of *ynfD*, *yjfN*, *yncJ*, or *yqaE* (Fig. 5A). However, the overexpression of *ynfD* resulted in an aberrant secreted protein profile that contained far more proteins than were seen with the control strain (Fig. 5A). Although the *ynfD*-expressing strain did not exhibit a growth defect (data not shown), analysis of membrane damage using a LIVE/DEAD BacLight kit (Invitrogen) demon-

TABLE 6 Compounds identified in a Biolog, Inc., Phenotype MicroArray comparing the Δ*yqaE*::Kn mutant to wild-type MC4100

Compound	Predicted target/function	Fold change[a]
Kanamycin	30S ribosomal subunit	395
Paromomycin	30S ribosomal subunit	361
100 mM sodium nitrite	Nitrite toxicity	180
Neomycin	30S ribosomal subunit	177
2-Hydroxy-1,4-naphthoquinone	Oxidizing agent	144
Josamycin	50S ribosomal subunit	129
Cefamandole nafate	Cell wall (cephalosporin)	116
5-Nitro-2-furaldehyde semicarbazone	Oxidizing agent, DNA damage inducing	100
Phenylmethylsulfonyl fluoride (PMSF)	Serine protease inhibitor	105
Sodium salicylate	Biofilm and capsule inhibition, chelator, prostaglandin synthetase inhibitor, Mar inducer	83
Trifluoperazine	Targets membrane, efflux pump inhibitor	75
Oxacillin	Cell wall (lactam)	70
Oxycarboxin	Respiratory enzymes	64
Cefoxitin	Cell wall (cephalosporin)	61

[a] Fold change of expression in the Δ*yqaE*::Kn mutant with respect to the wild-type (MC4100) strain according to Biolog analyses. A positive fold value indicates increased growth.

strated an increase in the number of cells with membranes that were permeable to propidium iodide when YnfD is present in excess quantities (Fig. 5B). Together, these data suggest that YnfD expression confers a leaky-membrane phenotype, causing cellular proteins to be released into the supernatant.

To further examine the functions of *ynfD*, *yjfN*, *yncJ*, and *yqaE*, knockout strains were subjected to Phenotype MicroArray analyses. For the most part, the results for the *ynfD*::Kn and *yjfN*::Kn mutants did not shed further light on their functions (data not shown). In contrast, the *yqaE*::Kn mutant gained resistance to 11 compounds unrelated to kanamycin, 9 of which have confirmed or potential cell envelope targets (Table 6), including cefamandole nafate and cefoxitin (cephalosporins that target the cell wall), oxacillin (a lactam that targets cell wall growth), 2-hydroxy-1,4-naphthoquinone and 5-nitro-2-furaldehyde semicarbazone (oxidizing agent), phenylmethylsulfonyl fluoride (PMSF) (which inhibits serine proteases), sodium salicylate (inhibits capsule and biofilm formation), and trifluoperazine (targets membrane, inhibits efflux pumps). Interestingly, the *yncJ*::Kn mutant gained resistance to chlorhexidine diacetate (targets electron transport; data not shown), while the *yqaE*::Kn mutant gained resistance to oxycarboxin (targets respiratory enzymes) (Table 6).

The leaky-membrane phenotype seen when YnfD is overexpressed (Fig. 5), coupled with the altered sensitivity of the *yncJ*::Kn and *yqaE*::Kn mutants to compounds that target the bacterial envelope (Table 6 and data not shown) and the Cpx regulation of these gene products (Fig. 4), suggests that YnfD, YncJ, and YqaE alter envelope-associated functions. Counterintuitively, our data predict that Cpx-mediated upregulation of these genes should lead to increased susceptibility to agents that act negatively on cell envelope components and functions, including proteins, the cell wall, and respiration. Although at present we have no explanation for these data, one intriguing possibility supported by our data is that Cpx-inducing cues result in a damage to the inner membrane that, if unchecked, may lead to lethal damage in the presence of "normal" activities at this cellular site, including respiration, secretion, and transport. Such damage could include the generation of harmful reactive oxygen species and irreversibly aggregated proteins. In this case, it would benefit the cell to inhibit activities at the inner membrane, which our data indicate is a general feature of Cpx-mediated adaptation. Such a response could also lead to increased sensitivity to some treatments affecting the envelope by limiting the activities of efflux pumps and transporters while enhancing resistance to bactericidal antibiotics by diminishing respiration. We propose that these changes are necessary to prevent the exacerbation of envelope damage in situations where the Cpx response is induced.

ACKNOWLEDGMENTS

We thank the staff of the Alberta Transplant Centre for Applied Genetics and the Department of Biological Sciences Molecular Biology Service Unit for assistance and advice with RNA isolation, cDNA labeling, microarray hybridization and analysis, and Q-PCR analysis.

T.L.R. is supported by an Alberta Heritage Foundation for Medical Research Senior Scholar Award. This work was supported by operating grants from the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes for Health Research.

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