

The Cpx Envelope Stress Response Regulates and Is Regulated by Small Noncoding RNAs

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The *Escherichia coli* genome encodes approximately 30 two-component systems that are required for sensing and responding to a variety of environmental and physiological cues. Recent studies have revealed numerous regulatory connections between two-component systems and small noncoding RNAs (sRNAs), which posttranscriptionally regulate gene expression by base pairing with target mRNAs. In this study, we investigated the role of sRNAs in the CpxAR two-component system, which detects and mediates an adaptive response to potentially lethal protein misfolding in the Gram-negative bacterial envelope. Here, we showed for the first time that sRNAs are members of the Cpx regulon. We found that CpxR binds to the promoter regions and regulates expression of two sRNA genes, *cyaR* and *rprA*. We also investigated the roles that these sRNAs play in the Cpx response. Cpx repression of *cyaR* expression creates a feed-forward loop, in which CpxAR increases expression of the inner membrane protein YqaE both directly at the transcriptional level and indirectly at the translational level. Moreover, we found that RprA exerts negative feedback on the Cpx response, reducing Cpx activity in a manner that is dependent on the response regulator CpxR but independent of all of RprA's previously described targets. sRNAs therefore permit the fine-tuning of Cpx pathway activity and its regulation of target genes, which could assist bacterial survival in the face of envelope stress.

Two-component systems (2CSs) are the primary means by which bacteria sense and respond to changes in their surroundings (1). Bacterial genomes frequently encode dozens of 2CSs, each of which detects a unique stimulus and performs a unique physiological role. Of the approximately 30 2CSs encoded in the *Escherichia coli* genome (2), the CpxAR 2CS is among the best characterized (reviewed in reference 3). The Cpx 2CS consists of the inner membrane (IM)-localized histidine kinase (HK) CpxA and the cytoplasmic response regulator (RR) CpxR. CpxA possesses two opposing enzymatic activities (4). In the presence of an inducing signal, CpxA acts as a kinase to phosphorylate CpxR at a conserved aspartate residue, thereby permitting CpxR to bind to DNA and modulate transcription. In the absence of an appropriate signal, CpxA acts as a CpxR~P phosphatase, keeping CpxR dephosphorylated and therefore inactive.

The molecular nature of the signal sensed by CpxA remains unknown; however, several cues that induce the Cpx pathway have been identified. These include alkaline pH (5), alterations to the composition of the IM (6, 7), and ectopic expression of pilins such as PapE, PapG, and BfpA in the absence of their cognate chaperones (8, 9). All of these cues are expected to generate misfolded IM and/or periplasmic proteins; the Cpx system is therefore considered an envelope stress response (3). The Cpx pathway is also induced by overexpression of the outer membrane (OM) lipoprotein NlpE (10), which is believed to be an auxiliary regulator capable of sensing adhesion to hydrophobic surfaces (11). In accordance with the view of Cpx as an envelope stress response, many of the genes whose expression is most strongly increased by CpxR encode periplasmic protein folding and degrading factors, such as the protease/chaperone DegP (10), the disulfide bond oxidoreductase DsbA (12, 13), and CpxP, which functions as both a chaperone and a repressor of the Cpx response (14–16). CpxR also regulates a variety of other genes with envelope-related functions (3, 17); for example, expression of macromolecular complexes such as flagella and pili is repressed during the Cpx response, thereby reducing protein traffic to an already troubled periplasm.

2CSs can participate in regulatory networks by interacting with other types of regulators. Many such regulatory networks include small noncoding RNAs (sRNAs). sRNAs are regulatory molecules approximately 50 to 300 nucleotides in length (reviewed in references 18 and 19). The best-characterized type of sRNAs, *trans*-encoded sRNAs, act by base pairing with target mRNAs, using short regions of imperfect complementarity. This base pairing can have several different outcomes. sRNAs can negatively regulate expression of their target mRNAs by blocking ribosomal access to the mRNA's ribosome-binding site, thereby reducing translation, and/or by increasing degradation by RNases such as RNase E. Conversely, sRNAs can positively regulate mRNA expression by removing secondary structures in the mRNA that normally inhibit ribosome binding, thereby increasing translation, or by protecting the mRNA from degradation by RNases. Key to many of these activities is the RNA chaperone protein Hfq (reviewed in reference 20), which both stabilizes sRNAs and promotes annealing to their target mRNAs.

Interactions between 2CSs and sRNAs are numerous and can occur in both directions—2CSs can regulate the transcription of genes encoding sRNAs, while sRNAs can also regulate the translation and/or stability of mRNAs encoding 2CS components (reviewed in references 21 and 22). A prime example of a 2CS controlling the expression of sRNAs is EnvZ/OmpR, which activates

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the expression of three sRNA genes (*micF*, *omrA*, and *omrB*) and represses the expression of one sRNA gene (*micC*) (23–25). *OmrA* and *OmrB*, in turn, repress expression of the *ompR* mRNA, creating a negative feedback loop (26). Expression of mRNAs encoding 2CS proteins can also be regulated by sRNAs that are members of different regulons. Such is the case for the *phoP* mRNA, which encodes the RR of the PhoPQ 2CS. Expression of *phoP* is repressed by two sRNAs (*MicA* and *GcvB*), each of which is controlled by a different regulator (the alternative sigma factor σ^E and the transcription factors *GcvA* and *GcvR*, respectively), thereby allowing communication between these regulatory pathways (27, 28).

In this study, our aims were (i) to determine whether the Cpx regulon contains sRNAs and (ii) to determine whether any of these sRNAs regulate expression or activity of the Cpx 2CS. Preliminary evidence that CpxAR regulates the expression of sRNAs was obtained in a recent microarray examining changes in gene expression upon overexpression of NlpE (17). In this microarray, expression of several sRNA genes (*micF*, *omrA*, *omrB*, and *rprA*) was increased by NlpE overexpression, while expression of *cyaR* was repressed. Additional regulators and mRNA targets of these genes are already known and are summarized in Table S1 in the supplemental material. Interestingly, the majority of these sRNAs regulate the expression of mRNAs encoding envelope-localized proteins (indicated in bold in Table S1), which is in keeping with the role of the Cpx system as an envelope stress response. In the present work, we confirmed Cpx regulation of four of these sRNAs. We found that the Cpx response regulates the expression of *cyaR* and *rprA*. We show that CpxR binds to the promoters of these genes, consistent with a model in which it regulates their expression directly. We additionally found that these sRNAs endow the Cpx response with several regulatory network motifs, with *CyaR* participating in a feed-forward loop to regulate the IM protein *YqaE* and *RprA* participating in a novel feedback loop with CpxR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material. Unless otherwise stated, strains were cultured in Luria-Bertani broth (LB) at 37°C with aeration at 225 rpm. Where indicated, isopropyl- β -D-thiogalactopyranoside (IPTG) (Invitrogen) was added to a concentration of 0.1 mM. Antibiotics (Sigma) were added where appropriate at the indicated concentrations: amikacin (*Amk*), 3 μ g/ml; ampicillin (*Amp*), 100 μ g/ml; chloramphenicol (*Cam*), 25 μ g/ml; kanamycin (*Kan*), 30 μ g/ml (*E. coli* K-12 strains) or 50 μ g/ml (enteropathogenic *E. coli* [EPEC] strains); spectinomycin (*Spc*), 25 μ g/ml; tetracycline (*Tet*), 10 μ g/ml.

Strain and plasmid construction. *E. coli* K-12 mutants and overexpression strains were constructed by standard techniques for P1 transduction and transformation (29). Donor strains harboring mutations in *rcsC*, *mzrA*, *rpoS*, *csqD*, *ydaM*, and *ptsG* were obtained from the Keio library (30). Where indicated, the kanamycin resistance cassette contained within these mutations was removed by Flp/Flp recombination target (FRT)-mediated recombination (31) to produce markerless deletions. EPEC strains were transformed by electroporation as previously described (32).

sRNA-*lux* transcriptional reporters were constructed as previously described (33). Briefly, promoters of sRNA genes were amplified by PCR, using the primer sequences listed in Table S3 in the supplemental material. Purified PCR products and the pJW15 *lux* reporter vector (34) were digested with *Bam*HI and *Eco*RI, gel purified, and ligated together. Correct insertion of promoter sequences was verified by PCR and sequencing. In addition, sRNA-*lux* reporters were transformed into strains harboring

mutations in known regulators of each gene (*cyaA* for *cyaR-lux*; *ompR* for *micF-lux*, *omrA-lux*, and *omrB-lux*; and *rcsB* for *rprA-lux*); regulator mutations affected expression of all *lux* reporters as expected based on published results (24, 25, 35–37; also data not shown).

The *cyaR::Kan* mutation in strain SV514 was constructed by λ Red recombination (38). The FRT-flanked kanamycin resistance cassette was amplified from the Keio library using primers *cyaRKOFor* and *cyaRKORev* (see Table S3 in the supplemental material). The purified PCR product was electroporated into strain DY378, which encodes λ Red recombinase functions. *cyaR::Kan* mutations in kanamycin-resistant transformants were verified by PCR. The *cyaR::Kan* cassette was then transduced into *yqaE'-lacZ* reporter strain NRD397.

The sRNA overexpression plasmids were obtained from the Gottesman lab's library. All plasmids were shown by Northern blotting to cause accumulation of the encoded sRNA, as expected (39).

Luminescence assays. Activity of *lux* reporters was measured as previously described (40). Strains were cultured overnight in LB at 30°C with aeration and then subcultured 1:100 into fresh LB and grown at 37°C with aeration for 4 h (with IPTG induction after 2 h if necessary). Normalized luminescence was determined by dividing raw luminescence (in counts per second [cps]) by the optical density at 600 nm (OD_{600}) of the same culture. Luminescence assays were performed two or three times, with five replicate cultures each time.

Electrophoretic mobility shift assays (EMSAs). Maltose binding protein (MBP)-CpxR was purified from JM109(pMCR) as described previously (4) with a few modifications. First, cells were disrupted by passage through a French pressure cell once at 20,000 lb/in². Second, the crude extract was incubated with the amylose resin overnight with gentle agitation for batch protein purification. This mixture was then poured into a column for subsequent washing and elution steps. MBP-CpxR at the indicated concentration was incubated in the presence of acetyl phosphate (20 mM) at 37°C for 30 min in 15 μ l of binding buffer (10 mM Tris [pH 7.4], 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μ g/ml bovine serum albumin [BSA], 1 mM dithiothreitol [DTT], 20 mM potassium glutamate, 10 mM MgSO₄). Next, 1.5 pmol of purified, PCR-amplified promoter DNA was added and the mixture was incubated for another 30 min at 37°C. Reactions were stopped by the addition of 6 \times DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water). Reactions were electrophoresed on a 5% nondenaturing TBE polyacrylamide gel (Bio-Rad) in 1 \times TBE running buffer (89 mM Tris, 89 mM boric acid, 1 mM EDTA [pH 8.0]). DNA was visualized with an ethidium bromide stain.

β -Galactosidase assays. Strains to be assayed were cultured overnight in LB containing appropriate antibiotics at 37°C with aeration. For the $P_{BAD::yqaE'-lacZ}$ reporter experiments, strains were subcultured 1:200 in fresh LB with antibiotics and grown at 37°C with shaking for 6 h; arabinose was added to a final concentration of 0.01% 4 h postsubculture to induce reporter expression. For all other experiments, strains were subcultured 1:50 into fresh LB with antibiotics and grown at 37°C with shaking to early stationary phase (5 h). Strains harboring sRNA overexpression plasmids were induced with IPTG after 3 h. β -Galactosidase activity was measured as previously described (41), with 5 μ l of cell culture being added to 195 μ l of 1 \times Z buffer for strains carrying the *cpxP-lacZ* reporter gene due to high reporter activity. Each strain was assayed in triplicate.

Western blot analysis. Subcultures for whole-cell lysates of bacterial strains used for Western blot analysis were prepared by diluting overnight cultures 1:50 into 5 ml fresh LB containing appropriate concentrations of antibiotics. Cultures were grown to early stationary phase (5 h) at 37°C with shaking, with induction of expression plasmids after 3 h of growth by addition of IPTG. One-milliliter samples, standardized to the same optical density at 600 nm, were pelleted, and cell pellets were lysed in 50 μ l of 2 \times SDS-PAGE loading dye (125 mM Tris [pH 6.8], 20% glycerol, 10% β -mercaptoethanol, 6% sodium dodecyl sulfate, 0.2% bromophenol blue). Electrophoresis and blotting were performed as previously described (15) with rabbit anti-MBP-CpxR (1:10,000 dilution) or anti-

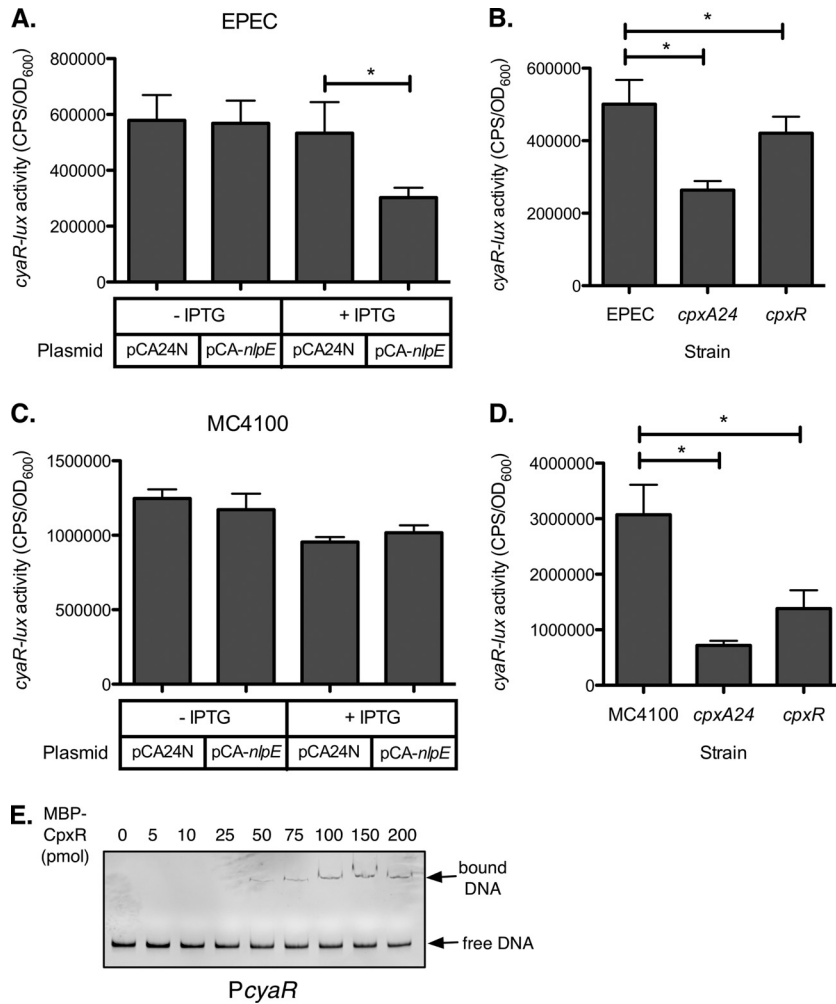


FIG 1 CpxR regulates expression of *cyarR*. (A and C) Luminescence assays comparing *cyar-lux* reporter expression in wild-type EPEC (A) or MC4100 (C) carrying vector control pCA24N or the overexpression plasmid pCA-*nlpE*. (B and D) Luminescence assays comparing *cyar-lux* reporter expression in wild-type, *cpxA24* (Cpx-activating mutation), and *cpxR* (Cpx-inactivating mutation) strains of EPEC (B) or MC4100 (D). Luminescence was normalized to the OD₆₀₀ of the culture. Data for luminescence assays represent the means and standard deviations of five replicate cultures. Asterisks denote a statistically significant difference from the relevant wild-type or vector control ($P < 0.05$, one-way ANOVA with Bonferroni's multiple-comparison test). (E) EMSA. A PCR product containing the *cyar* promoter region was incubated alone or with increasing concentrations of MBP-CpxR~P protein and then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining.

MBP-CpxA (1:50,000 dilution) primary antibodies and alkaline phosphatase-anti-rabbit secondary antibodies (Sigma; 1:25,000 dilution). Proteins were detected by chemiluminescence using a Bio-Rad ChemiDoc MP imaging system and an Immun-Star alkaline phosphatase chemiluminescence kit (Bio-Rad). Western blots were exposed using a cumulative signal acquisition mode, and the longest exposure in which no pixels were saturated was selected for band intensity analysis.

RESULTS

In order to confirm the preliminary microarray results indicating that the sRNA genes *cyarR*, *micF*, *omrA*, *omrB*, and *rprA* are members of the Cpx regulon (17), we examined the effect of Cpx pathway activation and inactivation upon expression of these genes using *lux* transcriptional reporters. We were able to confirm Cpx regulation, either direct or indirect, of four of the five genes: *omrA*, *omrB*, *cyarR*, and *rprA*; expression of the *micF-lux* reporter was not consistently altered by activation or inactivation of the Cpx response (data not shown). We also confirmed that expression of

both *omrA-lux* and *omrB-lux* was activated by the Cpx response indirectly via the Cpx-regulated connector protein MzrA and the EnvZ/OmpR 2CS as previously reported (reference 42 and data not shown). We therefore chose to focus on *cyarR* and *rprA*, as they represented potentially novel Cpx regulon members. Unless otherwise specified, we use the term "regulation" to include both direct (binding of CpxR to a gene's promoter) and indirect (changes in gene expression mediated through any means other than direct binding of CpxR to a gene's promoter) mechanisms of regulation throughout the remainder of this paper.

Cpx regulation of *cyarR*. In the microarray, Cpx activation via *nlpE* overexpression decreased *cyarR* expression to one-third of the uninduced control in enteropathogenic *E. coli* (EPEC), while no significant change in *cyarR* expression was observed upon *nlpE* overexpression in *E. coli* strain MC4100 (17). Using a *cyar-lux* transcriptional reporter, we confirmed that *nlpE* overexpression represses *cyarR* expression in EPEC (Fig. 1A) but has an effect

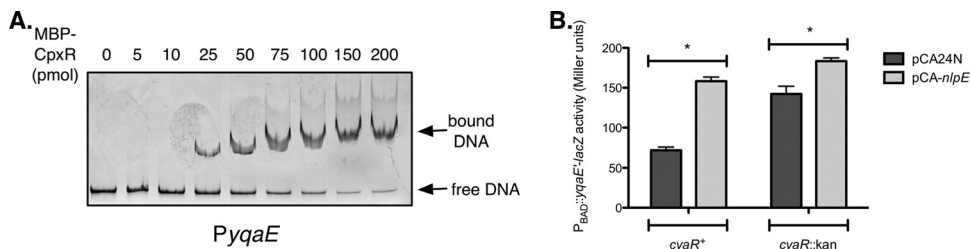


FIG 2 CyaR participates in a feed-forward loop regulating expression of *yqaE*. (A) EMSA. A PCR product containing the *yqaE* promoter region was incubated alone or with increasing concentrations of MBP-CpxR~P protein and then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining. (B) β -Galactosidase assay comparing expression of a $P_{BAD}::yqaE'$ -*lacZ* translational reporter in the presence of the vector control pCA24N or the *nlpE* overexpression plasmid pCA-*nlpE*, in strains harboring a wild-type copy of *cyaR* or a *cyaR::Kan* mutation. Data represent the means and standard deviations of three replicate cultures. Asterisks denote a statistically significant difference from the relevant vector control ($P < 0.05$, one-way ANOVA with Bonferroni's multiple-comparison test).

indistinguishable from that of the vector control in MC4100 (Fig. 1C). Activation of the Cpx response by a different means (using the gain-of-function allele *cpxA24*) repressed *cyaR-lux* activity in both EPEC and MC4100 (Fig. 1B and D, respectively). Interestingly, inactivation of the Cpx response through mutation of *cpxR* also decreased *cyaR-lux* expression (Fig. 1B and D), although the effect of the *cpxR* mutation was consistently smaller than the effect of the *cpxA24* mutation.

To explain the observation that *cyaR* expression is decreased both when the Cpx response is activated and when Cpx is inactivated, we hypothesized that Cpx might exert both direct and indirect regulatory effects on *cyaR*. *cyaR* is also known to be regulated by catabolite repression (35, 36). We therefore examined the effects of *cpx* mutations upon *cyaR-lux* expression under high-glucose conditions where cyclic AMP (cAMP) levels would be expected to be low, and therefore cAMP receptor protein (CRP)/cAMP would have little effect on *cyaR* expression. Figure S2A in the supplemental material shows that, under high-glucose conditions, the *cpxA24* mutation still represses *cyaR-lux* expression, while the *cpxR* mutation no longer has an effect, suggesting that the *cpxR* mutation affects *cyaR* expression via cAMP/CRP. We then searched the microarray results to find any Cpx-regulated genes that might affect levels of cAMP. Indeed, the *PtsG* and *UhpT* glucose transporters are negatively regulated by the Cpx response (17). In support of the hypothesis that the *cpxR* mutation affects *cyaR* expression via effects on cAMP levels, we found that deletion of *cpxR* did not affect *cyaR* expression in a *ptsG* mutant background (see Fig. S2B). We therefore believe that the reduced *cyaR* expression in the *cpxR* mutant is secondary to altered expression of glucose transporters in this strain.

We hypothesized that the repression of *cyaR* expression in the *cpxA24* mutant could be the result of direct binding of phosphorylated CpxR to the *cyaR* promoter. In order to examine this possibility, we performed an electrophoretic mobility shift assay (EMSA) to assess the ability of a purified, phosphorylated MBP-CpxR (MBP-CpxR~P) fusion protein to bind to the *cyaR* promoter *in vitro*. This MBP-CpxR fusion has previously been shown to complement a *cpxR* mutation (4). We found that addition of 50 pmol or more of MBP-CpxR~P to the *cyaR* promoter DNA caused the appearance of a distinct CpxR-DNA complex (Fig. 1E). The *cyaR* promoter had lower affinity for MBP-CpxR~P than the positive control, *cpxP*, which gave a shifted band with 25 pmol of protein (see Fig. S1A in the supplemental material), but higher affinity than the negative control, *rpoD*, which did not bind to

MBP-CpxR~P unless at least 100 pmol of protein was added (see Fig. S1B). We therefore hypothesize that CpxR weakly binds the *cyaR* promoter region, which is consistent with the relatively small change in expression of *cyaR* when the Cpx response was activated (Fig. 1A to D).

Cpx regulation of both *cyaR* and *yqaE* creates a feed-forward loop. We next turned to the question of the role of CyaR in the Cpx response. Many sRNAs exert feedback regulation on their own regulator (43); we examined whether CyaR might affect Cpx pathway activity by transforming a *cyaR* overexpression plasmid into a *cpxP-lacZ* reporter strain. No change in activity of the *cpxP-lacZ* reporter was observed when CyaR was overexpressed relative to the empty vector control (data not shown). sRNAs also participate in feed-forward loops, in which a regulator controls expression of a target gene both directly (by binding to its promoter) and indirectly, by regulating expression of a third gene, which is itself a regulator of the target gene (43). We identified a potential feed-forward loop consisting of CpxR, CyaR, and the IM protein YqaE. Transcription of the *yqaE* gene was previously shown to be activated by the Cpx response (17), while translation of *yqaE* is known to be repressed by CyaR (35). Combined with our results from Fig. 1, these data suggested that CpxR could increase expression of *yqaE* both directly, by binding to its promoter, and indirectly, by decreasing expression of *cyaR*.

In order to address whether CpxR directly regulates transcription of *yqaE*, we performed an EMSA. We found that MBP-CpxR~P binds to the *yqaE* promoter with similar affinity as to the *cpxP* positive control (Fig. 2A; see also Fig. S1A in the supplemental material), suggesting that the previously reported transcriptional regulation of *yqaE* is direct. To assess whether the Cpx response regulates translation of *yqaE*, we used a previously described $P_{BAD}::yqaE'$ -*lacZ* translational reporter (35). Transcription of this construct is driven by the arabinose-inducible P_{BAD} promoter and is therefore not subject to regulation by CpxR. When the Cpx response was activated by the plasmid pCA-*nlpE*, expression of the *yqaE* translational reporter increased approximately 2-fold relative to the vector control (Fig. 2B). The majority of the Cpx enhancement of *yqaE* translation was CyaR dependent, since pCA-*nlpE* increased reporter expression only ~1.2-fold in a *cyaR::Kan* mutant strain (Fig. 2B). Our data suggest that Cpx repression of *cyaR* expression gives rise to a coherent feed-forward loop, in which CpxR both directly and indirectly activates *yqaE* expression.

Cpx regulation of *rprA*. Microarray analysis showed that *rprA*

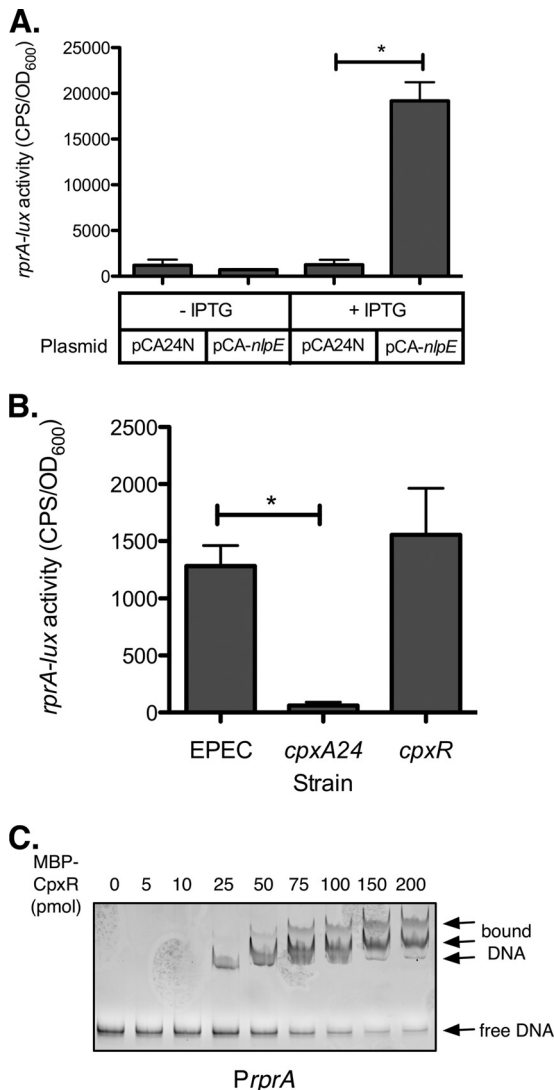


FIG 3 CpxR regulates expression of *rprA* in EPEC. (A) Luminescence assays comparing *rprA-lux* reporter expression in wild-type EPEC carrying vector control pCA24N or the overexpression plasmid pCA-*nlpE*. (B) Luminescence assays comparing *rprA-lux* reporter expression in wild-type EPEC and *cpxA24* (Cpx-activating mutation) and *cpxR* (Cpx-inactivating mutation) strains of EPEC. Luminescence was normalized to the OD₆₀₀ of the culture. Data for luminescence assays represent the means and standard deviations of five replicate cultures. Asterisks denote a statistically significant difference from the relevant wild-type or vector control ($P < 0.05$, one-way ANOVA with Bonferroni's multiple-comparison test). (C) EMSA. A PCR product containing the *rprA* promoter region was incubated alone or with increasing concentrations of MBP-CpxR~P protein and then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining.

expression was increased almost 8-fold when the Cpx pathway was activated by *nlpE* overexpression in EPEC, while the *rprA* transcript was not detected in the MC4100 microarrays (17). Therefore, we initially characterized the effect of activating and inactivating the Cpx response on *rprA-lux* activity in EPEC. In concordance with the microarray results, overexpression of *nlpE* activated expression of the *rprA-lux* reporter more than 15-fold relative to the vector control (Fig. 3A). Unexpectedly, activation of the Cpx response using the *cpxA24* mutation had the opposite

effect on the *rprA-lux* reporter, strongly repressing its expression (Fig. 3B). Inactivation of the Cpx response by mutation of *cpxR* had little effect on the *rprA-lux* reporter (Fig. 3B). EMSA showed that MBP-CpxR~P bound to the *rprA* promoter region (Fig. 3C), with similar affinity as for the *cpxP* promoter positive control (see Fig. S1A in the supplemental material). Three distinct shifted bands were observed when MBP-CpxR~P was added to the *rprA* promoter DNA (Fig. 3C), suggesting the presence of multiple CpxR binding sites in the promoter region.

To gain more insight into Cpx regulation of *rprA*, we repeated the *rprA-lux* experiments in MC4100 wild-type and *cpx* mutant strains; however, no reporter activity was observed in any of the strains (data not shown), consistent with previous findings by microarray (17). In an attempt to boost reporter activity to detectable levels, we introduced a $\Delta rcsC$ mutation. This HK mutation increases activity of the Rcs pathway and was previously shown to elevate *rprA* expression in the *E. coli* MG1655 background (37). Mutation of *rscC* has the additional benefit of eliminating any indirect effects of Cpx activation on Rcs signaling. In the MC4100 $\Delta rcsC$ background, we were able to detect a low level of activity of the *rprA-lux* reporter (Fig. 4). Critically, in the absence of a functional Rcs phosphorelay, both overexpression of *nlpE* and the *cpxA24* mutation increased *rprA-lux* activity (Fig. 4A and B, respectively). We therefore concluded that activation of the Cpx response enhances *rprA* expression, possibly as a result of direct binding of CpxR to the *rprA* promoter. Although we currently do not understand why the *cpxA24* allele leads to inhibition of *rprA* transcription in EPEC, we believe that unidentified connections between the Cpx and Rcs pathways, complex regulation involving multiple regulators, and/or strain differences may be involved (see Discussion).

RprA overexpression inhibits the Cpx response. To address the function of RprA in the Cpx response, we examined whether RprA can exert feedback regulation on the Cpx pathway. The effect of overexpression of RprA on Cpx pathway activity was measured using *cpxP-lacZ* and *degP-lacZ* reporters. The sRNA MicF, although not a member of the Cpx regulon (data not shown), was included in these experiments as a control because it has previously been shown to repress *cpxR* translation (44). Overexpression of RprA significantly repressed activity of both the *cpxP-lacZ* and the *degP-lacZ* reporter (Fig. 5) ($P < 0.05$, one-way analysis of variance [ANOVA] with Bonferroni's multiple-comparison test). Since *degP* is transcriptionally activated by both the Cpx 2CS and the σ^E stress response (10, 45), we also assayed expression of a uniquely σ^E -controlled transcriptional reporter, *rpoHP3-lacZ*, and found that it was not substantially inhibited by RprA overexpression (see Fig. S3 in the supplemental material). Surprisingly, MicF overexpression did not repress expression of any of the reporters (Fig. 5; see also Fig. S3).

We used Western blotting to examine the effects of the sRNAs on CpxA and CpxR protein abundance. The blots showed that overexpression of RprA did not affect the intracellular abundance of either of these proteins (Fig. 6). This result implies that repression of the Cpx pathway by RprA does not result from direct translational inhibition of either *cpxA* or *cpxR*. In agreement with previous results showing that MicF overexpression reduces translation of *cpxR* (44), we observed a significant decrease in CpxR protein levels upon overexpression of MicF (Fig. 6). We additionally found a previously unreported decrease in CpxA protein levels upon MicF overexpression (Fig. 6).

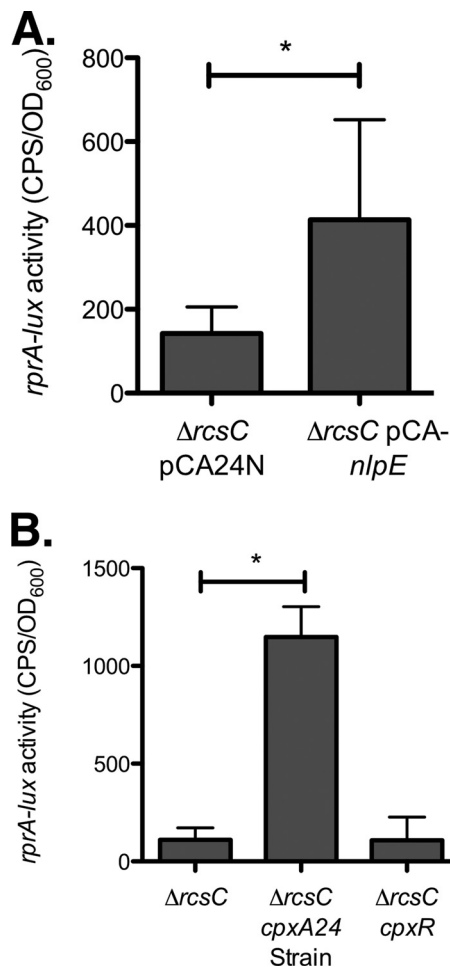


FIG 4 The Cpx response regulates *rprA* expression independently of the Rcs pathway in *E. coli* MC4100. (A) Luminescence assays comparing *rprA-lux* reporter expression in MC4100 $\Delta rcsC$ carrying vector control pCA24N or the overexpression plasmid pCA-*nlpE*. (B) Luminescence assays comparing *rprA-lux* reporter expression in $\Delta rcsC$, $\Delta rcsC$ *cpxA24* (Cpx-activating mutation), and $\Delta rcsC$ *cpxR* (Cpx-inactivating mutation) strains of MC4100. Luminescence was normalized to the OD₆₀₀ of the culture. Data for luminescence assays represent the means and standard deviations of five replicate cultures. Asterisks denote a statistically significant difference from the relevant wild-type or vector control ($P < 0.05$, one-way ANOVA with Bonferroni's multiple-comparison test).

Cpx pathway inhibition by RprA is independent of RprA's known targets and dependent on CpxR. In order to determine whether inhibition of the Cpx pathway by RprA occurred through one of RprA's known targets, *rpoS*, *ydaM*, and *csgD* (46–48), we deleted each of these individually and assayed for a disappearance of Cpx repression upon *rprA* overexpression. Overexpression of RprA in *E. coli* W3110 strains harboring deletions of *rpoS*, *ydaM*, or *csgD* still resulted in a decrease in *cpxP-lacZ* reporter activity (Fig. 7), suggesting that inhibition of the Cpx pathway by RprA does not occur via its regulation of these target genes.

Since RprA does not appear to inhibit the Cpx response by acting directly on the transcripts of *cpxA* or *cpxR*, or through any of its published targets, we sought to determine whether inhibition of Cpx activity by RprA involves sensing of an envelope-localized inducing cue by CpxA by assessing whether mutations to

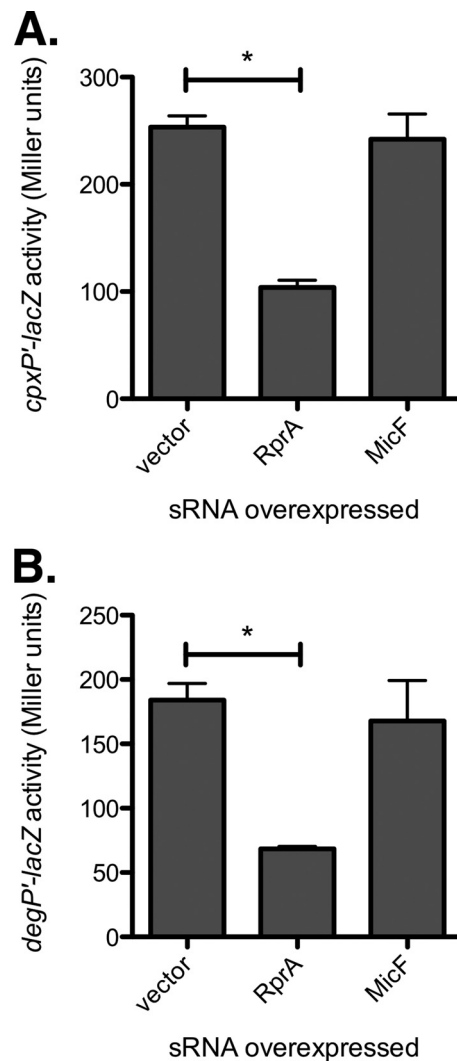


FIG 5 Overexpression of *rprA* inhibits Cpx pathway activity. Effect of *micF* and *rprA* overexpression on activity of the *cpxP-lacZ* (A) and *degP-lacZ* (B) transcriptional reporters. Asterisks denote a statistically significant difference from the vector control ($P < 0.05$, one-way ANOVA with Bonferroni's multiple-comparison test). Data represent the means and standard deviations of three replicate cultures.

cpxA, *cpxP*, or *cpxR* in *E. coli* W3110 abolished inhibition of the Cpx pathway by RprA. Experiments with a *degP-lacZ* reporter revealed that RprA overexpression still obviously repressed the Cpx pathway in *cpxA::Cam* and *cpxP::Kan* strains (Fig. 8). In contrast, although RprA overexpression in the absence of *cpxR* still led to a statistically significant decrease in *degP-lacZ* expression, this decrease was greatly diminished (Fig. 8). In fact, inhibition disappeared almost entirely in a *cpxR::Spc* strain (Fig. 8). These data suggested that inhibition by RprA occurs not through its regulation of some envelope-localized component sensed by CpxA but, rather, predominantly by signaling through CpxR.

It has previously been shown that phosphorylation of CpxR can occur in a CpxA-independent manner by the low-molecular-weight phosphodonor acetyl phosphate, a product of the Pta-AckA pathway (49). Thus, we investigated the possibility that Cpx inhibition by RprA occurs through the Pta-AckA pathway by de-

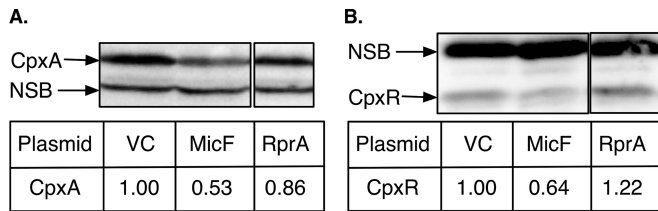


FIG 6 Overexpression of *rprA* does not decrease CpxA or CpxR protein levels. The effect of overexpressing MicF and RprA on CpxA and CpxR protein levels was measured by Western blotting with primary antibodies against either CpxA (A) or CpxR (B), with a nonspecific band (NSB) as a loading control. Quantification of CpxA and CpxR bands was performed using a ChemiDoc MP imager (Bio-Rad) with Image Lab software and is shown beneath the images.

letting both *pta* and *ackA* and measuring inhibition of the *cpxP-lacZ* reporter by RprA. No difference between the wild-type strain and the *pta-ackA::Tn10* strain was observed upon RprA overexpression (see Fig. S4 in the supplemental material). Therefore, RprA inhibits the Cpx pathway via CpxR in a Pta-AckA-independent manner.

DISCUSSION

Two-component systems and sRNAs are both widely used by bacteria to regulate gene expression in response to environmental changes. In recent years, many connections between these two types of regulators have been revealed, with at least six of *E. coli*'s 30 2CSs shown to regulate the expression of one or more sRNAs, and numerous sRNAs demonstrated to directly or indirectly regulate 2CS activity (21). In this work, we showed that the Cpx 2CS regulates the expression of at least four sRNA genes: *cyaR*, *rprA*, *omrA*, and *omrB* (Fig. 9). We found that CpxR regulates expression of *cyaR* and *rprA* in a manner consistent with direct binding to their promoters. These Cpx-regulated sRNAs create new regulatory motifs not previously identified within the Cpx response, including both feed-forward and negative feedback loops (Fig. 9).

Cpx regulation of sRNA expression. Regulation of both *cyaR* and *rprA* by the Cpx response is complex. In the case of *cyaR*, both activation and inactivation of the Cpx response decrease *cyaR* expression (Fig. 1). We believe that the decreased *cyaR* expression in the *cpxR* mutant results from secondary effects on glucose transporters, since mutation of *cpxR* does not affect *cyaR* expression

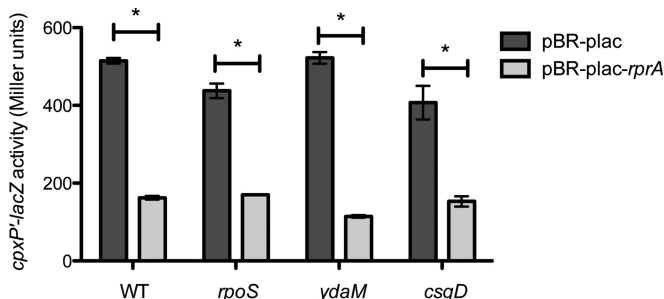


FIG 7 Inhibition of the Cpx pathway by RprA is not dependent on any of RprA's known target genes. β -Galactosidase assay showing the effect of *rprA* overexpression on activity of a *cpxP-lacZ* reporter in wild-type (WT) and *rpoS*, *ydaM*, and *csgD* mutant strains. Data represent the means and standard deviations of three replicate cultures. Asterisks indicate $P < 0.05$ (one-way ANOVA with Bonferroni's multiple-comparison test).

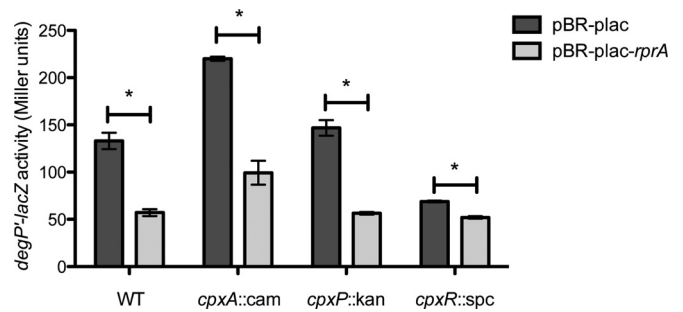


FIG 8 Inhibition of the Cpx pathway by RprA is CpxR dependent but CpxA independent. β -Galactosidase assay showing the effect of *rprA* overexpression on activity of a *degP-lacZ* reporter in wild-type (WT), *cpxA::Cam*, *cpxP::Kan*, and *cpxR::Spc* mutant strains. Data represent the means and standard deviations of three replicate cultures. Asterisks indicate $P < 0.05$ (one-way ANOVA with Bonferroni's multiple-comparison test).

either in high-glucose medium or in the absence of the glucose transporter *ptsG* (see Fig. S2 in the supplemental material). Microarray results suggest that when the Cpx response is inactivated in the *cpxR* mutant, expression of PtsG and UhpT increases (17), increasing the rate of glucose transport into the cell. The increased cellular glucose concentration would decrease cAMP production and thereby decrease *cyaR* expression. The role of the Cpx response in sugar transport is further supported by the recent finding that CpxR represses expression of the glucose 6-phosphate transporter UhpT in enterohemorrhagic *E. coli* (50). In contrast, activation of the Cpx response does not appear to repress *cyaR* expression via effects on catabolite repression, since the *cpxA24* mutation still reduces *cyaR-lux* activity under high-glucose conditions (see Fig. S2A). The simplest explanation for these results is that phosphorylated CpxR represses *cyaR* expression through direct binding to its promoter, since CpxR can bind the *cyaR* promoter *in vitro* (Fig. 1E). However, we cannot formally rule out indirect effects of Cpx pathway activation on *cyaR* expression.

Our initial experiments with *rprA* in EPEC showed that activating the Cpx response using two different methods produced opposite effects on *rprA* expression—*nlpE* overexpression increased *rprA-lux* activity, while the *cpxA24* mutation repressed *rprA* expression (Fig. 3). When these experiments were repeated in the MC4100 Δ *rscC* mutant background, we found that both *nlpE* overexpression and the *cpxA24* mutation increased *rprA-lux* expression (Fig. 4). Since CpxR also binds to the *rprA* promoter *in vitro* (Fig. 3C), we favor the explanation that binding of CpxR to the *rprA* promoter enhances *rprA* expression (although, again, we cannot exclude the possibility of indirect regulation at this time). In this case, the *rprA* repression observed in the EPEC *cpxA24* mutant could result from indirect effects on Rcs activity. In support of this idea, Evans and colleagues (51) have recently shown that the Cpx response affects Rcs activity in response to peptidoglycan alterations. Thus, it seems likely that the Cpx response can interact with the Rcs pathway through an unknown mechanism. In addition, *in silico* analysis indicates that other regulators in addition to RcsB and CpxR may bind to the *rprA* promoter region; we are currently investigating whether any of these other regulators may play a role in the complex, strain-dependent regulation of *rprA*.

CyaR-dependent feed-forward loop. Interactions between regulators can produce regulatory network motifs that have

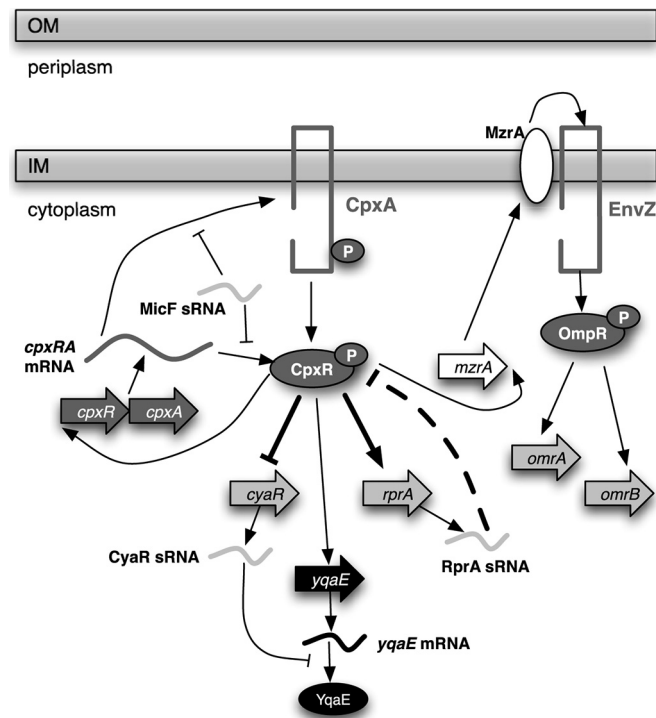


FIG 9 Model of regulatory connections between the Cpx two-component system and sRNAs. In the presence of envelope stress, the CpxA histidine kinase autophosphorylates and then phosphorylates the response regulator CpxR. CpxR directly regulates the transcription of two sRNA genes: *cyaR* transcription is repressed, while *rprA* transcription is either activated or repressed, depending on the growth phase. CpxR indirectly regulates the transcription of the sRNAs OmrA and OmrB by activating transcription of the inner membrane protein MzrA; MzrA physically interacts with EnvZ, increasing the activity of the EnvZ/OmpR two-component system, which activates *omrA* and *omrB* transcription. CpxR regulates the expression of the inner membrane protein YqaE in two ways: directly at the transcriptional level and indirectly at the translational level through repression of CyaR, creating a feed-forward loop. Two sRNAs affect expression or activity of the Cpx response: RprA indirectly represses activity of CpxR by an unknown mechanism (indicated by a dashed line), while MicF represses translation of the *cpxRA* mRNA. Boldface arrows and lines indicate regulatory connections described for the first time in this work. OM, outer membrane; IM, inner membrane; P, phosphate.

unique properties. One of these motifs is the feed-forward loop, in which a regulator controls the expression of a target gene both directly (by binding to its promoter) and indirectly (by regulating another regulator that also affects expression of the target) (43, 52). Feedforward loops can be classified as either coherent, if the direct and indirect routes of regulation are both positive or both negative, or incoherent, if one route is positive and the other negative (52). sRNAs have been shown to participate in both coherent and incoherent feed-forward loops (53). We have identified a coherent feed-forward loop, in which CpxR activates expression of *yqaE* both directly, by binding to its promoter and increasing *yqaE* transcription (Fig. 2A) (17), and indirectly, by repressing expression of *cyaR* and thereby increasing translation of *yqaE* (Fig. 2B) (35). A similar feed-forward loop involving the sRNA Spot42 was recently shown to reduce leaky expression of its target genes in the absence of an activating cue (54). Thus, CyaR could help to reduce leaky expression of *yqaE* under Cpx-inactive conditions. Although the function of YqaE is currently unknown, it was shown that a *yqaE* mutant was more resistant to several envelope-dam-

aging compounds in a Biolog phenotype microarray (17), suggesting that *yqaE* expression makes cells more sensitive to some toxic agents. Therefore, CyaR repression of *yqaE* could help to ensure that *yqaE* is expressed only when the Cpx response is fully activated.

sRNAs affecting Cpx pathway activity. Recent studies have identified numerous sRNAs that affect the expression or activity of 2CSs (21). Our data, combined with those of Holmqvist et al. (44), indicate that several sRNAs affect the Cpx pathway (Fig. 9), with RprA mediating feedback regulation and MicF possibly permitting other regulators to communicate with the Cpx response.

Our data demonstrate that RprA exerts negative feedback on the Cpx response without directly affecting expression of CpxR or CpxA. Overexpression of RprA decreased expression of two Cpx-regulated genes (Fig. 5) but did not affect the abundance of CpxR and CpxA proteins (Fig. 6). Furthermore, RprA overexpression did not affect activity of a *cpxR'*-*gfp* translational fusion containing the 5' untranslated region (UTR) and first 20 codons of *cpxR* (data not shown); however, the ability of RprA to interact with other regions of the *cpxR* coding sequence remains to be thoroughly tested. These results suggest that RprA modulates the activity of the Cpx pathway rather than its expression. Since signals can enter the Cpx pathway through numerous signaling components (3), we overexpressed RprA in mutants lacking the Cpx system components CpxP, CpxA, and CpxR in order to gain more information about the mechanism of RprA feedback regulation. This experiment showed that RprA's effects on the Cpx pathway were dependent on CpxR but not on CpxA (Fig. 8). Surprisingly, RprA-mediated repression was not dependent on the Pta-AckA pathway (see Fig. S4 in the supplemental material), which was previously shown to be the major CpxA-independent source of CpxR phosphorylation when cells are grown in the presence of excess glucose (5). Furthermore, RprA repression of Cpx activity was not dependent on any of the known RprA targets *rpoS*, *ydaM*, and *csgD* (Fig. 7), suggesting that additional targets of RprA regulation remain to be identified.

There are several mechanisms by which RprA could conceivably influence the activity of CpxR without altering its expression. One possibility is that RprA regulates a noncognate HK (i.e., not CpxA) that is capable of cross-phosphorylating CpxR. We do not favor this possibility, however, since cross talk between 2CSs has been shown to be unlikely to occur *in vivo* (55, 56). Another exciting possibility is that RprA regulates the expression of a novel auxiliary regulator that influences CpxR activity. Such auxiliary regulators can affect the activity of an RR by modulating its rate of phosphorylation or dephosphorylation or its ability to bind to DNA (57). Identification of the mechanism by which RprA influences Cpx pathway activity could therefore shed light on both the cellular role of this sRNA and on the regulation of 2CS activity.

Holmqvist and colleagues previously reported that MicF decreases expression of a *cpxR-gfp* translational fusion (44), in agreement with our finding that overexpression of MicF decreases the abundance of both CpxR and CpxA (Fig. 6). Curiously, despite this decrease in Cpx protein levels, overexpression of *micF* did not significantly decrease expression of the Cpx-regulated genes *cpxP* and *degP* (Fig. 5). We considered the possibility that MicF overexpression affects Cpx activity only under pathway-activating conditions, since gene regulation by CpxR depends not only on its abundance but also on its phosphorylation status. However, when we repeated the β -galactosidase assay from Fig. 5A in LB at pH 8,

which is an inducing condition for the Cpx pathway (5), we still found no effect of MicF overexpression on *cpxP-lacZ* activity (data not shown). Thus, the significance of MicF regulation of *cpxRA* is currently unclear. One possibility that we are currently investigating is that MicF overexpression has differential effects on Cpx regulon members—decreased levels of CpxR could have a larger effect on its ability to bind to low-affinity promoters than its ability to bind to high-affinity promoters like *cpxP*. If this hypothesis is correct, MicF could provide a mechanism for its regulators, including EnvZ/OmpR and Lrp, to restrict the size of the Cpx regulon to include only select targets.

In summary, we have demonstrated that sRNAs comprise a previously unrecognized part of the Cpx regulon, with roles in both feed-forward and feedback regulation. In addition, multiple sRNAs affect the expression or activity of CpxR. These results demonstrate that sRNAs link the Cpx response to a variety of other cellular regulators. Such regulatory connections may play an important role in *E. coli*'s ability to withstand envelope stress.

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