The Cpx envelope stress response of Escherichia coli regulates and is regulated by the small RNA RprA

by

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Abstract

When bacteria sense changes in their environment, they adapt by altering the expression of their genes in such a way that accommodates the change. To do this, bacterial cells can modulate the activity of their genes at either the level of transcription, or at the post-transcriptional level. Post-transcriptional gene regulation generally involves either a non-coding regulatory RNA element in the transcript of the gene being regulated (i.e. riboswitch), or a small regulatory RNA molecule. The latter type, small regulatory RNAs (sRNAs) have in recent years been shown to be involved in regulating a number of cellular processes (such as iron metabolism, motility, outer membrane porin biogenesis), and be expressed in response to a variety of environmental signals. One such environmental signal is the misfolding of envelope localized proteins, also known as envelope stress, which is detected by the Cpx two-component signal transduction system in the bacterium *Escherichia coli*. The Cpx envelope stress response consists of the inner membrane bound signal kinase CpxA, its periplasmic inhibitor CpxP, and the response regulator CpxR which acts as a transcription factor when activated. A recent microarray done by our group has suggested that induction of the Cpx envelope stress response leads to the altered expression of the sRNA genes *rprA* and *cyaR* which may help alleviate the stress through the regulation of their known targets, many of which encode envelope-localized structures. Here we confirm Cpx regulation of these two genes by showing that Cpx induction modulates activity of transcriptional reporter genes made with promoters of these genes. We also show that overexpression of one of these genes, *rprA,* promotes the Cpx-related phenotype of amikacin resistance and reduced motility. Furthermore, we show that overexpression of *rprA* causes repression of the Cpx response, forming a negative feedback loop which likely acts indirectly on

response regulator of the Cpx response, CpxR. This inhibition was found not to involve direct translational repression of *cpxR* but might involve modulation of its activity, which recent experiments have suggested may involve the glutamate-dependent acid stress resistance system (GDAR).

This work is dedicated to my mother and father, who always insisted I pursue what I was interested in without regard to what others would think (even them).

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LIST OF SYMBOLS, NOMENCLATURE, AND ABBREVIATIONS

2CS: two-component system

A_{420}: absorbance at 420 nm

Amk: amikacin

Amp: ampicillin

ATP: adenosine triphosphate

Cam: chloramphenicol

cAMP: cyclic adenosine monophosphate

CPS: counts per second

CRP: catabolite repression protein

DNA: deoxyribonucleic acid

EMSA: electrophoretic mobility shift assay

EPEC: enteropathogenic *Escherichia coli*

HUS: haemolytic uremic syndrome

IGR: intergenic region

IM: inner membrane

IPTG: isopropyl β-D-thiogalactoside

Kan: kanamycin

LB: Luria-Bertani

LPS: lipopolysaccharide

MBP: maltose-binding protein

mRNA: messenger RNA

OD_{600}: optical density at 600 nm

OM: outer membrane

OMP: outer membrane protein

PCR: polymerase chain reaction

PMF: proton motive force

RBS: ribosomal binding site

RNA: ribonucleic acid

RNase: ribonuclease

Rpm: rotations per minute

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Spec: spectinomycin

sRNA: small RNA

Tet: tetracycline

Tp: trimetroprim

WT: wild-type

CHAPTER 1

INTRODUCTION

1.1 *Escherichia coli*

Escherichia coli is a Gram-negative rod-shaped bacterium that lives in the large intestine of humans. Most strains of *E. coli* live as commensal organisms within the human host, metabolizing important micronutrients which the host cannot and playing an important role in the digestion of food in the intestinal tract. In fact, commensal strains of *E. coli* are some of the first bacteria to colonize the gut of newborn children, and once established represent a large proportion of the gut microbiome (1). Some strains of *E. coli*, however, live as human pathogens and can cause serious disease in either infants or adults depending on the strain. Enteropathogenic *E. coli,* for example, seldom causes disease in adults but is a leading contributor to the millions of infant deaths caused by diarrheal diseases in countries lacking proper water sanitation each year (2). Enterohemarrhogenic *E. coli*, on the other hand, causes diarrheal disease and occasionally haemolytic uremic syndrome (HUS) in people of all ages all over the world, although it is seldom fatal. Lab strain *E. coli* K12 is likely the best-studied organism in the world: much of its cellular physiology and architecture was elucidated in some of the earliest molecular genetics experiments. Since then it has been used as a model to study not only microbial genetics but also DNA replication in a broader sense, since the mechanisms of DNA replication are remarkably well-conserved across the kingdoms of life. Because of this, *E. coli* is commonly used as a model organism both for intestinal pathogens, but also for Gram-negative gene regulation and physiology in general, since there is ample information available regarding *E. coli*'s genome and proteome.

One interesting aspect of *E. coli*'s lifecycle (and more broadly of all enteric bacteria) is that in order for it to successfully colonize the human gut, it must survive a wide array of environmental threats before it even reaches the large intestine. The cell must first survive

outside of the human host, typically in soil or on abiotics surfaces (3), followed by passage through the mouth, throat, stomach, small intestine then large intestine. On this journey to its site of colonization the cell must survive dessication, fluctutations in temperature, antimicrobial peptides, osmotic stress, drastic changes in pH (in the stomach in particular), as well as elements of competition with other microbes. Understandably then, *E. coli* has evolved a variety of cellular systems which detect changes in its environment and adapt by causing an organized shift in gene expression. Depending on the threat or stimulus, this adaptation can come in many forms, from the expression of purpose-suited cell structures which accommodate changes in pH, for example, to more drastic lifestyle shifts such as the shift to stationary phase, or the production of biofilm. Regardless of the stimulus however, adaptation in *E. coli* (as in all bacteria) always involves organized changes in gene expression, whether it be at the transcriptional level or post-transcriptional level.

1.2 Post-transcriptional gene regulation by small RNAs

There are two main modes of gene regulation in bacteria: transcriptional regulation, and post-transcriptional. Both of these levels of regulation can be broken down further into sub-categories, but in general each level is characterized by a few important distinctions.

1.2.1 Transcriptional regulation.

Regulation at the transcriptional level typically works by preventing or allowing the initiation of transcription through physical interactions between a regulator (called a transcription factors) and the RNA polymerase and/or other transcription factors at the gene's promoter. Some

transcriptional regulation does not involve direct protein-protein contact between regulators, but rather involves the alteration of the physical conformation of the extended promoter, the classic example being the pleotropic regulator H-NS (4). Many transcription factors are part of two-component signal transduction systems, and act to alter the transcription of targets genes in response to stimulation by a sensor protein (called a signal kinase) which itself is induced by some external stimuli (which can be anything from nutrients to damaging agents or conditions).

1.2.2 Post-transcriptional regulation.

Post-transcriptional regulation involves a regulator which either enhances or inhibits the translation into protein of an mRNA transcript once it has already been made. This can be accomplished by a non-coding regulatory RNA structure inherent in the sequence of the gene which is being regulated (referred to as a riboswitch) or sometimes by small regulatory RNA molecules (sRNAs for short) which interact with a target RNA transcript through direct base-pairing. The literature review in this chapter will focus only on the latter type of post-transcriptional regulation, since this are the focus of the research discussed in this thesis. Regulation by riboswitches is reviewed elsewhere (5). While the specific factors that dictate what type of gene regulation (transcriptional vs. post-transcriptional) is used to mediate a particular process are difficult to determine, each type of regulation has distinct advantages. Transcriptional gene regulation tends to provide a more stable, long-lasting regulatory effect than post-transcriptional regulation, because the regulator itself is a protein and therefore more stable. Post-transcriptional regulation on the other hand is less energetically costly because the regulator itself does not need to be translated into a polypeptide, and provides a more rapid response for

the same reason. The past twelve years have heralded an explosion of research on post-transcriptional gene regulation by sRNAs, and it has become evident that these molecules play a role in regulating almost all cellular processes in bacteria.

1.2.3 History of Small RNA Research

The first small non-coding RNA (sRNA) to be characterized was discovered by researchers who were attempting to demonstrate the mechanism of copy-number control of the *E. coli* R1 plasmid. Copy number control mechanisms typically involve an activator of replication which is characteristically stable, and a repressor of replication which is unstable (allowing for rapid plasmid replication following cytokinesis but a decline of replication thereafter). What they found was that the repressor of replication for R1 was in fact a *cis*-encoded sRNA (aptly named RNA I) which, through its antisense binding of the *copB* transcript inhibits plasmid replication (6). The first chromosomally and *trans*-encoded sRNA gene to be discovered was *micF*, whose gene product regulates expression of the outer membrane porin OmpF, and whose expression is induced upon changes in osmolarity (7). This was a significant step forward for understanding RNA regulators, because as a *trans*-encoded sRNA, MicF is not expressed in direct conjunction with its regulated target, and its mechanism of target binding showed that regulation by an sRNA does not require perfect complementarity with the target. By 2001, the significance and magnitude of sRNA regulation in bacteria had started to become apparent, with roughly 11 sRNA genes having been identified in the inter-genic regions (IGRs) of *E. coli* (8), all of which had been discovered by happenstance within the context of the system that each group focused on – no high throughput study had yet been conducted to identify novel

sRNAs. In 2001 however, a group led by Susan Gottesman conducted a study to systematically identify sRNA genes in the IGRs of *E. coli* by comparing highly conserved non-annotated regions in closely related species as well as by using whole-genome expression microarrays to look for short transcripts with no known gene product (9). This study alone identified 17 novel sRNA species, most of which were also found to interact with the Hfq protein (9). More recently, sensitive high throughput RNA-seq techniques have permitted the discovery of sRNAs on the basis of their co-immunoprecipitation with Hfq (10), which helped identify not only novel sRNAs located in intergenic regions but surprisingly also cryptic sRNAs located within the coding sequence of protein-coding genes (11). Because of this recent surge in sRNA discovery we are now aware of roughly 100 (12) sRNAs in *E. coli* alone, although not all of them have been ascribed roles in the cell yet.

1.2.4 The Role of Hfq

There are two major obstacles to regulation by sRNAs: the first is the relatively low *in vivo* stability of these sRNA molecules due to degradation by cellular RNases, and the second is the difficulty with which one might assume the sRNA molecule finds and physically associates with its target mRNA molecule within the cell. Both of these problems are thought to be overcome through the interaction with the Sm-like RNA chaperone Hfq, with which nearly all *trans*-encoded sRNAs are thought to associate (13, 14). Deletions of the *hfq* gene in both *Salmonella* and *E. coli* have been shown to cause a decrease in intracellular levels of various sRNAs (15, 16), as well as to abolish the regulatory effects on sRNAs targets (17). The decrease in stability of sRNAs in Δ*hfq* strains is thought to be due to increased signaling for degradation

by RNase E in the absence of Hfq, which normally protects sRNAs from degradation, possibly by binding to the same sequences that RNase E recognizes (16, 18). Hfq is also thought to aid in the association of sRNA-mRNA partners by binding both molecules and bringing them into proximity with one another (13) via multiple RNA binding domains on either of Hfq's structural faces (19, 20). Other evidence suggests that Hfq serves to increase annealing rates of an sRNA and its target mRNA by altering the secondary structure of the two partner molecules, thus accelerating regulation of the transcript (21), and simultaneously stabilizing the sRNA-mRNA pair once it has formed (22). Another role which has been proposed for Hfq is the acceleration of degradation of sRNA-mRNA duplexes through a direct interaction with RNase E (23). There is strong evidence for such a direct interaction between the two proteins: they have been shown to co-immunoprecipitate (24), and this interaction is dependent on the same binding domain that RNase E uses to interact with the degradosome RNA helicase RhlB (25).

1.2.5 Mechanisms of Target Regulation

Trans-encoded sRNAs can exert either a negative or a positive regulatory effect on the expression of their target gene(s), and this is almost always accomplished by binding the target mRNA transcript and altering availability of the transcript to the ribosome and/or cellular RNAses. There is a small subset of sRNAs which effect their target genes by directly affecting the activity of their protein products (26), but this review will focus solely on sRNAs which act at the post-transcriptional but not post-translational level. The largest class of sRNAs works by inhibiting the translation of their target transcript (13), but they are not characteristically distinct from positive regulatory sRNAs, as it has become evident that some sRNAs can affect some of

their targets negatively and some positively. Such is the case for RprA, which positively influences the translation of *rpoS* (27, 28), but negatively affects the translation of *csgD* and *ydaM* (29, 30). Conversely, some sRNA-regulated mRNAs are regulated in both negative and positive manners by their various sRNA regulators, such as *rpoS,* which is positively regulated by DsrA, RprA, and ArcZ (22, 31) and negatively regulated by OxyS (17).

Negative regulation. Negative regulation by an sRNA is thought to be due by and large to the occlusion of the ribosomal binding site (RBS) of the mRNA by base-pairing of the sRNA at or near that sequence (13), the classic example being that of OxyS, whose binding motif on the *flhA* transcript overlaps the ribosomal-binding site (32), thus preventing initiation of translation by the ribosome (Figure 1). While inhibition of translation by sRNAs is primarily attributed to this RBS sequestration mechanism, a role for RNase E has also been established for negative regulation by sRNAs such as MicC, which has been shown to induce degradation of the *ompD* transcript by RNase E (33). Degradation of an sRNA-mRNA transcript by RNAse E is thought to contribute to, but is not sufficient for negative regulation by most inhibitory sRNAs (24). However, induced RNase-E digestion of an mRNA has also been found to be the primary mode of regulation by some sRNAs, such as RybB which targets multiple *omp* genes using this mechanism (34). The mechanism of negative regulation by an sRNA can sometimes be less obvious, however, as is the case for translation inhibition of *fur* and *ompN* by RyhB, which binds the *fur* transcript far upstream of the translational initiation site (35), but the *ompN* transcript within the 5' end of the transcript's coding sequence (36).

Positive regulation. Positive regulation by an sRNA typically involves the sRNA binding and destabilizing a secondary structure in the 5' UTR of the transcript which normally precludes

binding by the ribosome, thereby freeing the ribosomal binding site (Figure 1). This regulatory mechanism, commonly referred to as an anti-antisense binding motif is found in the regulation by many positive regulatory sRNAs, including GlmZ, DsrA, RprA and the Qrr sRNAs (37-40). The four redundant Qrr sRNAs in *Vibrio cholerae* were found to disrupt an inhibitory stem loop in the 5' UTR of the *vc0939* transcript, and the effect of expressing these sRNAs is the same as that of mutating the stem loop (40). Other evidence suggests that sRNAs can positively regulate their target transcript by stabilizing it and making it less amenable to degradation by RNase E, as has been shown for DsrA's interaction with *rpoS* (28, 38), as well as for the group A *Streptococcus* transcript *ska* which is bound by the FasX sRNA at its 5' end, to protect it from digestion by exonucleases (41).

1.2.6 Cell Functions Regulated by Small RNAs

sRNAs have been shown to be involved in regulating a plethora of bacterial cellular functions such as motility (30), biofilm formation (42, 43), iron metabolism (35) and general stress responses (17, 38). While many of the cell functions regulated by sRNAs seem entirely unrelated, a high proportion of these sRNAs regulate their respective target genes in response to an environmental stressor (13) or signal, often in response to induction of a two-component signal transduction system, as is the case with RprA, MicA, MicF, OmrA and OmrB (42, 44-47). The benefit of this might be that each of these cell signal systems can alter the expression of its regulon in both a slow, more permanent manner via transcriptional regulation as well as in a rapid but transient manner through sRNA regulation, which together allow both an immediate and long-lasting effect on the signaling system's target genes.

A number of sRNAs are known to take part in various stress responses including envelope stress (eg. OmrA/B), oxidative stress (eg. OxyS) and acid stress response (eg. GadY, ArrS) (48-51). One can hypothesize that sRNAs make ideal regulators in stress responses, again due to their rapid and efficient mode of target regulation. Additionally, select stress response systems receive regulatory input from multiple sRNAs, each canonically associated with different cell systems. For example the stationary phase sigma factor and general stress response factor σ^S (encoded by *rpoS*) is subject to regulation by no less than four sRNAs (RprA, DsrA, ArcZ, and GcvB), each of which acts in response to different environmental stimuli (27, 31, 52). Therefore not only does sRNA-mediated regulation of *rpoS* lend itself to rapid changes in abundance of the sigma factor, but these rapid changes can be incurred by various environmental stressors for which the cell would benefit from increased σ^S activity. There are also scenarios in which a single gene is regulated by several sRNAs, each of which is transcriptionally activated by different stress responses. A good example of this is the now well-studied curli regulatory gene *csgD,* which is regulated at the post-transcriptional level by OmrA, OmrB, GcvB, McaS, RprA, and RydC (53, 54). In this case *csgD* is post-transcriptionally repressed in response to osmotic stress (OmrA/B), nutritional stress (GcvB), and envelope stress (RprA), demonstrating that biogenesis of curli is not only tightly controlled by post-transcriptional regulation, but that this tight regulation can occur in response to a variety of environmental cues.

As alluded to above, the glutamate-dependent extreme acid stress resistance pathway encoded by the *gad* genes (GDAR) is under the control of at least two sRNAs. The GDAR pathway is the most powerful of four acid resistance pathways in *E. coli (55)*, and it relies on the conversion of exogenous glutamate to γ-butyric acid (GABA) by the GadAB decarboxylase enzyme, and the subsequent transport of GABA out of the cell through the antiporter GadC

(which also uptakes fresh glutamate into the cell) (56). Since the conversion of glutamate to GABA consumes one proton, this results in a net increase in cytoplasmic pH. The pathway is known to be regulated by at least three transcription factors: GadE, GadW, and GadX (57). GadE directly controls the transcription of *gadA, gadB* and *gadC (58)*, and GadW and GadX both activate the transcription of *gadE* as well as *gadA, gadB* and *gadC* (57). One of the GDAR regulon's sRNAs is GadY, which is a *cis*-encoded sRNA coded opposite the 3' UTR of *gadX* (upstream of *gadW*). Opdyke *et al*. (2004) demonstrated that overexpression of *gadY* causes accumulation of the *gadX* transcript, suggesting that expression of this sRNA increases stability of the *gadX* transcript (50). Later work revealed that *gadX* and *gadW* are in fact encoded in an operon, and GadY acts to induce RNase III cleavage of this short-lived transcript to produce the mature *gadX* and *gadW* transcripts which, in this way, accumulate to a much higher abundance and thus are expressed to a higher level (59). The second sRNA known to regulate the GDAR pathway is ArrS, whose overexpression was found to paradoxically decrease abundance of the full-length T3 transcript of the GDAR transcriptional regulator gene *gadE* while at the same time increase survival to pH 2 challenge (60). Further investigation revealed that ArrS binding to the 5' UTR of the full length T3 *gadE* transcript induces its cleavage by RNase III thereby processing it to the shorter T2 transcript which is then more readily translated by the ribosome, and this results in higher *gadE* expression and thus activity of the acid resistance pathway (60).

Interestingly, a large number of sRNAs modulate the expression of envelope-localized structures. Each major outer membrane porin gene in *E. coli* (*ompA, ompC,* and *ompF*) is regulated in part by sRNAs (7, 61, 62), which allows rapid changes in porin representation in the envelope in response to various inducing cues. Surface structures involved in motility and biofilm formation are inhibited by the sRNA McaS in response to changes in nutrient availability

(30), and numerous other sRNAs regulate early biofilm formation through their post-transcriptional control of *csgD*, which encodes the master regulator of curli, the thin amyloid appendages involved in cell-cell adhesion and early biofilm development (29, 43, 63). Nutrient transporters are also subject to regulation by sRNAs, such as GcvB which regulates several transporters (64) and SgrS which inhibits translation of the *ptsG* gene that encodes a glucose-specific phosphoenolpyruvate phosphotransferase (65). Whether or not there is in fact any pervasive evolutionary tendency for envelope localized structures to be regulated post-transcriptionally by sRNAs remains to be investigated, although the number of sRNA-regulated envelope genes suggests that this may be the case. From an evolutionary perspective this makes sense in pathogenic bacteria: many surface exposed structures are the targets of immune cells, phages, and colicins produced by competing bacteria, and therefore it's advantageous in this niche to be capable of rapidly removing these structures from the cell surface.

sRNAs also play an important role in the regulation of genes whose products occupy the cytoplasm, namely genes involved in metabolism. The most well-studied example of an sRNA that regulates metabolism is Spot42, which represses translation of galactose catabolism gene *galK* in glucose-rich conditions, but under glucose-poor conditions *spot42* is transcriptionally repressed by CRP-cAMP (66) and *galK* is allowed to be expressed. What makes Spot42 repression of *galK* ideal is that while *galK* (which encodes galactokinase) is not necessary in glucose-rich conditions, expression of *galE* and *galT,* which are encoded upstream in the same operon, is necessary even when the cell isn't consuming galactose, and translation of these genes is unaffected by Spot42 activity (67). More recent research has revealed a new subset of Spot42 target genes not involved in galactose metabolism, half of which are transcriptionally regulated by CRP-cAMP (67). One example of these is *fucI*, whose product is an L-fucose isomerase

required for fucose catabolism. *fucI* transcription is stimulated by activated CRP-cAMP and its translation is repressed by Spot42. Again, Spot42 itself is transcriptionally repressed by CRP-cAMP, and therefore in glucose-deplete conditions transcriptional inactivation of *fucI* is paired with translational repression, completing a coherent feedforward loop (68) and demonstrating a growing number of genes in bacteria pair transcriptional regulation with post-transcriptional regulation by an sRNA.

Another well-studied example of sRNA regulation of metabolism is RyhB, which is a key regulator of both iron storage as well as uptake in conditions of iron depletion (69). RyhB is among the large host of genes that are transcriptionally repressed by the master iron uptake regulator Fur bound to Fe^{2+} in iron-rich conditions, but is de-repressed under iron-poor conditions when Fur is unbound to its iron cofactor (70). RyhB then acts to combat iron starvation by repressing translation of a number of genes encoding non-essential iron-requiring proteins, notably the succinate dehydrogenase operon *sdhCDAB* and the NADH dehydrogenase operon *nuoA-N* (71). More recent research has demonstrated that RyhB positively regulates translation of two target genes: *shiA* and *cirA* (72, 73). The former encodes a transporter of shikimate, a precursor of the siderophore enterobactin, and the latter encodes a catechol siderophore receptor. Translation of both of these genes has been demonstrated to be inhibited by binding of Hfq, but stimulated upon binding by RyhB. In the case of *cirA*, evidence suggests that in the absence of RyhB, Hfq binds the *cirA* transcript near the site of translation initiation and occludes the 30S subunit of the ribosome, however in the presence of RyhB the sRNA binds the 5' UTR of *cirA* +15/+16 relative to the translation initiation region (TIR) and prevents Hfq from interfering with translation initiation (73). Since *cirA* transcription is repressed by Fur-Fe^{2+} in iron-rich conditions but activated by the Fur-repressed RyhB in iron-poor conditions, this is yet

another example of an sRNA participating in a coherent feed-forward loop to efficiently increase expression of *cirA* upon transcriptional de-repression by Fur. All in all, RyhB expression effectively increases intracellular iron levels by stimulating production of two siderophore receptors and by inhibiting production of non-essential iron-using proteins in order to free up iron for essential iron-using proteins.

Virulence is another process that appears to be heavily influenced by sRNA regulation in various bacterial species. In pathogenic *Vibrio cholerae* strains, both the major virulence transcription factor HapR, as well as the quorum sensing regulator LuxR are regulated by the four redundant sRNAs Qrr1-4 (40, 74). In *Salmonella*, the InvR sRNA is the first one in that species to be found on a pathogenicity island, and is thought to aid in the elaboration of the Type 3 secretion system by repressing the expression of another bulky envelope structure, OmpD (75). Importantly, *hfq* mutants in various pathogenic bacteria including *V. cholerae, P. aeruginosa, L. monocytogenes* and *L. pneumophila* exhibit reduced virulence when compared to wild-type (76-79), suggesting that sRNA regulation in these species is important for the expression of virulence determinants. Unsurprisingly, various sRNAs have been found to impact bacterial survival within a host cell as well (80), likely because the taxing requirements for bacterial survival within a host cell requires drastic alterations in gene expression.

1.2.7 Advantages of Small RNA Regulation

What advantages does regulation by an sRNA provide over regulation by a transcription factor? For one, sRNA genes are typically much smaller and therefore less energetically costly than protein-coding genes, and this, paired with the fact that they do not require translation into a

mature polypeptide allows them to produce their functional gene products much more quickly. Since the target molecule of the sRNA gene is an mRNA transcript, the regulation is also theoretically less leaky, because less of the target gene products will be produced once the regulator (the sRNA) reaches its target. Pairing transcriptional inhibition of a target by a transcription factor with post-transcriptional inhibition by an sRNA allows for even tighter regulatory control as well, because the transcription factor maintains more stable, long-term regulation while the sRNA prevents the translation of any transcripts which may have leaked through, and sometimes irreversibly represses translation by inducing degradation of the transcript by RNase E . Additionally, regulation of a target transcript by an sRNA will by its nature allow it to sometimes be epistatic over regulation of that same gene by a transcription factor, since action by the sRNA is downstream of that of the transcription factor (8). Another theory is that regulation by sRNAs may allow for a type of cross-talk wherein expression levels of a given sRNA or subset of sRNAs may affect the activity of other sRNAs within the cell as they each try to compete for binding by Hfq (81), although whether or not this dynamic is relevant under natural physiological conditions remains to be seen. Finally, it may be that regulation at the post-transcriptional level by sRNAs allows additional regulation of the target without complicating the promoter region by the addition of new transcriptional regulation. Boehm and Vogel have suggested that the 5' UTR of the *csgD* mRNA may function analogously to the complex *csgD* promoter at the post-transcriptional level through its regulation by RprA, OmrA and B, GcvB, and McaS (42).

1.2.8 Small RNA Identification

Identifying novel sRNA genes can be a challenge for a number of reasons. Principally they are much smaller than protein-coding genes, and have much lower sequence conservation between species, sometimes with only small segments of them being highly conserved (82). Whereas countless candidate protein-coding genes have been identified in *E. coli* simply by identifying highly-conserved ORFs in inter-genic regions in its genome, more in-depth bioinformatics analyses are required to identify potential sRNA genes. Identification of candidate sRNA genes is generally accomplished by looking for orphan σ^{70} promoters, rho-independent terminator sequences and stretches of sequence conservation between species (83). These types of analyses are, however, severely limited by the amount of sequence information that is available for whatever organism the research is being conducted on, and as such they are well suited to the needs of groups working with *E. coli*, *Salmonella,* or *P. aeruginosa,* for example. Trouble can arise, however, when sRNA genes have very low levels of sequence homology between species, and thus are not likely to be identified in bioinformatic screens. A novel approach to identifying such cryptic sRNA genes has been to use Hfq co-immunoprecipitation followed by tiling microarray analysis to pull down and identify sRNA species which interact with Hfq (84). This Hfq co-IP approach has even succeeded in identifying putative sRNA genes which reside in the 3' UTRs of other genes (11), a finding which raises the exciting possibility that there may be sRNAs embedded in genes with which they share a regulatory function (85). More research needs to be done to characterize this novel class of sRNAs; little is known about how, for example, these sRNAs interact with Hfq but the corresponding full-length transcript of the gene they are embedded in does not. Importantly, it would have been impossible to identify these particular sRNA genes by bioinformatic approaches because any sequence conservation at these regions would be attributed to the genes whose 3' UTRs the sRNAs reside in.

1.3 The Cpx envelope stress response

1.3.1 The Gram-negative Bacterial Envelope

In Gram-negative bacteria such as *E. coli* and *Salmonella*, the cell envelope is comprised of an inner phospholipid bilayer membrane and an outer membrane which is heavily modified with polysaccharides and lipoproteins to comprise the lipopolysacchararide layer. In between these two membranes is the space called the periplasm, which houses a thin layer of peptidoglycan and a plethora of proteins which function to monitor and maintain structures that reside in the envelope. These structures include flagella, pili, curli, secretion machineries and outer membrane porins, many of which serve crucial functions to the cell. Perturbations to the cell's envelope can therefore compromise the cell's viability, and thus detecting and responding to perturbations is of the utmost importance to the cell. There are a variety of stressors to the envelope that are detected by signaling systems called two-component signal transduction systems. Two-component systems, as is suggested by their name, are typically comprised of two parts: a dimeric inner-membrane localized sensor kinase with domains both in the periplasm (or sometimes only in the inner membrane) for signal sensing and in the cytoplasm for downstream signaling, and a dimeric cytoplasmic response regulator which, once activated by the sensor kinase via phosphate transfer, usually acts as a transcription factor to activate and repress the expression of genes in its target operons. Coordinated regulation of a two-component system's target genes following induction of the system generally results in an organized response to whatever danger to the cell the two-component system was sensing. One two-component system

that exists in a variety of Gram-negative bacteria is the CpxAR envelope stress response, which is thought to mainly detect and respond to the aggregation of misfolded proteins in the envelope, but is now known to detect some signals not associated with misfolded envelope proteins.

1.3.2 Discovery and Characterization of the Cpx Two-component System

The Cpx two-component signal transduction system (Figure 2) was identified in the early 1980's when a group discovered that mutations in two loci in the *E. coli* chromosome resulted in reduced elaboration of the conjugative F pilus (86), which they named *cpxA* and *cpxB* (conjugative plasmid expression A and B). The authors showed that mutations in *cpxA* indeed resulted in reduced levels of the pilus protein TraT (86). Later this same group discovered that mutations in *cpxA* and *cpxB* resulted in reduced OmpF porin levels (87), which led them to speculate that the Cpx gene products were involved in the regulation of envelope-localized structures. The first clue that the Cpx proteins may comprise a two-component system came in 1986 when the Silverman group determined the polypeptide sequence of CpxA which revealed that it bears high sequence similarity to the sensor kinase EnvZ of the EnvZ-OmpR two-component system (88). In 1993, the DNA segment upstream of *cpxA* was found to encode CpxR, the cognate response regulator of CpxA, which the authors determined bears significant homology to OmpR-family response regulators (89).

The role of the Cpx two-component system in detecting and responding to envelope stress was proposed by the Silhavy group after it was found that constitutively activated alleles of *cpxA* could suppress the toxic effects of certain LamB fusion proteins which are known to aggregate and cause damage in the periplasm (90). This suppression was found to be dependent

on the presence of a wild-type copy of the *degP* gene, which encodes a periplasmic serine protease (91) that had previously been shown to be under transcriptional control of the envelope stress sigma factor σ^E (92) and to be necessary for degradation of other periplasm-localized toxic fusion proteins (93). Cpx regulation of the *degP* gene was later confirmed by fusion gene reporter assays, making it the first characterized member of the Cpx regulon (94). Further work showed that expression of *dsbA,* which encodes a periplasmic disulfide bond oxidase required for proper folding of various envelope proteins (95) and *ppiA,* encoding a periplasmic peptidyl-prolyl cis/trans isomerase (96), are up-regulated by activated CpxR. Together the knowledge of this limited Cpx regulon led researchers to believe that this is a two-component system whose purpose is to alleviate envelope stress caused by the misfolding of envelope-localized proteins. Although the classical view of the Cpx envelope stress response is that the pathway is by and large responsible for the detection and amelioration of envelope stress caused by the misfolding/mislocalization of envelope-localized proteins, recent data suggest that additional signals might induce the pathway. A recent expression microarray done by our group has shown that while many of the genes deemed to be Cpx-regulated indeed encode protein folding and degradation factors, the regulon also contains a plethora of other genes that function in diverse physiological functions such as aerobic respiration, antibiotic resistance, nutrient transport, cell wall structure and post-transcriptional control of other regulatory circuits (97). Additionally, transcriptome analyses of the Cpx regulon in *Vibro cholerae* and *Haemophylus ducreyi* have revealed that Cpx regulation of *degP*, one of the principle envelope protein quality control agents of the Cpx regulon, is not well conserved among closely related bacteria (98, 99). This might suggest that the detection of misfolded envelope proteins is not the primary role for the Cpx response in Gram-negative, and that this may be an adaptation unique to *Escherichia coli*.

1.3.3 Signal Transduction of CpxA/R

CpxA and R constitute an EnvZ/OmpR-like two-component system (88, 89) and as such its signal transduction mechanism bears much resemblance to others in this family. Detection of an inducing cue by CpxA's conserved periplasmic loop is followed by a conformational change in the transmembrane HAMP linkers of the dimer (100) leading to autophosphorylation at a conserved histidine residue in the cytoplasmic domain (101). Once phosphorylated, CpxA loses its phosphatase activity and gains kinase activity, allowing it to phosphotransfer to its cognate partner CpxR at a conserved aspartate residue, at which point CpxR gains promoter binding ability and exerts its regulatory effects on target operons. In the absence of an inducing cue, CpxA exhibits phosphatase activity, maintaining CpxR in its inactive form (101). The mechanism by which phosphorylated CpxR (designated CpxR~P) gains promoter-binding ability upon phosphorylation is not well understood, however a consensus sequence for CpxR-binding has been generated based on DNA-footprint analyses of well characterized Cpx-regulated genes (102), and like other response regulators CpxR may either activate or repress transcription from a given promoter (103). In its basal state, it is thought that CpxA is bound and inhibited by the periplasmic protein CpxP, which is transcribed divergently from the *cpxRA* locus (104, 105). Through this action it is thought that CpxP maintains CpxA in its phosphatase form, thereby keeping CpxR de-phosphorylated and inactive (104). A direct interaction between CpxA and CpxP has yet to be proven, although this model is strongly favoured due to data which show that artificial tethering of CpxP to the inner membrane allows it to maintain its inhibitory function after spheroplasting, whereas a native, periplasmic CpxP loses its inhibitory function (106).

Furthermore, it has been speculated due to structural analyses of the CpxP and CpxA proteins that the CpxP dimer harbors a conserved highly basic concave face that may be responsible for interacting with a stretch of negatively charged amino acids in the periplasmic loop of CpxA (107). Additionally, mutations near a conserved LTXXQ motif localized at the poles of CpxP's bowl-like tertiary structure have been shown to abolish CpxP's ability to inhibit the pathway (107), making this region another possible candidate for direct interaction with CpxA. Release from inhibition by CpxP involves its degradation by the periplasmic protease DegP, which frees CpxA of CpxP and thus allows it to adopt its kinase form (108), and this has been shown to happen upon addition of various inducing cues such as alkaline pH and aggregation of Pap pilus subunits (108, 109). As much as this makes an appealing model for how inducing signals physically cause activation of CpxA, release from inhibition by CpxP is not actually sufficient for activation of CpxA. Indeed, deletion of *cpxP* does not induce the pathway to the same degree as induction by *nlpE* overexpression (110), thus it seems that either there are additional inhibitors of the Cpx pathway redundant with CpxP or that the biochemical mechanism of CpxA induction does not act solely by the release of CpxA from its inhibitor.

1.3.4 Signal Sensing by CpxA

The majority of signals detected by the Cpx system are input through CpxA, although the exact biochemical nature of signal detection by CpxA is not yet understood. It is well documented that various mutations designated as *cpxA** mutations, some of which affect a specific section of CpxA's periplasmic loop make the sensor kinase signal-blind and lock it in its kinase state (91, 101), suggesting that signal input does occur through this periplasmic loop.

Some point mutations within a 32 amino acid stretch located in the middle of the periplasmic loop have been shown to partially abolish signal detection by CpxA, although none of these mutations alone were sufficient for a complete signal-blind phenotype (Malpica and Raivio, in preparation). Again, while a direct interaction between CpxA and CpxP has not yet been shown, there is genetic evidence to suggest that inhibition of CpxA by CpxP requires this same periplasmic loop (104). CpxP itself presents another possible location of signal input, as conditions such as alkaline pH which induce degradation of CpxP by DegP lead to the pathway's activation (108), suggesting that once degradation by DegP stops and CpxP is allowed to accumulate, inhibition of the pathway would resume. Thus, CpxP may serve to adjust activity of the Cpx response in a negative feedback capacity (104) by monitoring the response to envelope stress through its interaction with DegP.

Another candidate for signal input is the outer membrane lipoprotein NlpE, whose overexpression has been shown to cause Cpx pathway activation in a manner dependent on the periplasmic loop of CpxA (90). While NlpE overexpression is used by researchers as an inducer of the Cpx pathway (97), signaling to CpxA by NlpE in normal physiological conditions is thought to occur upon surface adhesion by the cell (111). As with signaling by misfolded proteins, the exact mechanism of signaling to CpxA by NlpE remains somewhat enigmatic, although structural studies have suggested that signaling to CpxA may occur through a conformational change in the linker domain that separate NlpE's N and C terminal domains, allowing the C terminal domain to extend toward the inner membrane and thus potentially interact with CpxA's periplasmic domain (112). Each of these possible locations of signal input is located in the envelope and CpxA-dependent, although CpxA-independent activation of the pathway has been observed. Increased activation by CpxR~P of Cpx-regulated genes upon entry

to stationary phase was determined to be CpxA-independent (113). This effect could also be mimicked by exposing the cells to either excess levels of glucose or pyruvate, both of which are associated with late stationary phase. This was not found to be due to increased phosphorylation of CpxR by the cytoplasmic phosphate donor acetyl-phosphate, although is thought to involve the AckA/Pta pathway nonetheless (114).

1.3.5 Inducing Cues of the Cpx Pathway

The first physiological inducing cue discovered for the Cpx system was the aggregation of misfolded proteins in the envelope, although due to more recent studies it is thought that there may be other sources of signal input, all of which are depicted in Figure 1.3. The toxicity caused by LamB fusion proteins such as LamB-LacZ-PhoA (which are known to form aggregates in the periplasm (115)) was shown to be suppressed by mutational induction of the Cpx pathway. Furthermore, this phenotype was shown to be mediated by degradation of these fusion proteins through elevated expression of periplasmic proteases upon induction of the Cpx response (91, 94). Expression of pilus proteins PapE and PapG in the absence of their cognate chaperone protein causes their misfolding in the periplasm, and this too was found to be capable of inducing the Cpx pathway in a CpxA-dependent manner as measured by increased activity of *cpxP-lacZ* and *degP-lacZ* fusions (116). Subunits of *E. coli*'s bundle-forming pilus (Bfp) when overexpressed in a non-native strain background in which they likely lacked proper folding factors were also found to induce activation of the Cpx pathway (117). While the accumulation of each of these subsets of misfolded envelope proteins all cause activation of the Cpx response, there are yet other envelope proteins such as specific PapA fusions which, when overexpressed

and misfolded, do not cause activation of the pathway (118). This begs the question of whether there is a specific sequence or structure in these misfolded aggregates which is sensed by CpxA and/or its auxiliary factor CpxP. Deletion of the N-terminal region of PapE was found to abolish activation of the pathway upon overexpression of the protein, however the overexpression of this N-terminal region alone was not sufficient to activate the pathway (116). Thus, a hypothetical Cpx-detected peptide sequence or structure has not yet been determined as being capable of Cpx induction.

Alkaline pH is a condition which has frequently been used as an inducer of the Cpx pathway by researchers, although the exact mechanism by which this condition is sensed by the pathway is not well understood (105). The involvement of pH in the Cpx response was originally proposed after it was found that the Cpx pathway induces expression of the *virF* virulence gene in *Shigella sonnei* in more alkaline conditions (119). It was subsequently found that both *cpxR* and *cpxP* mutants exhibited heightened sensitivity to alkaline conditions, and that a change in pH from 7 to 8 was sufficient to induce an increase in activity from a *cpxP-lacZ* reporter (105). It is thought to be unlikely that CpxA is sensing changes in pH directly; a more likely explanation is that the elevated pH induces damage and/or misfolding to envelope-localized proteins which in turn are sensed by CpxA (105). Disulfide bonds are known to be oxidized above a pH of 8 (120), thus one explanation could be that the alkaline conditions cause alterations in the secondary structure of envelope localized proteins through the oxidation of important disulfide bonds, and that this is sufficient to cause induction of the Cpx response. This theory is supported by data which show that the Cpx pathway causes the upregulation of a variety of protein folding factors and proteases.

As mentioned above, overexpression of the outer membrane lipoprotein NlpE induces the Cpx pathway in a CpxA-dependent manner (90). It is thought that NlpE may play a role in the activation of the Cpx response which occurs upon surface adhesion because deletion of *nlpE* causes an attenuation of Cpx induction upon adhesion to hydrophobic glass beads (111). In agreement with this finding, deletion of either *cpxR* or *nlpE* causes a reduction of surface attachment by cells, suggesting that there may be components of the Cpx regulon which are responsible for stable surface attachment, and therefore absence of Cpx induction causes a reduction in attachment (111). Another explanation for these data may be that surface adhesion causes an increase in envelope protein misfolding and stress, and therefore a working Cpx pathway is required in a damage-control capacity during surface adhesion by maintaining important envelope components during this stressful event through the upregulation of the protein folding factors DegP, DsbA and PpiA, etc. (111).

Since in *Escherichia coli* the Cpx two-component system detects and responds primarily to perturbations which cause the misfolding of envelope proteins, it was long hypothesized that this function would hold true in closely related organisms. Instead, recent transcriptomic work has revealed that in at least two other gammaproteobacteria, *Haemophilus ducreyi* and *Vibrio cholera*e, the Cpx pathway may in fact play a different role. The Cpx pathway in *Haemophilus ducreyi* influences the transcription of various virulence factors (99) but does not affect expression of the major protein folding and degradation factors known to play a role in amelioration of envelope stress in *E. coli* (121). The overlap that exists between the Cpx regulon in *E. coli* and that in *H. ducreyi* is one of target protein localization; in both organisms the pathway represses production of bulky, energy hungry envelope localized structures such as virulence factors, and indeed, the Cpx pathway in enteropathogenic *E. coli* (EPEC) represses

production of virulence determinants such as the bundle-forming forming pilus (BFP) (117). It is also of note that in *H. ducreyi,* as in *E. coli,* induction of the Cpx response reduces the expression of a subset of nutrient transporters and metabolism genes, supporting the concept that the Cpx pathway might have a conserved role in the regulation of energy production. The Cpx response in *Vibrio cholerae* on the other hand is somewhat diverged from that of *E. coli* in terms of its major inducing cues and target operons. The pathway in this organism has been found to be induced by excess chloride ions (122) and iron depletion (98) rather than alkaline pH or overexpression of the outer membrane lipoprotein *nlpE,* although some evidence suggests that aberrant disulfide bonds might activate the pathway in *V. cholerae* (122). Interestingly, Acosta *et al.* demonstrated that induction of the Cpx response in *V. cholerae* by excess chloride ions could in fact be suppressed by addition of exogenous iron sulfate, strengthening the idea that the Cpx pathway might primarily be responsible for the sensing of iron deficiency-related stresses in this organism (98). Indeed, a transcriptomic approach determined that the Cpx regulon in *V. cholerae* contains a variety of genes encoding proteins involved in siderophore biosynthesis and transport (98). Finally, deletion of two TolC-dependent efflux pumps or TolC itself also significantly induced the Cpx pathway in *V. cholerae,* and again this could be suppressed by addition of exogenous iron (98). This last finding is particularly interesting because while the Cpx pathway in *E. coli* is not induced by iron depletion, deletion of TolC does induce pathway activity (unpublished data), raising the exciting possibility that a substrate of a TolC-dependent efflux pump might signal to the Cpx pathway in both organisms. While the Cpx pathway in *E. coli* has not been found to detect perturbations to iron homeostasis, interestingly it has been found to be induced by zinc depletion (Wong and Raivio, in preparation), suggesting perhaps a wider role for the Cpx pathway in the detection of metal limitation.

1.3.6 Target Operons of the Cpx System

Along with the classic Cpx targets which encode envelope protein folding factors and proteases, the Cpx response is now known to modulate the expression of an expansive number of genes whose products function in a variety of cell systems that involve structures localized to the cell's envelope (although many non-envelope localized proteins are also Cpx-regulated). Activation of the Cpx system leads to a concerted regulatory response which results both in the direct amelioration of envelope stress through the destruction or repair of misfolded envelope subunits as well as the decreased elaboration of envelope structures whose presence hinders the return to envelope homeostasis. Together these actions remedy whatever damage was sensed by the system and allow the cell to survive and grow in the presence of stressful conditions.

As mentioned earlier, activation of the Cpx response results in a strong increase in expression of a number of envelope protein folding and degradation factors, namely DegP (94, 123), DsbA (95), PpiA (96), and the chaperone Spy (106, 124). DegP is known to induce the degradation of pilin subunits (125), which is consistent with the findings that overexpression of select Pap subunits causes induction of the Cpx response (116). In addition to its protease activity DegP has also been shown to exhibit chaperone activity at higher temperatures, increasing the stability of the malodextrin amylase MalS (123) as well as aiding in correct folding of bundle forming pilus subunits in enteropathogenic *E. coli (126)*. DsbA is responsible for catalyzing the proper formation of disulfide bonds of envelope-localized proteins (127), and PpiA is a peptidyl-prolyl cis/trans isomerase involved in proper envelope protein folding (96). The protein Spy is a periplasmic chaperone which bears a high degree of homology with CpxP

and is highly up-regulated upon induction of the Cpx response (106). Finally, it has been shown that in addition to its inhibitory role in the Cpx pathway, CpxP is also capable of acting as protease adapter by enhancing the binding and degradation of misfolded PapE and PapG by DegP (109), and that it may exhibit chaperone activity in the context of bundle-forming pilus biogenesis (128). Through the up-regulation of these proteins, the Cpx response alleviates envelope stress caused by the accumulation of misfolded proteins both by causing their degradation and by reducing the degree of protein misfolding.

As previously mentioned, a recent microarray conducted in our lab revealed a host of previously unknown Cpx-regulated genes (97). Among the affected genes were three cell wall modification genes: *ygaU, ldtD* and *slt,* respectively encoding a hypothetical protein with a LysM domain associated with cell wall hydrolysis enzymes, an L,D-transpeptidase which synthesizes DAP-DAP crosslinks in peptidoglycan, and a lytic transglycosylase which cleaves between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues in peptidoglycan. This finding raised the exciting possibility that the Cpx pathway might enact changes in peptidoglycan structure in conditions of envelope stress in order to help cope with damage to the envelope. Indeed, Bernas-Cabal *et al.* demonstrated that all three of these genes were transcriptionally upregulated upon induction of the Cpx response, and that this regulation was most likely through direct binding by CpxR (129). Interestingly, it was also found that induction of the Cpx response causes changes in peptidoglycan composition, while deletion of the response regulator *cpxR* did not have a significant impact, suggesting that induction of the Cpx response causes adaptation in the form of peptidoglycan structure changes, but basal Cpx pathway activity does not play a role in the maintenance of the cell wall. While direct biochemical evidence that perturbations to the cell wall induce the Cpx pathway have yet to be

demonstrated, there is some indirect genetic evidence to suggest such an interaction might exist. Evans *et al*. found that simultaneous deletion of four penicillin binding proteins (PBPs) was sufficient to induce activity of the Cpx pathway in addition to the Rcs phosphorelay (130). This group was not, however, able to identify the peptidoglycan constituent responsible for signaling to either pathway, and thus the biochemical mechanism of this signaling remains elusive.

In addition to up-regulating the expression of various chaperones and proteases, the Cpx system has long been known to repress the expression of genes involved in swarming and swimming motility as well as chemotaxis (103), and recently it has been shown that Cpx induction causes more than a two-fold decrease in the expression of *flhC*, which encodes one half of the master flagella biosynthesis regulator $FlhC_2D_2$ (97), thereby reducing elaboration of flagella indirectly. Curli, which are the thin amyloid fimbrae produced on the cell's surface in early biofilm formation and cell-cell adhesion events (131) are repressed during Cpx induction as well, both directly by the active CpxR~P (46, 132) and indirectly by the Cpx-regulated sRNA RprA and through its activation of the OmrA and B sRNAs by activating the EnvZ/OmpR two-component system (97).While the purpose for the down regulation of both the processes of motility and biofilm formation is unclear, it may simply involve a general reduction of bulky, energy-dependent structures in the envelope during a time of stress. Flagellar motility for example is highly dependent on the proton motive force, therefore a decrease in the activity of flagella in a time of envelope stress may serve to shift energetic resources away from the function of motility in order to return the cell to a state of energetic homeostasis.

Furthermore, recent microarray analyses have shed considerable light on previously unknown members of the Cpx regulon (97). These experiments have shown that a large number of inner membrane transporters have their expression repressed upon induction of the Cpx

response, including an iron transporter, multiple sodium transporters and peptide transporters. Another interesting finding was that a number of genes whose products are involved in electron transport, the TCA cycle and oxidative phosphorylation are down-regulated upon induction of the Cpx response. The inhibition of aerobic respiration functions potentially influences antibiotic resistance. CpxA* mutants have long been known to exhibit resistance to the aminoglycoside antibiotic amikacin (91) although the mechanism of this increase in resistance has remained somewhat unclear. One hypothesis is that Cpx upregulation of the gene *yccA*, which encodes an inhibitor of the protease FtsH, is the cause of this amikacin resistance. Since aminoglycosides act by interfering with translation through the Sec translocon, which in turn causes degradation of Sec translocon componenets by FtsH, increased expression of *yccA* is capable of partially counteracting the toxicity mediated by aminoglycosides by repressing FtsH (133). Cpx regulation of *yccA* may not account entirely for this resistance phenotype, however. Deletion of several newly discovered Cpx-repressed genes, including cytochrome oxidase, NADH dehydrogenase and succinate dehydrogenase (all of which are components of the electron transport chain) led to increased resistance to treatment with amikacin and hydroxyurea (97). Resistance to aminoglycoside antibiotics like amikacin can be mediated in (broadly) two ways: factors which reduce the proton gradient across the inner membrane, and factors which reduce translocation of newly translated proteins across the Sec translocon (134). Repression of NADH dehydrogenase and succinate dehydrogenase could in fact reasonably be assumed to reduce both cellular proton motive force (PMF) as well as protein secretion to the envelope, as their participation in the electron transport chain shuttles protons into the periplasm, and they themselves are highly abundant proteins which require translocation through the Sec translocon. Whether or not the amikacin resistance endowed upon Cpx pathway induction is dependent

solely on its regulation of these genes and the exact mechanism of this resistance are still to be determined.

1.4 Research on the small RNAs in the Cpx Regulon

Transcriptomic data have also revealed that the expression of two sRNA genes is directly altered upon induction of the Cpx response (97). These are *cyaR* and *rprA*, and between them they regulate at the post-transcriptional level a variety of genes involved in various cellular processes, although they both regulate at least one envelope-localized structure. CyaR, which is transcriptionally activated by the catabolite repression regulator CRP-cAMP (135), was originally identified in 2008 as a regulator of the small outer membrane porin gene *ompX* (136) and since then it has also been implicated in the regulation of the quorum sensing gene *luxS* (137) as well as the NAD synthase gene *nadE* (137). RprA was originally identified in a screen for multicopy suppressors of a *dsrA* mutant that would restore *rpoS* translation (27) in the absence of DsrA. Transcription of *rprA* is dependent on the RcsBCD phosphorelay system (27) and interestingly the stability of RprA has been found to be osmolarity-dependent (138), which is consistent with its regulation by the Rcs pathway which responds to changes in osmolarity. RprA is now known to enhance the translation of the stationary phase sigma factor σ^S (27), as well as repress the translation of both the master curli transcription factor gene *csgD* and an associated adenylate cyclase enzyme *ydaM* (29). More recent work however has suggested a role for RprA in the response to acid stress, insofar as its overexpression leads to improved survival to extreme acid challenge (139). This phenotype was found to be dependent on *gadX*, which encodes a

transcriptional activator of the glutamate-dependent acid resistance pathway (GDAR), although more work needs to be done to characterize the exact mechanism of this regulation.

The focus of this research is the characterization of both the Cpx regulation of these two sRNA genes (*rprA* and *cyaR*), and the effect that these genes might have on the Cpx regulon or on activity of the pathway itself (Figure 1.4). A previous student in the lab, Stefanie Vogt, constructed transcriptional *lux* fusion reporters of the reporters of both of these genes in the pJW15 plasmid (Table 1), and using these, regulation of *rprA* and *cyaR* by the Cpx pathway observed in our microarray (97) was confirmed upon induction of the Cpx response by overexpression of *nlpE* or by mutational induction of the response. *cyaR* transcription was repressed ~2-fold in the *cpxA24* background, and *rprA* transcription was activated more than 5-fold upon Cpx induction by *nlpE,* and this activation was contingent on a functional copy of *rcsB*. While CyaR was not found to have any effect on the Cpx pathway, overexpression of *rprA* was found to cause a ~2-fold repression of the pathway, forming a negative feedback loop. This repression was found not to involve direct repression through translational inhibition of either *cpxR* or *cpxA*, as accumulation of their respective protein products was not affected. Interestingly, repression was found to be independent of CpxA entirely but dependent on CpxR, suggesting that RprA is not inhibiting the pathway by affecting an envelope-localized signal sensed by CpxA, but rather is modulating activity of CpxR. The inhibition was also not found to involve changes in acetyl phosphate, as deletion of the pathway responsible for production of acetyl phosphate (*ackA ptA*) did not abrogate RprA's ability to inhibit the Cpx pathway. Finally, repression of the response was found not to be dependent on any of RprA's known target genes, but at least somewhat dependent on a functional copy of *gadE*, which encodes the transcription factor responsible for activation of the glutamate dependent acid resistance pathway (GDAR).

This led us to propose that RprA might repress the Cpx response by positively regulating the GDAR pathway through the *gadE* gene, which in turn has an inhibitory effect on CpxR. Concurrent with this hypothesis, *rprA* overexpression was found to significantly increase activity of a *PgadE::lux* transcriptional fusion, but strangely was not found to increase abundance of the *gadE* transcript as seen by qRT-PCR experiments. Another complication is the fact that overexpression of *gadE* by itself is not sufficient to replicate Cpx pathway repression by RprA. Whether or not RprA's effect on *gadE* transcription is dependent on either σ^S, which is known to activate the GDAR pathway, or *gadX*, with which RprA has been proposed to interact (139), has yet to be determined. Also yet to be determined is the mechanism by which an increase in GDAR pathway activity might inhibit activity of the CpxR response regulator. Overexpression of *rprA* was also found to increase amikacin resistance, which is a phenotype long associated with constitutive activation of the Cpx response (91), and to decrease motility, consistent with recent observation that Cpx pathway induction represses transcription of the flagella regulatory genes *flhDC*. Together these results suggest that RprA may promote at least two known Cpx-related phenotypes and act as a feedback inhibitor of the Cpx response by modulating activity of CpxR, perhaps through regulation of the GDAR pathway.

1.5 Figures

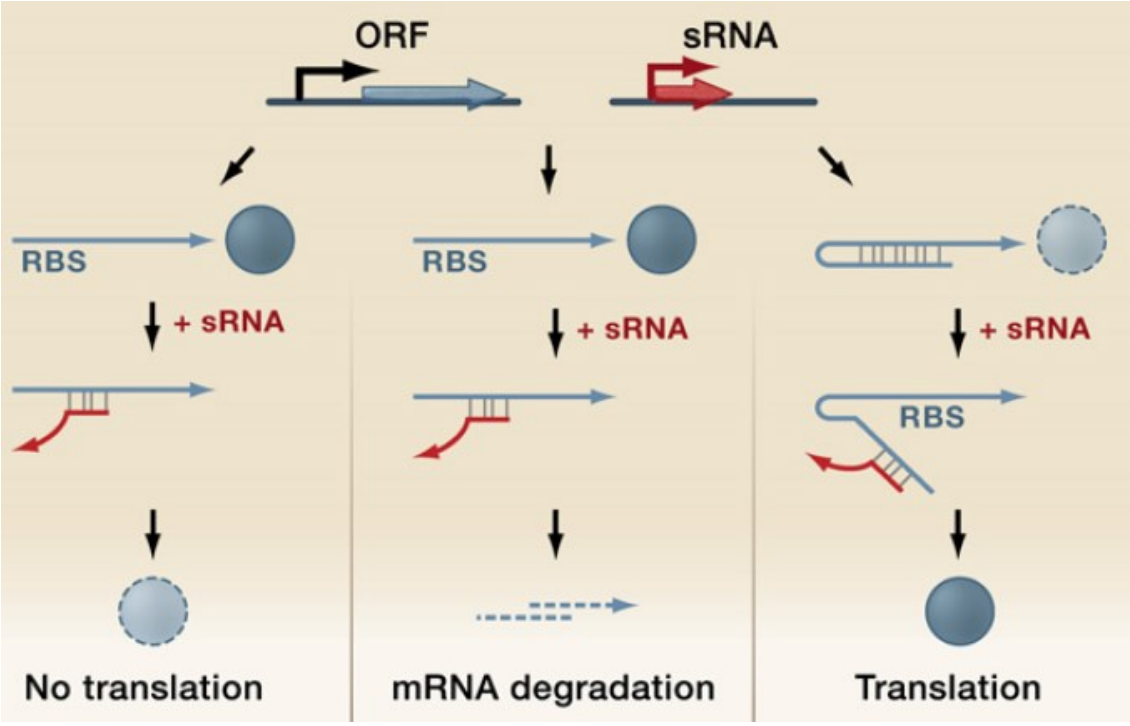

Figure 1.1: General mechanisms of regulation by *trans*-encoded sRNAs. Binding usually results in the occlusion of the RBS from the ribosome and/or the induction of degradation of the transcript by RNases (negative regulation) or the de-sequestration of the RBS by a secondary structure upon sRNA binding (positive regulation). Figure adapted from Waters and Storz (2009).

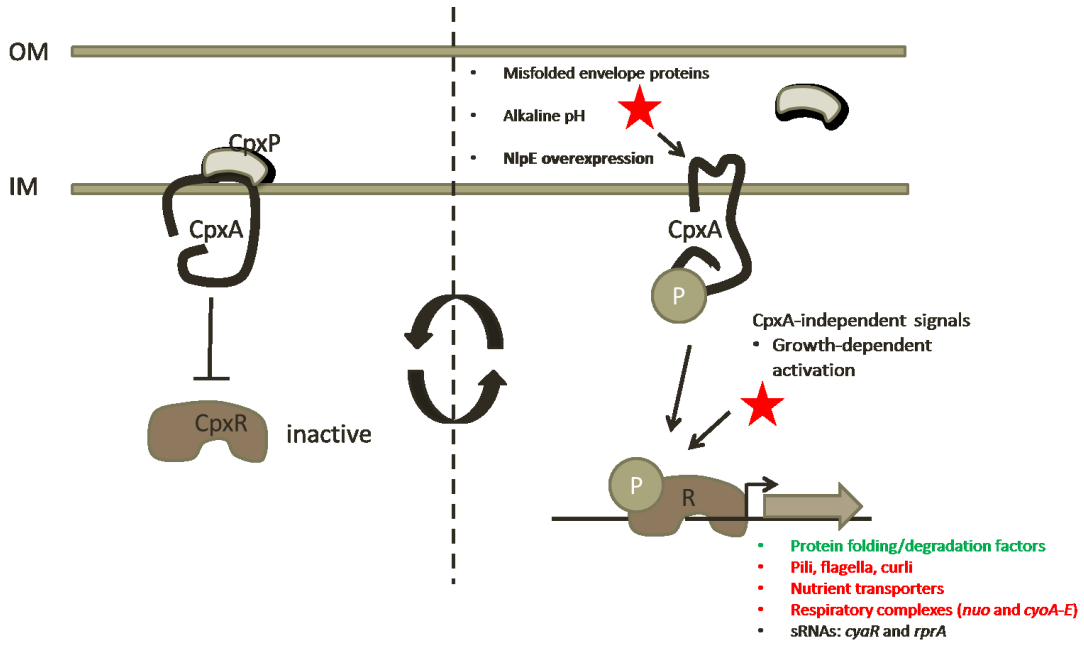

Figure 1.2: An abbreviated view of the Cpx envelope stress response. Induction of the system leads to the phosphotransfer to CpxR by CpxA and subsequent regulation of a number of genes encoding protein folding and degradation factors as well as envelope structures and sRNA regulators.

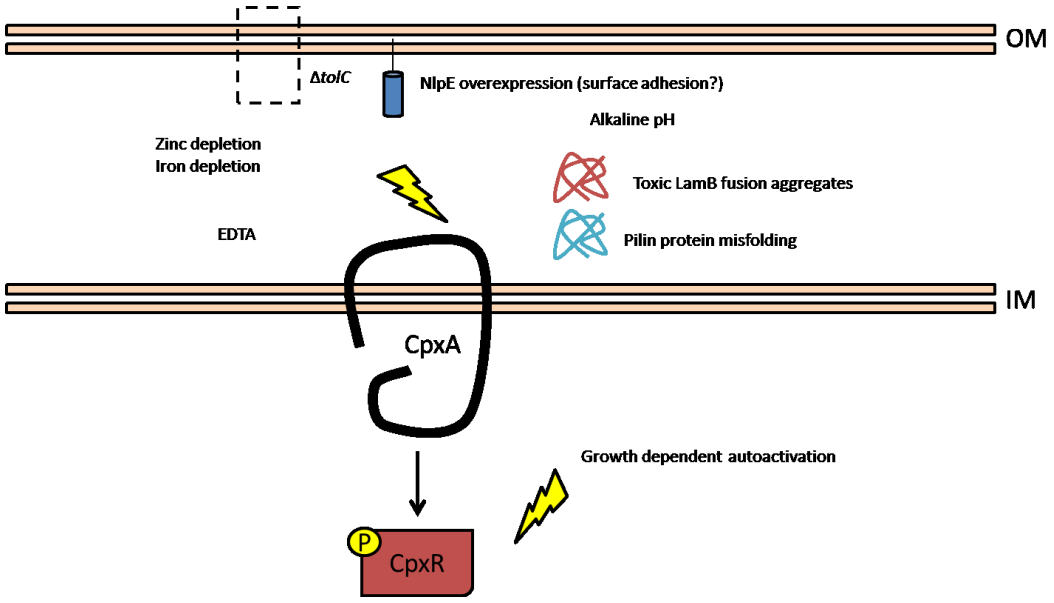

Figure 1.3: Inducing cues of the Cpx envelope stress response. The Cpx pathway is activated by a wide range of physiological signals including expression of pilin proteins in the absence of their cognate chaperone, tripartite fusion expression, alkaline pH, *nlpE* overexpression, deletion of the efflux component TolC, zinc/iron depletion, and EDTA. The majority of these signals are thought to cause the misfolding of envelope proteins.

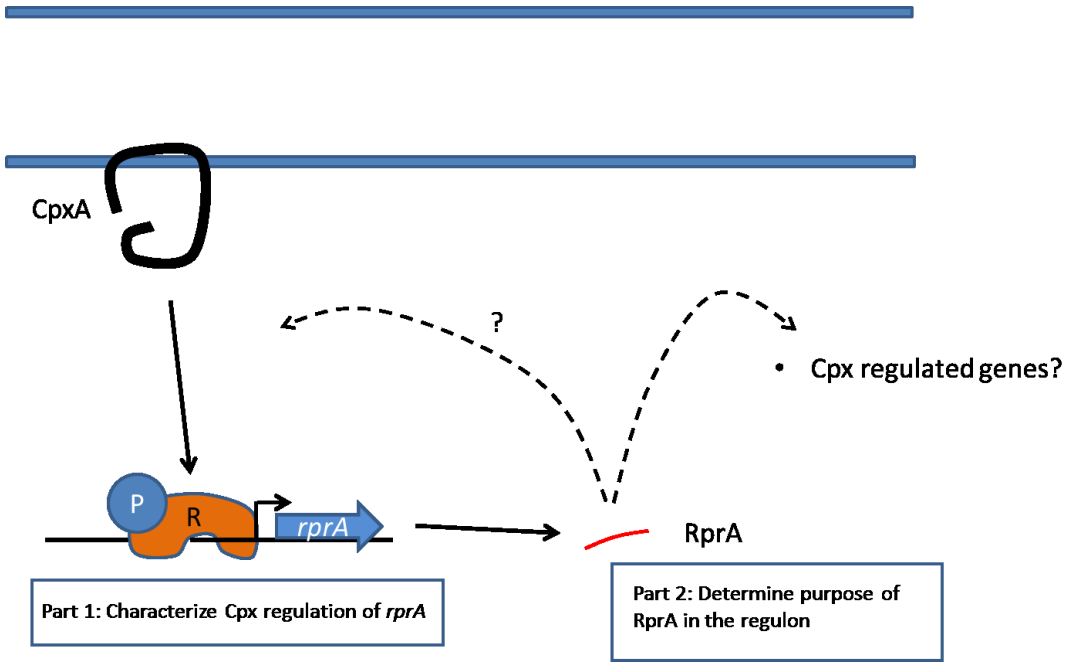

Figure 1.4: Research goals of project. This project set out to define the regulation of *rprA* (as well as *cyaR*) by the Cpx envelope stress response, as well as define the role of RprA in the Cpx regulon, whether it be to act as a feedback regulator of the Cpx response or to regulate Cpx-related phenotypes.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Media and Growth conditions

Bacteria were grown in Luria-Bertani broth (LB) containing the following ingredients: 1% tryptone (BD), 0.5% NaCl, 0.5% Bacto-yeast extract (BD), and 1.5% Agar (BD). Cultures were grown aerobically at 37°C while shaking at 225 rpm unless temperature sensitive, in which case they were grown at 30°C. Plated cultures were grown aerobically at 37°C or 30°C in a standing incubator. If relevant, broth contained 30 μg/mL kanamycin, 50 μg/mL spectinomycin, 25 μg/mL chloramphenicol, 1 μg/mL amikacin or 100 μg/mL trimethroprim.

2.2 Reporter Genes

β-galactosidase reporter genes were in single copy on the chromosome, integrated at the λRS88 site. Unless otherwise stipulated, these transcriptional reporters contained DNA from roughly 200 bp upstream of the transcriptional start site to 20 codons within the coding region of the gene. Luciferase reporter genes were constructed in the low copy-number, kanamycin-resistant plasmid pSC101-derivative plasmid pJW15, which contained the *lux* operon in the multiple cloning site. These reporters, again, contained DNA from roughly 200 bp upstream of the transcriptional start site to 20 codons with the coding region. GFP reporter genes were constructed in the high copy-number, chloramphenicol-resistant plasmid pXG10, which contains a *gfp* ORF in the multiple cloning site. These reporters contaed DNA from the transcriptional start site to 20 codons within the coding sequence, and this sequence was inserted downstream of a P*tet*^{O} constitutively active promoter and upstream of the *gfp* ORF.

2.3 Preparation of P1-*vir* lysates

Overnight cultures of donor strains were made by inoculating 5 mL of LB broth containing 30 μg/mL kanamycin, 50 μg/mL spectinomycin, 25 μg/mL chloramphenicol, 1 μg/mL

amikacin, 100 µg/mL trimethroprim or no antibiotic with single colonies of *E. coli* donor bacteria then cultures were incubated overnight at 37°C while shaking at 225 rpm. The next day donor bacteria were subcultured 1:50 into 5 mL fresh LB medium containing 0.2% glucose and 5 mM $CaCl_2$ and were then grown for roughly 30 minutes at 37°C while shaking at 225 rpm. 100 µL of P1 vir lysate made from *E. coli* MC4100 was then added and the cultures were incubated at 37°C while shaking for an additional 2 hours or until the samples had cleared entirely. 100 µL of chloroform was then added and the tubes were vortexed vigorously for 10 seconds, then the lysates were centrifuged at 4000 rpm for 10 minutes to pellet cell debris. Roughly 3 mL of supernatant was then carefully transferred to sterile screw-capped tubes, to which 100 µL of chloroform was added. Lysates were then either immediately used for transduction into recipient bacteria or stored at 4°C.

2.4 P1-*vir* mediated transduction

Overnight cultures of recipient bacteria were made by inoculating 5 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 25 µg/mL chloramphenicol, 1 µg/mL amikacin, 100 µg/mL trimethroprim or no antibiotics with single colonies of *E. coli* recipient strain, then cultures were incubated overnight at 37°C while shaking overnight at 225 rpm. The next day overnight cultures were centrifuged at 4000 rpm for 10 minutes then resuspended in 2.5 mL dH_2O containing 5 mM $CaCl_2$ and 10 mM $MgSO_4$. 100 mL of recipient cell solution was then mixed with 100 mL of donor P1 *vir* phage lysate (or 10^{-1} dilutions of donor P1 lysate) and was incubated at 30°C for 30 minutes without shaking. 1 mL of fresh LB medium containing 10 mM citrate was then added to the solutions which were then incubated for an additional 45 minutes at 37°C without shaking. Cell solutions were then centrifuged at 4000 rpm for 10 minutes then resuspended in 100 µL 1M citrate. 100 µL of cell

solutions were then plated on selective media, and plates were incubated at 37°C overnight (or 30°C for *cpxA24* strains). The next day single colony isolates were re-struck onto fresh selective media and grown overnight at 37°C to confirm resistance to ensure isogeneity.

2.5 Plasmid isolation

Isolation of all plasmids referenced was done using a Sigma-Aldrich GenElute™ Plasmid Miniprep kit (Sigma-Aldrich) according to its published protocol. 5 mL of overnight culture containing plasmid of interest was resuspended in 200 µL of Lysis Solution by inverting 6-8 times. The solution was then neutralized with 350 µL of Neutralization/Binding Solution and mixed by inverting 4-6 times. The solution was then centrifuged at 12,000g for 10 minutes. The column was then prepared by addition of 500 µL Column Preparation Solution and was then centrifuged at 12,000g for 1 minute. The cell lysate was then added to this column which was then centrifuged at 12,000g for 1 minute. The flow-through was then discarded and 750 µL Wash Solution was added to the column, which was centrifuged at 12,000g for 1 minute. The flow-through was discarded and the column was centrifuged once more at 12,000g for 1 minute. Finally the DNA was eluted with 50 µL milliQ H_2O by centrifuging at 12,000g for 1 minute into a fresh tube. Plasmid DNA was stored at -20˚C.

2.6 Calcium chloride-mediated transformation

Overnight cultures of recipient strain bacteria were made by inoculating 5 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 25 µg/mL chloramphenicol, 1 µg/mL amikacin, 100 µg/mL trimethroprim or no antibiotics with single colonies of recipient strain *E. coli* and were grown at 37°C while shaking overnight at 225 rpm. The next day recipient bacteria were subcultured 1:50 into fresh LB medium containing

relevant antibiotics and grown to an OD_{600} of 0.2 (roughly 1.5 hours). Cultures were then centrifuged at 4000 rpm for 10 minutes, resuspended in 1 mL of Magic Formula (0.1M $CaCl_2$, 0.1M MOPS in dH_2O), then let to sit on ice for at least 30 minutes. Cultures were again centrifuged for 10 minutes at 4000 rpm, then were resuspended in 200 µL fresh Magic Formula. 1-5 µL of plasmid DNA was then added to the cultures, which were then allowed to sit on ice for an additional 10 minutes. Cultures were then heat-shocked at 42°C for 30 seconds then 1 mL of fresh LB was added to each tube. Cell mixtures were then allowed to recover from heat shock and express newly acquired resistance genes for 1 hour at 37°C while shaking at 225 rpm. Following recovery, 100 µL of each mixture was plated on selective media and incubated overnight at 37°C (or 30°C for *cpxA24* strains). The next day single colony isolates were re-struck onto fresh selective media and grown overnight at 37°C to confirm resistance to ensure isogeneity.

2.7 Electroporation-mediated transformation

Overnight cultures of recipient strain bacteria were made by inoculating 50 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 25 µg/mL chloramphenicol, 1 µg/mL amikacin, 100 µg/mL trimethroprim or no antibiotics in a 250 mL Erlenmeyer flask with single colonies of recipient strain *E. coli* and were incubated at 37°C overnight while shaking at 225 rpm. The next day recipient bacteria were subcultured 1:50 into fresh LB medium in a new Erlenmeyer flask containing relevant antibiotics and were grown at 37°C while shaking at 225 rpm to an OD_{600} of 0.5 (roughly 1.5 hours). Cultures were then transferred to sterile 50 mL conical tubes and were centrifuged at 4000 rpm for 5 minutes, then were resuspended in 2 mL of fresh LB, then transferred to sterile microfuge tubes (1 mL per tube). These tubes were then heat-shocked at 50°C for 30 seconds in a water bath, then

immediately put on ice for 2 minutes. Cultures were then centrifuged at 13,200 rpm for 1 minute, the supernatant was aspirated, and the pellets were washed with 1 mL ice-cold glycerol. This wash step was then repeated three times: once with 1 mL cold glycerol, then with 500 µL cold glycerol, then with 200 µL. Cells were centrifuged once more at 13,200 rpm for 1 minute, and were resuspended in 200 µL cold 10% glycerol. 50 µL of solution was then transferred to a 500 µL microfuge tube then 1-5 µL of plasmid DNA was added. This solution was then quickly transferred to a sterile 2 mm electroporation cuvette, which was then placed on ice for 5 minutes. The cuvette was then placed in the BioRad electroporation chamber and electroporated on EC2 setting. To each cuvette was then added 1 mL fresh room temperature LB broth, and this cell solution was then carefully transferred to a glass culture tube and recovered by incubating at 37°C while shaking at 225 rpm for 1 hour. 100 µL of each electroporation culture was then plated on selective media and incubated at 37°C overnight (or 30°C for *cpxA24* strains). The next day single colony isolates were re-struck onto fresh selective media and grown overnight at 37°C to confirm resistance to ensure isogeneity.

2.8 β-galactosidase assays

Overnight cultures were made by inoculating 2 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 25 µg/mL chloramphenicol, 1 µg/mL amikacin, 100 µg/mL trimethroprim or no antibiotics with single colonies of *E. coli* strains to be assayed and were grown at 37°C while shaking overnight at 225 rpm. The next day bacteria were subcultured 1:50 from overnight cultures into fresh LB medium containing the same concentrations of relevant antibiotics, and were grown at 37°C (or 30°C for *cpxA24* strains) while shaking at 225 rpm for 5 hours unless otherwise specified. If bacteria being assayed harboured an arabinose-inducible or IPTG-inducible expression plasmid, expression was induced

by addition of 0.2% arabinose or 0.1 mM IPTG after three hours of growth unless otherwise specified. Subsequently cells were centrifuged at 4000 rpm for 10 minutes and resuspended in 2 mL 1X Z-buffer (0.03M $Na_2HPO_4 \cdot 7H_2O$, 0.02M $NaH_2PO_4 \cdot H_2O$, 5 mM KCl, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.27% β-mercaptoethanol in dH_2O) at which point 250 µL of each culture was transferred to a 96-well plate and the OD_{600} was measured with the Wallac Victor^2 Multilabel counter plate reader (Wallac). The remaining cells were then lysed with 20 µL 1% SDS and 40 µL chloroform and allowed to sit for 10 minutes. 5 µL of each culture was then transferred to 150 µL 1X Z-buffer in a 96-well plate (195 µL for *PcpxP::lacZ* strains), then 50 µL 10 mg/mL ONPG was added to each well and the A_{420} was measured with the plate reader. β-galactosidase activity was calculated in Miller units by the formula:

$$\text{Specific activity (Miller units)} = (A_{420} \cdot 60{,}000) / OD_{600}$$

or

$$\text{Specific activity} = (A_{420} \times 600{,}000) / OD_{600} \text{ for } \textit{PcpxP::lacZ} \text{ strains}$$

Activity values represented in these assays are averages of three biological replicates, and error bars represent the standard deviation between these replicates. Each experiment was repeated at least once and representative results are presented.

2.9 Luciferase assays

Overnight cultures were made by inoculating 2 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 1 µg/mL amikacin, 25 µg/mL chloramphenicol, 100 µg/mL trimethroprim or no antibiotics with single colonies of *E. coli* strains to be assayed and were grown at 37°C while shaking overnight at 225 rpm. The next day bacteria were subcultured 1:50 from overnight cultures into fresh 200 µL LB medium containing

the same concentrations of relevant antibiotics in a 96-well luciferase plate, and were grown at 37°C (or 30°C for *cpxA24* strains) while shaking at 225 rpm for 4 or 6 hours. If bacteria being assayed harboured an arabinose-inducible or IPTG-inducible expression plasmid, expression was induced by addition of 0.2% arabinose or 0.1 mM IPTG two hours before reporter gene activity measurement unless otherwise specified. Activities of the transcriptional luciferase reporter (CPS) as well as cell density (OD_{600}) were then measured using the Wallac Victor^2 Multilabel counter plate reader (Wallac). Luminescence values for each culture were then calculated as follows:

Specific activity(X) (AU) =

(luminescence(X) – luminescence (blank)) / (OD_{600}(X) – OD_{600}(blank))

Activity values represented in these assays are averages of three biological replicates, and error bars represent the standard deviation between these replicates. Each experiment was repeated at least once and representative results are presented.

2.10 Preparation of whole-cell lysates

Overnight cultures of strains to be assayed were prepared by inoculating 5 mL of LB medium containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 1 µg/mL amikacin, 25 µg/mL chloramphenicol, 100 µg/mL trimethroprim or no antibiotics with single colonies of *E. coli* and were incubated at 37°C overnight while shaking at 225 rpm. The next day bacteria were subcultured 1:50 into fresh LB medium containing relevant antibiotics and were grown at 37°C while shaking at 225 rpm for 5 hours unless otherwise stated. If necessary, cultures were induced with 0.1 mM IPTG two hours before culture collection. OD_{600} of cultures were then determined and an equivalent amount of cells equaling that of 1 mL of the

least-dense culture was then transferred to a sterile microfuge tube, which was then centrifuged at 13,200 rpm for 2 minutes. Pellets were then resuspended in 100 µL of 2X SDS-PAGE Sample Buffer (0.125M pH 6.8 Tris, 20% glycerol, 10% β-mercaptoethanol, 6% SDS, 0.2% bromophenol blue in dH_2O). These lysates were then either used immediately or stored at -20°C for future use.

2.11 SDS-PAGE

Acrylamide gel glass plates were cleaned thoroughly with dish soap and hot water, rinsed with distilled water then with 95% ethanol prior to use. Once dry, acrylamide gel plates were assembled in the gel casting unit. A resolving gel solution was then prepared according to the BioRad solutions book (10.2% 29:1 acrylamide, 0.39M pH 8.8 Tris, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED in MilliQ H_2O for a 10% acrylamide gel) (BioRad). The resolving gel solution was then quickly injected into the gel casting unit with a sterile 10 mL syringe. The resolving gel was then covered with either 0.1% SDS for acrylamide concentrations equal to or lower than 10%, or isopropanol for acrylamide concentrations 12% or higher. Once solidified (15 minutes later), 0.1% SDS or isopropanol was then poured off, and a stacking gel solution was prepared according to the BioRad solutions book (4.95% 29:1 acrylamide, 0.125M pH 6.8 Tris, 0.1% SDS, 0.1% APS, 0.1% TEMED in MilliQ H_2O) (BioRad). The stacking gel was then quickly injected on top of the resolving gel with a sterile 10 mL syringe, and the comb was inserted. Once dry (15 minutes later), the gel unit was submerged in 1X Tris-Glycine solution (0.302% Tris base, 1.88% glycine, 0.1% SDS in dH_2O) in a BioRad electrophoresis chamber (BioRad). Whole-cell lysates of interest were then boiled at 100°C for 5 minutes then 10 µL of each was carefully loaded in the SDS-PAGE gel, which was then electrophoresed at

100V for 1-2 hours. The acrylamide gel was then either stained with coomassie for analysis or transferred to a nitrocellulose membrane for Western blot analysis.

2.12 Western blot analysis

Acrylamide gels were carefully transferred from the electrophoresis apparatus to a black and white electrotransfer casing against a nitrocellulose membrane and flanked by filter paper and sponges soaked in 1X electroblotting buffer (20% methanol, 10% 10X electroblotting buffer (3.0275% Tris base, 14.413% glycine in dH_2O, pH 8.3) in dH_2O). This casing was then fitted into an electrophoresis chamber (black side to the negative electrode), and the chamber was filled with 1X electroblotting buffer. Transfer of proteins was done at 10V overnight at room temperature. The next day, the nitrocellulose membrane was transferred to a clean Tupperware container and covered in 50 mL of 2.5% MTS (2.5% w/v skim milk powder, 50 mL 10X TS (9% NaCl, 1.211% Tris base) in dH_2O). The membrane was then blocked in this solution for 1.5 hours at room temperature while rotating gently. The blocking solution was then discarded and 50 mL of 2.5% MTS containing 5 µL rabbit α-CpxR-MBP or rabbit α-CpxA-MBP antibody was added to the membrane, which was then incubated while rotating gently for 1 hour. This solution was then discarded and the membrane was washed with 50 mL of Wash Solution (10% 10X TS, 0.5% Tween 20 in dH_2O) 4 times for 30 minutes, then once more overnight while rotating at room temperature. The next day the Wash Solution was discarded and 50 µL of 2.5% MTS containing 10 µL α-rabbit::AP antibody was added to the membrane, which was then incubated for 1 hour at room temperature while gently rotating. Next the membrane was again washed with 50 µL of Wash Solution 4 times for 30 minutes while rotating at room temperature, then once more rotating overnight at room temperature. The next day the membrane was transferred to a sheet of cellophane and 2.5 mL of BioRad ChemiStar chemiluminescence development solution

(BioRad) was added to the membrane, which was allowed to sit at room temperature for 10 minutes. The membrane was then imaged with a BioRad Chemiluminescence imager (BioRad). Each Western blot experiment was done at least twice and experiments shown are representative results.

2.13 Coomassie analysis

Acrylamide gels from SDS-PAGE were carefully transferred to a clean Tupperware container, to which was added 50 µL of Coomassie blue solution (0.45% methanol, 10% glacial acetic acid, 0.25% Coomassie brilliant blue in dH_2O). This was then incubated at room temperature while gently rotating overnight. The next day the dye was discarded and gel was covered in 50 µL of Destain solution (0.45% methanol, 10% glacial acetic acid in dH_2O), and the gel was incubated this way for 1 hour at room temperature while gently rotating. This solution was changed twice throughout the hour. Subsequently the gel was transferred to a sheet of cellophane and was imaged with the BioRad Chemilumescence imager (BioRad). Each Coomassie experiment was done at least twice and experiments shown are representative results.

2.14 Flagella isolation

Overnight cultures of strains to be assayed were made by inoculating 150 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin or no antibiotics with single colonies of *E. coli* and were grown at 37°C while shaking at 225 rpm overnight. The next day, cultures were centrifuged for 20 minutes at 7000 rpm and 4°C, then resuspended in 20 mL 1X PBS. These were then homogenized with a homogenizer (Polytron) at 4°C on low for 4 minutes each. Each sample was then supplemented with ammonium sulfate to a final concentration of 17% w/v, and was allowed to precipitate overnight at room temperature. The next day, precipitates were centrifuged at 12,000 rpm and 4°C for 30 minutes, then resuspended in 750 µL 1X PBS and

transferred to sealed dialysis tubing and dialyzed in a container of 1X PBS overnight. The following day the contents of the dialysis tubes were transferred to sterile microfuge tubes and either stored at -20°C or were immediately prepared for analysis by SDS-PAGE. If samples were used immediate they were centrifuged at 13,200 rpm at room temperature for 20 minutes. The supernatant was aspirated and the pellet was resuspended in 100 µL 2X SDS-PAGE Sample Buffer (recipe above) and boiled at 100°C for 5 minutes prior to gel loading.

2.15 Motility assays

Overnight cultures of strains to be assayed were prepared by inoculating 5 mL of LB medium containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 1 µg/mL amikacin, 25 µg/mL chloramphenicol, 100 µg/mL trimethroprim or no antibiotics with single colonies of *E. coli* and were incubated overnight at 37°C while rotating at 225 rpm. The next day, 2 µL of each overnight culture was injected into a 0.3% agar plate (0.3% agar, 0.5% yeast extract, 0.5% NaCl, 1% Tryptone in dH_2O) containing relevant antibiotics and 0.1 mM IPTG if required. These plates were then grown statically at 37°C overnight for 14 hours. The next day the swim diameter for each strain was measured and recorded. Each strain was assayed in biological triplicate, and swim diameters presented are average values of said replicates, with error bars representing standard deviation between replicates. Each motility experiment was done at least twice, and results shown are representative results.

2.16 GFP assays

Overnight cultures of strains to be assayed were prepared by inoculating 2 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 1 µg/mL amikacin, 25 µg/mL chloramphenicol, 100 µg/mL trimethroprim or no antibiotics with

single colonies of *E. coli* and were grown at 37°C overnight while shaking at 225 rpm. The next day cultures were subcultured 1:50 into fresh LB medium in a 96-well plate containing relevant antibiotics and were grown for 5 hours at 37°C while shaking at 225 rpm, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently the OD_{509} as well as OD_{600} were measured for each culture with the Wallac Victor^2 Multilabel counter plate reader (Wallac), and an activity for each was determined by the following formula:

$$\text{Specific GFP activity (X) (AU)} = (A_{509}(X) - A_{509}(\text{blank})) / (OD_{600}(X) - OD_{600}(\text{blank}))$$

Each strain was assessed in biological triplicate and the activity values presented are averages between said replicates, with the error bars representing standard deviations between replicates. Results shown are representative results of at least two independent experiments.

2.17 Transposon mutagenesis

Overnight cultures of AE902 *E. coli* were prepared by inoculating 50 mL of LB broth containing 100 µg/mL ampicillin with single colonies of AE902. The next day this culture was electroporated with 1 µL of Epicenter EzTn::tp Tn5 transposon (Epicenter) as described elsewhere in this section. The next day, trimethroprim resistant colonies were screened for luciferase activity.

2.18 Luciferase-based screen

Trimethroprim-resistant colonies from the transposon mutagenesis were used to inoculate wells of a 96-well plate containing 200 µL LB broth with 100 µg/mL ampicillin and 0.1 mM IPTG. Six of the wells were inoculated with single colonies of AE901 and AE902 *E. coli* for reference. Screen plates were grown at 37°C while rotating at 225 rpm for 4 hours, at which point the CPS values and OD_{600} of screen clones were measured with the Wallac Victor^2

Multilabel counter plate reader (Wallac), and the luminescence values were calculated for each clone as described elsewhere in this section. Subsequently 100 µL of each culture was transferred to a fresh 96-well plate containing 100 µL of 20% glycerol in each well, and this plate was stored at -80°C for later use.

2.19 Determination of transposon insertion sequence

To determine the site of Tn5 transposon insertion, a nested degenerate PCR was utilized to amplify the DNA sequences flanking the transposon. First, single colonies of screen clones were boiled and used as template DNA for a first-step PCR using primers ARB-6, RP-1 and FP-1. This PCR had two sets of cycles: the first had a dissociation temperature of 94˚C (2'), an annealing temperature of 30˚C (30''), and an extension temperature of 72˚C (1.5'), and was repeated 7 times. The second cycle had a dissociation temperature of 94˚C (2'), an annealing temperature of 45˚C (30''), and an extension temperature of 72˚C (1.5'); this was repeated 30 times. The products of this first PCR were then used as the template of a second PCR, which used the ARB-2 primer which is complementary to the ends generated by the ARB-6 primer, combined with RP-1 and FP-1 primers complementary to the transposon ends. This protocol had an extension temperature of 94˚C (2'), an annealing temperature of 60˚C (30''), and an extension temperature of 72˚C (1.5'), and these were repeated 35 times. The products of this PCR were used as the template for Big-Dye sequencing (Invitrogen) to identify the site of transposon insertion.

2.20 Growth curves

Overnight cultures of strains to be assayed were prepared by inoculating 2 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 1

μg/mL amikacin, 25 μg/mL chloramphenicol, 100 μg/mL trimethroprim or no antibiotics with single colonies of *E. coli* and were grown at 37°C (or 30°C for *cpxA24* strains) while shaking at 225 rpm overnight. The next day bacteria were subcultured 1:50 into 2 mL fresh LB medium containing relevant antibiotics in 24-well plate and were grown for 8 hours at 37°C (or 30°C for *cpxA24* strains) while shaking at 225 rpm. Each hour the OD_{600} of these cultures was measured using the Wallac Victor^2 Multilabel counter plate reader (Wallac). Each strain was analyzed in biological triplicate, and growth values shown are average values between said replicates, and the error bars shown represent standard deviations between replicates. Results shown are representative results of at least two independent experiments.

2.21 RNA isolation

Isolation of total cellular RNA was done using the Trizol™ reagent (Roche). All work was done using filter-tips for pipettes, and all surfaces and equipment were treated with RNAse ZAP (Ambion). Overnight cultures of strains to be assayed were prepared by inoculating 5 mL of LB broth containing 100 μg/mL ampicillin, 30 μg/mL kanamycin, 50 μg/mL spectinomycin, 25 μg/mL chloramphenicol, 1 μg/mL amikacin, 100 μg/mL trimethroprim or no antibiotics with single colonies of *E. coli* and were grown at 37°C while shaking at 225 rpm overnight. The next day cultures were centrifuged at 4000 rpm and were resuspended in 1 mL Trizol reagent and mixed by pipetting. 100 μL of 1-bromo-3-chloropropane was added to each sample, which were then vortexed 3 times for 15 seconds, and let to stand for 10 minutes. Samples were then centrifuged for 15 minutes at 4°C and 12,000 rpm. The aqueous (top) phase of each sample was then transferred to a fresh 1.6 mL RNase-free tube, and 500 μL ice-cold isopropanol was added and the samples were vortexed for 15 seconds and let to stand for 10 minutes at 4°C. Samples were then centrifuged at 4°C and 12,000 rpm for 10 minutes, and the supernatants were aspirated

carefully. Pellets were then washed in 75% RNase-free EtOH and pulse-vortexed 3 times, and samples were centrifuged for 10 minutes at 4°C and 12,000 rpm. The pellets were again washed in fresh 75% EtOH, and then centrifuged once more for 10 minutes at 4°C and 12,000 rpm, and the supernatant was carefully removed. The samples were then allowed to air-dry at room temperature for 15 minutes to evaporate residual EtOH. Finally samples were resuspended in 20 µL RNase-free milliQ H_2O, had their rough concentrations determined with a nano-drop and were stored at -80°C.

2.22 Northern blots

RNA samples were prepared for agarose gel electrophoresis by mixing 1 µg of RNA with Loading Buffer (50% deionized formamide, 6.14% formaldehyde, 10% 10X MOPS, 10% RNase-free glycerol, 0.05% bromophenol blue in RNase-free milliQ H_2O) in a 1:2 ratio of RNA to Loading Buffer (volume:volume). These samples were denatured in a 65°C heat block for 10 minutes, then immediately chilled on ice for 1 minute. Samples were then loaded into 3% RNase-free agarose gel (10% 10X MOPS, 1.5g agarose, 2% formaldehyde), which was covered in 1X MOPS and run at 40V for 5 hours. The gel was then soaked in 20X SSC (3M NaCl, 300 mM sodium citrate, pH 7.0 in RNase-free MilliQ H_2O) at room temperature twice for 15 minutes, then transferred to nylon membrane overnight by traditional osmotic transfer. The next day the membrane was transferred to a piece of filter paper soaked in 2X SSC (0.3M NaCl, 30 mM sodium citrate, pH 7.0) and was exposed to UV light for 5 minutes in a fume hood, then rinsed with RNase-free milliQ H_2O, then allowed to dry for 4 hours. DIG-U-labelled RNA probes against *cpxP* and its 3' UTR "*stnc870*" were then generated by *in vitro* transcription, first by making the following master mix (per tube): 4 µL RNase-free milliQ H_2O, 4 µL template DNA (PCR product of either *cpxP* or only *stnc870*), 4 µL labelling mix (Roche), 4 µL

transcription buffer (Roche), 2 µL T7 RNA Polymerase (Roche), 2 µL RNase OUT (Invitrogen). These samples were incubated at 37°C for 15 minutes, and then 1 µL DNase I (Invitrogen) was added to each tube. These were then stored at -20°C until needed. The nylon membrane was then incubated while rotating at 68°C covered in warm DIG Easy Hyb solution (Roche) for 30 minutes. This solution was then discarded and the membrane was incubated in DIG Easy Hyb solution containing 100 ng of either α*cpxP*-DIG probe or α*stnc870*-DIG probe overnight at 68°C. The next day the membrane was transferred to an RNase-free container with 20 mL Low Stringency Buffer (2X SSC containing 0.1% SDS) and was incubated at 68°C for 10 minutes, then this was repeated with fresh buffer. The membrane was then incubated at 68°C with 20 mL High Stringency Buffer (0.1X SSC containing 0.1% SDS) twice for 15 minutes. The membrane was then transferred to a new container with 50 mL Washing Buffer (0.1M maleic acid, 0.15M NaCl, 0.3% Tween 20, pH 7.5) and allowed to rotated at room temperature for 2 minutes. This was discarded and the membrane was incubated with 50 mL Blocking Solution (10% 10X Blocking Buffer (Roche) in Maleic acid buffer) for 30 minutes while rotating at room temperature. This was removed and the membrane was incubated with 20 mL Antibody Solution ($1:10^4$ dilution of anti-DIG-AP in Blocking Solution) at room temperature for 30 minutes. This buffer was removed and the membrane was incubated with 50 mL aliquots of Washing Buffer for 15 minutes twice. The membrane was then incubated in 20 mL Detection Buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 3 minutes at room temperature while rotating. Finally, the membrane was transferred to a hybridization bag, to which was added 20-30 drops of CDP-Star solution (Roche) and the membrane was let to stand for 5 minutes. The membrane was then visualized with the chemiluminescence imager. Results shown are representative of two independent experiments.

2.23 QRT-PCR

cDNA libraries of strains to be assayed were generated by reverse transcription of whole cell RNA samples isolated by techniques explained elsewhere in this section. 1 µg of RNA was first mixed with 1 µL DNase I reaction buffer (Invitrogen), 1 µL 1U/µL DNase I (Invitrogen), and was filled to 10 µL with RNase-free milliQ H_2O. This was done twice for each RNA sample. Solutions were then incubated for 15 minutes at room temperature, then 1 µL of 25 mM EDTA (Invitrogen) was added and solutions were incubated at 65°C for 10 minutes. Next 2 µL of Master Mix 1 was added to each tube (124.5 ng/µL random hexamer primers (Invitrogen), 5 mM dNTP mix in RNase-free milliQ H_2O), and samples were incubated at 65°C for 5 minutes, then chilled on ice for 5 minutes. To each tube was then added 7 µL Master Mix 2 (4 µL 5X First-Strand Buffer (Invitrogen), 2 µL 0.1M DTT (Invitrogen), 1 µL RNase OUT (Invitrogen)), and the samples were incubated for 2 minutes at 25°C. Next, to one half of the samples, 1 µL Reverse Transcriptase Superscript II (Invitrogen) was added, and the other half 1 µL RNase-free milliQ H_2O was added. The samples were then reverse transcribed in a thermocycler with the following run parameters: 25°C for 10 minutes, 42°C for 50 minutes, then 70°C for 15 minutes. Samples were then stored at -20°C until further use. Next, 300 nM solutions of *gadA*(F+R), *gadE*(F+R) and *rpoD*(F+R) primers were prepared, and 250 pg/µL solutions of template cDNA samples were prepared. Next, 7.5 µL of Master Mix (5 µL 2X Dynamite Master Mix (a proprietary mix developed, and distributed by the Molecular Biology Service Unit (MBSU), in the department of Biological Science at the University of Alberta, Edmonton, Alberta, Canada. It contains Tris (pH 8.3), KCl, $MgCl_2$, Glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye, SYBR Green (Molecular Probes) as the detection dye, and an antibody inhibited Taq polymerase. To obtain this Mastermix, please contact the MBSU at 780-492-1066.), 2.5 µL *rpoD, gadA* or *gadE* primer

mix) was added to each well of a qPCR plate, and 2.5 µL of template DNA mixture was added to each well. The clear adhesive film was applied to the plate, which was then vortexed for 30 seconds on medium speed and centrifuged for 2 minutes at 2000 rpm. Finally the plate was loaded into the qPCR machine and analyzed for cDNA abundance. Each strain was analyzed in biological triplicate, and each biological replicate was measured in technical triplicates. Results shown are average values of said replicates, and error bars shown represent standard deviations between replicates. Results shown are representative results of at least two independent experiments.

2.24 Amikacin resistance assays

Overnight cultures of strains to be assayed were prepared by inoculating 2 mL of LB broth containing 100 µg/mL ampicillin, 30 µg/mL kanamycin, 50 µg/mL spectinomycin, 25 µg/mL chloramphenicol, 1 µg/mL amikacin, 100 µg/mL trimethroprim or no antibiotics with single colonies of *E. coli* and were grown at 37°C while shaking at 225 rpm overnight. The next day bacteria were subcultured 1:50 from overnight cultures into fresh LB medium containing either 3 µg/mL, 1.5 µg/mL, 0.75 µg/mL, 0.375 µg/mL or 0 µg/mL amikacin and 0.1 mM IPTG in a 96-well plate and were grown for 8 hours at 37°C (or 30°C) while shaking at 225 rpm. After each hour the OD_{600} of the cultures was measured with the Wallac Victor^2 Multilabel counter plate reader (Wallac). Each strain was assayed in biological triplicate and growth values shown are average values between replicates, with the error bars representing standard deviation between replicates. Results shown are representative results of at least two independent experiments.

2.25 Acid challenge assays

Overnight cultures of strains to be assayed were prepared by inoculating 5 mL of LB broth (or 2 mL for the β-galactosidase assay variation) containing 100 μg/mL ampicillin, 30 μg/mL kanamycin, 50 μg/mL spectinomycin, 25 μg/mL chloramphenicol, 1 μg/mL amikacin, 100 μg/mL trimethroprim or no antibiotics with single colonies of *E. coli* and were grown at 37°C (or 30°C for *cpxA24* strains) while shaking at 225 rpm overnight. The next day bacteria were subcultured 1:50 into fresh LB medium containing relevant antibiotics and were grown for 4 hours at 37°C (or 30°C for *cpxA24* strains) while shaking at 225 rpm, then were centrifuged at 4000 rpm for 10 minutes. Next, pellets were resuspended in 5 mL (or 2 mL for the β-galactosidase assay variant) of LB medium equilibrated to pH 2.9 with 1M HCl and were grown for 1 hour at 37°C (or 30°C for *cpxA24* strain) while shaking at 225 rpm. Next, cultures were centrifuged at 4000 rpm for 10 minutes and were then either resuspended in 2 mL 1X Z-buffer and then treated as a β-galactosidase assay, or were resuspended in 5 mL LB medium (pH 7), then serial diluted at increments of 10^{-1} down to 10^{-7}. Next 10 μL of these were spotted onto non-selective media and grown overnight at 37°C (or 30°C for *cpxA24* strains). The next day these plates were visualized with a colony counter and relative survival to acid challenge was assessed. Each strain was analyzed in biological triplicate, and results shown are representative of at least two independent experiments.

2.26 Polymerase Chain Reaction (PCR)

All PCR experiments were done using a Master-Mix by the following formula (per 50μL reaction): 22 μL MilliQ H_2O, 10 μL betaine, 8 μL 5mM dNTPs, 5 μL 10X PCR buffer, 2 μL 50 mM $MgSO_4$, 1 μL F primer, 1 μL R primer, 1 μL Taq polymerase. Unless otherwise stipulated, the protocol used had a 94˚C dissociation step (2’), an annealing step of variable temperature

(30’’), and a 72˚C extension step (variable time), and these were repeated 30 times. DNA was then stored at -20˚C.

2.27 Strains, plasmids, and reporter genes

Strain or plasmid	Description	Source or reference
Bacterial strains		
MC4100	F⁻ *araD139 Δ(argF-lac)* U169 *rpsL150* (Str^r) *relA1 flhD5301 deoC1 ptsF25 rbsR*	(1)
W3110	F⁻ λ⁻ IN(*rrnD-rrnE*) *rph-1*	(2)
2K1056	W3110 *lacZ⁻*	Lab strain
TR10	MC4100 *cpxA24*	(3)
TR49	MC4100 λRS88[*degP'-lacZ*$^+$]	(4)
TR50	MC4100 λRS88[*cpxP'-lacZ*$^+$]	(4)
TR51	MC4100 *cpxR::spec*	(3)
TR71	MC4100 λRS88[*rpoHP3'lacZ+]*	(5)
AE101	MC4100 (pBR-Plac)	This study
AE102	MC4100 (pBR-Plac-micF)	This study
AE103	MC4100 (pBR-Plac-omrA)	This study
AE104	MC4100 (pBR-Plac-omrB)	This study
AE105	MC4100 (pBR-Plac-rprA)	This study
AE106	MC4100 (pBR-Plac-cyaR)	This study
AE107	TR49 (pBR-Plac)	This study
AE108	TR49 (pBR-Plac-micF)	This study
AE109	TR49 (pBR-Plac-omrA)	This study

AE110	TR49 (pBR-Plac-omrB)	This study
AE111	TR49 (pBR-Plac-rprA)	This study
AE112	TR49 (pBR-Plac-cyaR)	This study
AE113	TR50 (pBR-Plac)	This study
AE114	TR50 (pBR-Plac-micF)	This study
AE115	TR50 (pBR-Plac-omrA)	This study
AE116	TR50 (pBR-Plac-omrB)	This study
AE117	TR50 (pBR-Plac-rprA)	This study
AE118	TR50 (pBR-Plac-cyaR)	This study
AE207	TR50 *rprA::kan*	This study
AE208	TR50 *omrAB::kan*	This study
AE209	TR50 *cyaR::cam*	This study
AE507	TR49 *rpoS::kan* (pBR-Plac)	This study
AE508	TR49 *rpoS::kan* (pBR-Plac-rprA)	This study
AE509	TR50 *rpoS::kan* (pBR-Plac)	This study
AE510	TR50 *rpoS::kan* (pBR-Plac-rprA)	This study
AE614	2K1056 λRS88[*cpxP'-lacZ*$^{+}$]	This study
AE615	AE614 *yrfG::kan* (pBR-Plac)	This study
AE616	AE614 *ymgC::kan* (pBR-Plac)	This study
AE617	AE614 *sulA::kan* (pBR-Plac)	This study
AE618	AE614 *purH::kan* (pBR-Plac)	This study
AE619	AE614 *ddpX::kan* (pBR-Plac)	This study
AE620	AE614 *csgD::kan* (pBR-Plac)	This study

AE621	AE614 *ygeD::kan* (pBR-Plac)	This study
AE622	AE614 *livK::kan* (pBR-Plac)	This study
AE623	AE614 *ygg::kan* (pBR-Plac)	This study
AE624	AE614 *sufI::kan* (pBR-Plac)	This study
AE625	AE614 *yrfG::kan* (pBR-Plac-rprA)	This study
AE626	AE614 *ymgC::kan* (pBR-Plac-rprA)	This study
AE627	AE614 *sulA::kan* (pBR-Plac-rprA)	This study
AE628	AE614 *purH::kan* (pBR-Plac-rprA)	This study
AE629	AE614 *ddpX::kan* (pBR-Plac-rprA)	This study
AE630	AE614 *csgD::kan* (pBR-Plac-rprA)	This study
AE631	AE614 *ygeD::kan* (pBR-Plac-rprA)	This study
AE632	AE614 *livK::kan* (pBR-Plac-rprA)	This study
AE633	AE614 *ygg::kan* (pBR-Plac-rprA)	This study
AE634	AE614 *sufI::kan* (pBR-Plac-rprA)	This study
AE907	MC4100 Δ*ptsG*	This study
AE908	MC4100 Δ*uhpT*	This study
AE910	TR51Δ*ptsG*	This study
AE911	TR51Δ*uhpT*	This study
AE886	MC4100 Δ*rcsC*	This study
AE887	TR10 Δ*rcsC*	This study
AE888	TR51Δ*rcsC*	This study
AE613	2K1056 λRS88[*degP'-lacZ*$^{+}$]	This study
AE614	2K1056 λRS88[*cpxP'-lacZ*$^{+}$]	This study

AE663	AE614 *rpoS*::kan^r	This study
AE664	AE614 *ydaM*:: kan^r	This study
AE666	AE614 *ΔcsgD*	This study
AE667	AE613 *cpxA*:: cam^r	This study
AE668	AE613 *cpxP*:: kan^r	This study
AE669	AE613 *cpxR*:: spc^r	This study
AE670	AE614 *pta-ackA*::Tn*10* (tet^r)	This study
AE890	MC4100 (pGX10[*cpxR'-'gfp]*) (pBR322)	This study
AE891	MC4100 (pGX10[*cpxR'-'gfp]*) (pBR-rprA)	This study
AE892	MC4100 (pGX10[*cpxR'-'gfp]*) (pBR-cyaR)	This study
AE893	MC4100 (pGX10[*cpxR'-'gfp]*) (pBR-omrA)	This study
AE407	TR50 (pBR-dsrA)	This study
AE523	AE614 *csgDEFGAB::kan* (pBR322)	This study
AE524	AE614 *csgDEFGAB::kan* (pBR-rprA)	This study
AE918	TR50 *gadA::kan* (pBR322)	This study
AE919	TR50 *gadA::kan* (pBR-rprA)	This study
AE920	TR50 *gadE::kan* (pBR322)	This study
AE921	TR50 *gadE::kan* (pBR-rprA)	This study
AE922	TR50 *gadC::kan* (pBR322)	This study

AE923	TR50 *gadC::kan* (pBR-rprA)	This study
AE924	TR50 *gadW::kan* (pBR322)	This study
AE925	TR50 *gadW::kan* (pBR-rprA)	This study
AE926	TR50 *gadX::kan* (pBR322)	This study
AE927	TR50 *gadX::kan* (pBR-rprA)	This study
AE928	MC4100 (pJW15[*PgadE'-lux]*) (pBR322)	This study
AE929	MC4100 (pJW15[*PgadE'-lux]*) (pBR-rprA)	This study
Plasmids		
pCA24N	Vector control from ASKA library; Cam^r	(6)
pCA-*nlpE*	IPTG-inducible *nlpE* overexpression vector from ASKA library; Cam^r	(6)
pJW15P*cyaR*	pJW15 luminescence reporter plasmid containing *cyaR* promoter; Kan^r	(7)
pJW15P*rprA*	pJW15 luminescence reporter plasmid containing *rprA* promoter; Kan^r	(7)
pBR-plac	pBR322 expression vector containing IPTG-inducible P_{lacO-1} promoter; Amp^r	(8)
pBR-Plac-micF	pBR-plac-based IPTG-inducible *micF* overexpression vector; Amp^r	(8)
pBR-Plac-rprA	pBR-plac-based IPTG-inducible *rprA*	(8)

	overexpression vector; Amp^r	
pBR-Plac-omrA	pBR-plac-based IPTG-inducible *omrA* overexpression vector; Amp^r	(8)
pBR-Plac-omrB	pBR-plac-based IPTG-inducible *omrB* overexpression vector; Amp^r	(8)
pBR-Plac-cyaR	pBR-plac-based IPTG-inducible *cyaR* overexpression vector; Amp^r	(8)
pXG10	pSC101-based *gfp* translational fusion vector, with constitutively expressed Tet^O promoter	(9)
pXG10(*cpxR*)	pSC101-based *gfp* translational fusion vector, with constitutively expressed Tet^O promoter, containing *cpxR* 5' UTR	This study
Reporter genes		
PcpxP::lacZ	Chromosomal, single copy *PcpxP'-'lacZ* integrated at λRS88	(4)
PdegP::lacZ	Chromosomal, single copy *PdegP'-'lacZ* integrated at λRS88	(4)
PrpoHP3::lacZ	Chromosomal, single copy *PrpoHP3'-'lacZ* integrated at λRS88	(4)
PcyaR::lux	Low copy-number pSC101-derived plasmid containing *PcyaR'-'lux*	(7)
PrprA::lux	Low copy-number pSC101-derived	(7)

	plasmid containing *PrprA'-'lux*	
cpxRUTR::gfp	High copy-number pXG10 plasmid containing *cpxR'-gfp*	This study

2.28 Bibliography

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CHAPTER 3

THE CPX ENVELOPE STRES RESPONSE REGULATES TWO sRNA GENES

3.1 Introduction

A microarray done in our lab in recent years (1) suggested that several small RNA genes have their expression modulated upon induction of the Cpx response: *rprA, cyaR, micF, omrA,* and *omrB.* We reasoned that *omrA, omrB,* and *micF* are indirectly affected by Cpx induction, because all three are activated by the EnvZ/OmpR pathway (2, 3), which has shown to be activated by CpxR though positive regulation of the EnvZ-activator MzrA (4). We do have some interest in the interplay between the Cpx pathway and MicF, as overexpression of MicF has been shown to decrease activity of a *cpxR::GFP* translational reporter (5) and it is not known under what conditions MicF acts to repress activity of the Cpx response. However, in the case of this project we limited our scope to sRNA genes which might be directly regulated by CpxR at the transcriptional level. In order to determine whether or not the remaining genes, *rprA* and *cyaR* are directly Cpx-regulated, we utilized luminescent reporter genes to examine the impact of *cpx* mutations that activate and abolish Cpx pathway activity on the expression of *cyaR* and *rprA*. Additional EMSA analyses were used to address whether regulation was direct or indirect.

3.2 *cyaR* transcription is directly repressed by CpxR

A transcriptional *PcyaR::lux* reporter gene was generated using the pJW15 plasmid by Dr. Stefanie Vogt (6). These plasmids were transformed into strains containing either the *cpxA24* allele that constitutively activates the Cpx pathway or the *cpxR::spec* mutation that abolishes Cpx pathway activity. Next, these strains were subcultured from overnight cultures into LB medium containing antibiotics selective for the reporter plasmid and grown to and OD_{600} of 0.8, at which point the luminescence emitted from each strain was measured using a plate reader. It was found that activation of the Cpx response in the *cpxA24* mutant repressed activity of the reporter roughly 2-fold (Figure 3.1). Next, virtual DNA footprinting was done to determine the

likelihood that this downregulation occurred upon direct promoter binding by CpxR (www.prodoric.de/vfp), but this program revealed that there was not in fact a strong consensus CpxR binding site in the *cyaR* promoter. Regardless, a fellow student in the lab, Randi Guest, conducted electrophoretic mobility shift assays (EMSAs) to more directly assay for CpxR binding of purified PCR-generated *cyaR* promoter DNA. It was found that CpxR phosphorylated with 20 mM acetyl phosphate bound the *cyaR* promoter moderately well at a titer of 50 pmol purified CpxR (6); for comparison, phosphorylated CpxR bound the *cpxP* promoter at a titer of 25 pmol CpxR, but bound the negative control *rpoD* promoter DNA at a titer of 100 pmol. Therefore it was concluded that CpxR indeed binds the *cyaR* promoter to mediate direct translational repression, although this binding must involve an atypical binding site since there was no consensus CpxR binding site found.

Paradoxically, activity of the *PcyaR::lux* reporter was also diminished ~2-fold in the Cpx-inactive *cpxR::spec* strain (Figure 3.1), suggesting that *cyaR* transcription might be subject to multiple layers of regulation by the Cpx response. The latter effect was, however, abolished by the addition of 2.8% glucose to the growth media, suggesting cAMP-CRP may play a role in the *cyaR* repression seen in the *cpxR* mutant. This hypothesis is supported by results from the microarray done in our lab which in fact reveal that two glucose transporters may have their expression decreased upon induction of the Cpx response: *uhpT* and *ptsG*. We therefore hypothesized that an increase in the expression of these transporter genes in the *cpxR* mutant (due to de-repression) may lead to an increase in glucose import and therefore decreased cAMP-CRP activity, which may account for the decrease in *cyaR* transcription in the *cpxR* mutant. Mutations were made in both of these genes and/or *cpxR*, and luciferase reporter gene assays were done with cultures grown in LB medium to an OD_{600} of 0.8 to determine if the repression

phenotype of the *cpxR* mutant was epistatic over mutations in *uhpT* or *ptsG*. The mutation in *ptsG* reduced *PcyaR::lux* activity by a moderate but not statistically significant degree ($P<0.05$, one-way ANOVA), but this was not seen for mutations in *uhpT* (Figure 2). The *ptsG cpxR* double mutant did not exhibit a reduction in reporter gene activity which was any more profound than either the *cpxR* or *ptsG* single mutants, suggesting perhaps that they act in the same pathway to reduce *cyaR* transcription. Altogether these data do not rule out a role for Cpx regulation of *ptsG* in the regulation of *cyaR,* but do not strongly support it either.

3.3 The Cpx response directly regulates transcription of *rprA*

We next sought to investigate potential direct transcriptional regulation of *rprA* using the same approach. Direct Cpx regulation of *rprA* seemed an appealing model as both RprA and the Cpx pathway negatively regulate the master curli biogenesis transcription factor gene *csgD* (which would form a coherent feedforward loop), and both RprA and the Cpx pathway are most highly active during stationary phase. In order to confirm Cpx regulation of *rprA* expression, a transcriptional *PrprA::lux* fusion in the pJW15 plasmid was constructed (Stefanie Vogt) (6) and activity of this reporter was assayed in *E. coli* MC4100 upon overexpression of the outer membrane lipoprotein gene *nlpE* as has previously been described (7). These strains were subcultured 1:50 from overnight cultures and grown in LB medium containing antibiotics selective for the reporter plasmid and the *nlpE* overexpression plasmid to an OD_{600} of 0.8, at which point luminescence of each culture was measured with a plate reader. Cpx pathway induction by *nlpE* overexpression was found to activate *PrprA::lux* expression >5-fold, suggesting that the Cpx pathway indeed activates *rprA* transcription (Figure 3.3). Importantly, it was found that in a strain bearing a mutation in *rcsB,* which encodes the response regulator of the Rcs phosphorelay that activates *rprA* transcription (8), induction of the Cpx pathway no longer

activated *rprA* transcription (Figure 3.3). Activation of *rprA* transcription by Cpx therefore seems to be contingent on existing activation by RcsB. Next, similarly to how we examined *cyaR*, we asked whether or not this transcriptional activation of *rprA* involved direct binding of its promoter by CpxR. Virtual footprinting (www.prodoric.de/vfp) suggested the existence of at least two near-consensus CpxR binding sites near the -35 site of the *rprA* promoter, providing a strong basis for potential direct binding by CpxR. Concurrent with this, EMSAs performed by Randi Guest demonstrated strong binding to the promoter of *rprA* by phosphorylated CpxR (6), as CpxR phosphorylated with 20 mM acetyl phosphate supershifted PCR-generated *rprA* promoter DNA at a titer of 25 pmol CpxR, which is the same titer required to shift the positive control DNA, *cpxP* promoter DNA. Again, for reference, phosphorylated CpxR only bound negative control *rpoD* promoter DNA at a titer of 100 pmol CpxR. Based on these data we conclude that phosphorylated CpxR indeed directly binds to the *rprA* promoter to mediate transcriptional upregulation, as measured by the transcriptional activity of our *PrprA::lux* reporter.

3.4 Conclusions and future directions

Taken together, these data suggest that CpxR directly regulates both the *rprA* and *cyaR* sRNA genes, and that this regulation involves CpxR binding the promoters of these genes. There do remain, however, several questions pertaining to the nature of the regulation of each of these genes. In regards to *cyaR,* work needs to be done to better characterize its paradoxical regulation by CpxR, specifically to better explain why its activity is reduced in both a Cpx-activated mutant and a Cpx-null mutant. While we still suspect its reduced activity in the *cpxR::spec* mutant is caused by de-repression of the *ptsG* transporter gene and thus reduced CRP-cAMP activity, in order to cement this, an experiment needs to be done to determine the dependence of this effect

on CRP-cAMP itself. Theoretically, addition of lactose in glucose-depleted media should restore the *cyaR* activity in the *cpxR::spec* mutant, since this would elevate CRP-cAMP activity, and this will be tested in the future. As for *rprA*, our findings do suggest direct transcriptional activation of this gene by CpxR, although it's interesting that Cpx activation was not sufficient for activity of the *rprA* reporter gene. Our hypothesis is that *rprA* promoter binding by phosphorylated CpxR enhances *rprA* transcription, and it interests us to determine whether or not this involves direct protein-protein binding between CpxR and RcsB, as it has been previously reporter that RcsB binds some promoters as a heterodimer to stimulate transcription (9). The latter theory could be tested using a bacterial two-hybrid system to assay for a potential direct interaction between CpxR and RcsB at the *rprA* promoter, as has previously been described (10). As mentioned earlier, we did not test for direct regulation of *omrA, omrB, micF,* or *rybB* since the perceived Cpx regulation of these genes could be explained by indirect Cpx activation/repression of these genes through Cpx control of their respective transcriptional regulators. It's worth mentioning, however, that direct Cpx regulation of these genes cannot actually be ruled out simply because they are also indirectly Cpx-regulated; that is to say they could easily be both directly regulated by CpxR and indirectly regulated by CpxR (through, for example, *mzrA* in the case of *omrA/B*). The results of the microarray which suggest up/downregulation of these sRNA genes might therefore warrant some investigation into the potential direct transcriptional regulation by CpxR (akin to the experiments we conducted with *cyaR* and *rprA*).

3.5 Figures

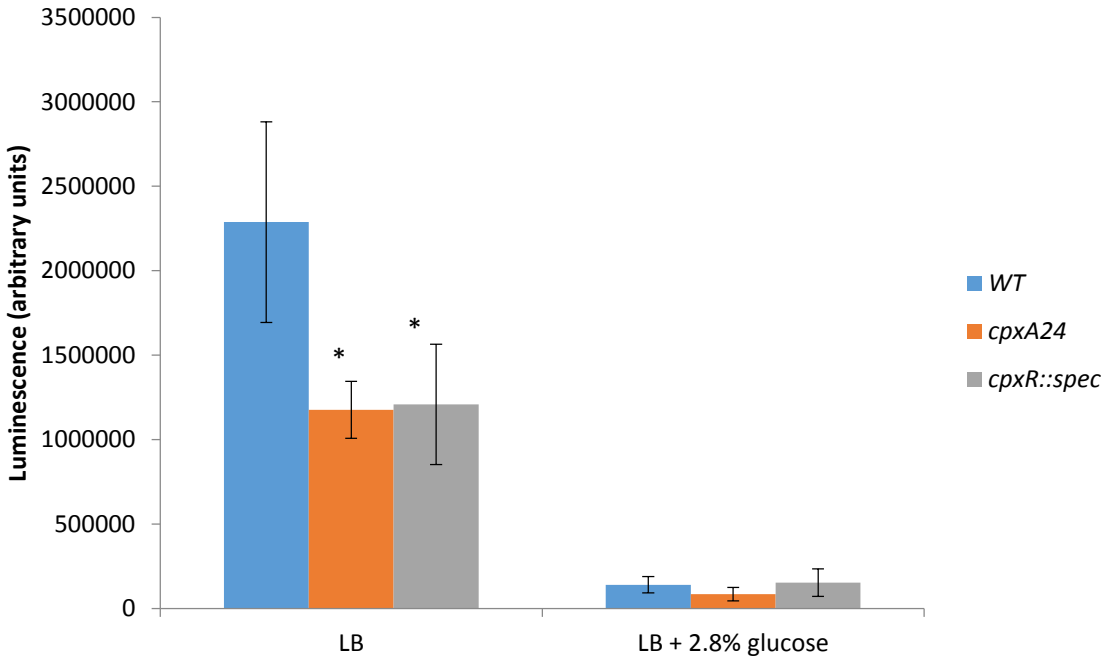

Figure 3.1: Cpx pathway induction represses *cyaR* transcription. Strains bearing either constitutively active Cpx pathway allele *cpxA24* or a *cpxR::spec* null allele and the pJW15-*PcyaR::lux* plasmid were subcultured 1:50 from overnight culture into fresh LB medium with or without 2.8% glucose, and grown for 6 hours at 37^{0}C while shaking in a 96-well plate. *PcyaR::lux* activity was then measured for each strain by recording luminescence absorbance values (CPS – counts per second) and adjusting for cell density. Bars represent average values between three replicates, and error bars represent standard deviation between said replicates. Results are representative of two independent experiments. Asterisks denote a significant difference compared to wild-type ($P<0.05$, two-way ANOVA).

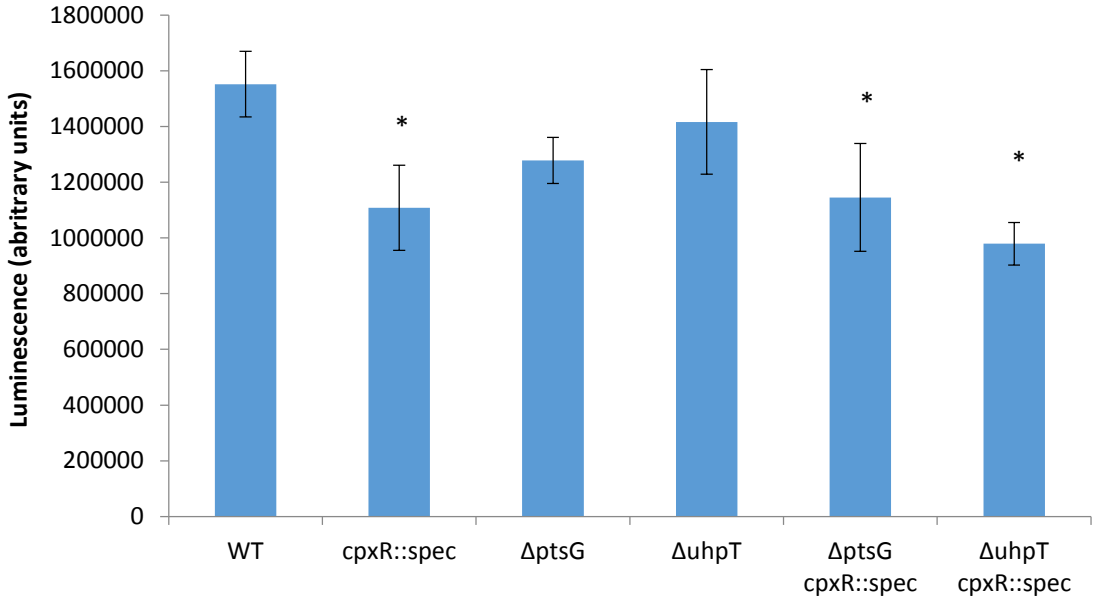

Figure 3.2: Deletion of *cpxR* or *ptsG* reduces *cyaR* transcription. Strains carrying the pJW15-*PcyaR::lux* transcriptional reporter plasmid and bearing single or double mutations *cpxR::spec, ΔptsG, ΔuhpT* were subcultured 1:50 from overnight culture into fresh LB medium in a 96-well plate and grown at 37^0C while shaking for 6 hours. *PcyaR::lux* transcriptional activity was then measured for each strain by recording CPS absorbance values and adjusting for cell density. Bars represent average values between three replicates, and error bars represent standard deviation between said replicates. Results are representative of two independent experiments. Asterisks denote significant difference compared to wild-type ($P < 0.05$, one-way ANOVA).

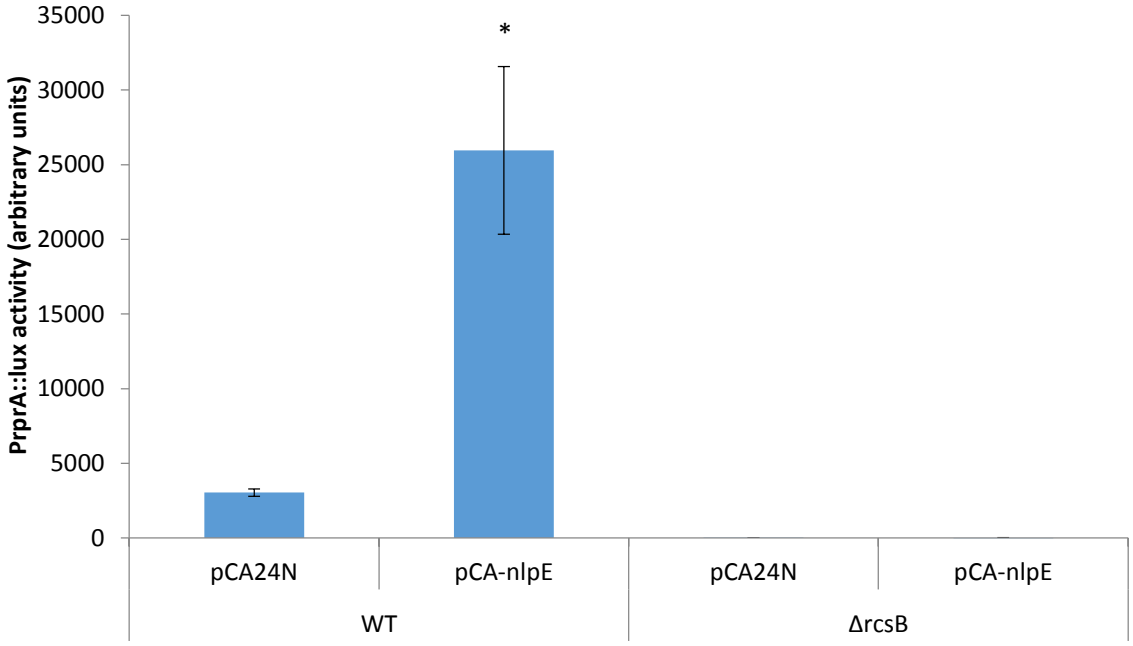

Figure 3.3: The Cpx response activates *rprA* transcription in an *rcsB*-dependent manner. Strains bearing the pCA-*nlpE* overexpression vector or pCA24 control vector were subcultured 1:50 from overnight cultures into fresh LB medium in a 96-well plate and grown at 37^0C while shaking for 6 hours. *nlpE* overexpression was induced by addition of 0.1 mM IPTG upon subculturing. Subsequently *PrprA::lux* activity was measured for each strain by recording CPS absorbance values and adjusting for cell density. Bars represent average values between three replicates, and error bars represent standard deviation between said replicates. Results are representative of two independent experiments. Asterisk denotes statistical significance compared to vector control pCA24N for a given strain ($P<0.05$, two-way ANOVA).

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CHAPTER 4

THE SMALL RNA RPRA REPRESSES THE CPX ENVELOPE STRESS RESPONSE

4.1 Introduction

Some sRNAs whose expression is controlled by a two-component system participate in feedback loops to influence the activity of their own regulator (1, 2). The OmrA and OmrB sRNAs, for example, act by directly basepairing the 5' UTR of the *ompR* mRNA, which encodes their own transcriptional activator, thereby repressing its translation by the ribosome (2). These two sRNAs can be described as having broadly two functions, then: one is to prevent the EnvZ/OmpR envelope stress response from exerting too severe a regulatory cascade, in favour of a more metered response. A good analogy for this type of feedback loop is the governor installed on the engine of high-performance cars, which prevents the engine from achieving revs beyond a set limit, for the purpose of safety and integrity of the engine. The other function of these sRNAs is to promote a response to envelope stress, in keeping with the function of the EnvZ/OmpR TCS as a whole. This is clearly shown by the array of large envelope-localized structures whose expression is repressed by OmrA: *ompT* (encoding a porin), *cirA* (encoding a siderophore tranporter), *fecA* (a citrate transporter), *csgD* (controller of curli production) and *flhDC* (flagella regulator) (3-5). This is why OmrA and OmrB make excellent models of flexible post-transcriptional regulators; not only do they repress EnvZ/OmpR regulon members at post-transcriptional speed, but they also act to keep activity of their own activator in check, which demonstrates just how well small RNAs can fine-tune a cellular process such as the response to envelope stress. Here we present data which suggest that RprA, too, is an sRNA which acts in a negative feedback loop to repress the activity of its own regulator, although unlike OmrA and OmrB, RprA does not repress activity of the Cpx response through conventional direct basepairing with the *cpxR* transcript, but rather modulates its activity through a mechanism we suspect involves the glutamate-dependent acid resistance pathway (GDAR).

4.2 The small RNA RprA inhibits the Cpx pathway

We sought to determine whether the directly Cpx-regulated sRNAs *rprA, cyaR,* as well as three indirectly Cpx-regulated sRNAs *omrA, omrB* and *micF* affected activity of the Cpx pathway. *E. coli* MC4100 bearing a chromosomal *PcpxP::lacZ* transcriptional reporter gene (which responds strongly to Cpx pathway activity) was transformed with pBR322 plasmids containing these genes (2), then these strains were subcultured 1:50 from overnight cultures into LB medium containing 0.1 mM IPTG for plasmid induction and were grown to an OD_{600} of roughly 1.0. Next, activity of the reporter gene was measured by conventional β-galactosidase assay in a plate reader (for more details, see Chapter 2). Only *rprA* overexpression was found to have an effect on the reporter, leading to a >2-fold reduction in *PcpxP::lacZ* activity (Figure 4.1). We also examined whether strains carrying deletions of these sRNAs impacted Cpx pathway activity (Figure 4.2). Deletion of *rprA, omrA, omrB,* or *micF* had no measurable effect on activity of the *PcpxP::lacZ* reporter gene in the absence an inducer of the Cpx pathway (Figure 4.2). This observation suggests that basal RprA levels do not influence basal Cpx pathway activity. It's also possible that RprA's effect on the Cpx pathway may be redundant with other sRNA genes, as has commonly been observed in other cases (6-8). To determine whether RprA impacted *cpxP* expression specifically or had more general effects on the Cpx response, we also measured the effect of overexpressing *rprA* on another Cpx-responsive reporter gene, *PdegP::lacZ. rprA* overexpression also repressed activity of this Cpx and σ^E-responsive reporter by a comparable amount (Figure 4.3). Finally, to examine whether this phenotype was specific to the Cpx response and was not dependent on the σ^E pathway, we also tested RprA's ability to inhibit an exclusively σ^E-responsive reporter, *rpoHP3::lacZ. rprA* overexpression had no

measurable effect on this reporter gene, suggesting that RprA indeed represses Cpx activity but not σ^E activity (Figure 4.4).

4.3 RprA does not inhibit the Cpx response by affecting CpxR or CpxA protein levels

The simplest explanation for an inhibitory effect of RprA on the Cpx response is that it may interact with and directly influence the mRNAs encoding CpxR and/or CpxA. To investigate this possibility, we assayed for changes in CpxR or CpxA protein levels in strains either overexpressing *rprA* or an empty vector control. Western blots with α-CpxR-MBP (maltose binding protein) or α-CpxA-MBP secondary antibodies were done on whole-cell lysates collected from cultures of *E. coli* MC4100 bearing the pBR-*rprA* plasmid or pBR322 empty vector grown for 5 hours in LB medium containing antibiotics selective for the plasmid, with induction of the plasmid by 0.1 mM IPTG. These blots revealed that CpxA and CpxR protein levels are not altered upon *rprA* overexpression (Figure 4.5), suggesting therefore that Cpx pathway inhibition by RprA does not involve direct translational repression of either the *cpxA* or *cpxR* transcripts. Interestingly, overexpression of *micF* caused a moderate decrease in CpxR protein levels, in keeping with previous findings that *micF* overexpression represses *cpxR::GFP* translation (1). *micF* overexpression also decreased CpxA protein levels moderately, which has not been previously observed, although it is unsurprising since *cpxR* and *cpxA* are thought to be co-transcribed and thus MicF could feasibly increase turnover of full length transcript.

To confirm these findings, a *cpxR*::GFP translational fusion was generated by cloning the 5' UTR (untranslated region) of *cpxR* into the pXG10 vector (9). This reporter contained the 5' UTR of *cpxR* – from the +1 bp to roughly 20 codons within the coding sequence, and this was downstream of a tet^O constitutively expressed promoter, and upstream of and in frame with the GFP coding sequence. This plasmid was transformed into *E. coli* MC4100 bearing pBR-*rprA*,

pBR-*micF* as a positive control, pBR-*omrA* as a negative control, or an empty vector, and these strains were then subcultured from overnight cultures 1:50 into fresh medium selective for each plasmid, and were then grown for 5 hours, at which point the GFP fluorescence was measured using a plate reader (A_{395}). Troublingly, we found that *micF* overexpression did not have as profound an effect on the reporter as has previously been observed (Figure 4.6) (1). *rprA* overexpression had a small but statistically significant inhibitory effect on the reporter (Figure 4.6), and given these data we can't confidently rule out direct translational repression of *cpxR* by RprA, although given that this inhibition is not reflected in the Western blot data and *micF* overexpression did not reliably repress the reporter to the degree that we expected, we interpret the results of this experiment cautiously.

4.4 The small RNA RprA does not inhibit the Cpx response through any of its known targets

At this time, RprA is known to regulate the expression of three genes: *rpoS*, encoding the stationary phase sigma factor σ^S, *csgD*, encoding the master transcriptional regulator of curli biosynthesis, and *ydaM,* encoding an adenylate cyclase whose activity affects both RpoS and CsgD. In order to determine if RprA regulation of any of these gene targets may be responsible for its down-regulatory effect on the Cpx response, we made deletions of each of these genes and conducted β-galactosidase assays to determine if their removal abolished Cpx pathway inhibition by RprA. Strains bearing these deletions and overexpressing *rprA* from the pBR322 plasmid were subcultured 1:50 into LB medium containing antibiotics selective for the plasmid and 0.1 mM IPTG and were grown for 5 hours, at which point activity of the *PcpxP::lacZ* transcriptional reporter was measured with a plate reader. Deletion *rpoS, csgD* or *ydaM* did not abolish repression of Cpx activity by *rprA* overexpression (Figure 4.7). Since Cpx pathway activity has

been linked to stationary phase (10), we sought additional verification that RprA was not acting on the Cpx response via σ^S. To do this, we examined the effect of overexpressing another sRNA that is known to regulate translation of the *rpoS* mRNA, DsrA (11). We overexpressed *dsRA* from a pBR322 plasmid and examined its impact on *PcpxP::lacZ* transcriptional reporter activity, using the same growth conditions and experimental parameters as we did with *rprA*. We found that overexpression of *dsrA* did not repress activity of the *PcpxP::lacZ* reporter, but rather increased its activity modestly (Figure 4.8). Since both RprA and DsrA stimulate *rpoS* translation, this finding makes it wholly unlikely that RprA acts on the Cpx pathway through regulation of σ^S.

Interestingly, *rprA* overexpression in an insertional *csgD::kan* mutant caused a striking increase in Cpx pathway activity, although this was not found in a clean Δ*csgD* mutant (Figure 4.7). Furthermore, deletion of genes under the positive control of CsgD (*csgEFG, csgAB*) did not mimic the effect of the insertional *csgD::kan* mutant (Figure 4.9). The fact that mutation of *ydaM,* whose product regulates CsgD activity positively (12) also did not affect RprA inhibition of the Cpx response further argues that the effect of the *csgD::kan* allele is an anomaly. At this time, we do not understand why the *csgD::kan* insertional mutation lead to such strong activation of the Cpx response.

4.5 Predicted targets of RprA are not involved in its inhibition of the Cpx response

Because Cpx pathway repression by RprA was determined not to involve direct repression of either *cpxA* or *cpxR* and was not dependent on any of RprA's known target genes, it was proposed that RprA must inhibit the Cpx pathway through regulatory action on an uncharacterized target gene. Our first foray into determining the identity of this unknown target involved use of the sRNA target prediction program sTarPicker (13), which was used to predict

potential RprA binding partners through which RprA might exert an effect on the Cpx pathway (Table 4.1). Deletions of the top ten predicted target genes from this program (*yrfG, ymgC, sulA, purH, ddpX, csgD, ygeD, livK, ygg,* and *sufI*) were generated by P1 *vir*-mediated transduction with Keio collection deletions (14) in *E. coli* MC4100 bearing a *PcpxP::lacZ* transcriptional reporter and the effect of these deletions on Cpx pathway inhibition by RprA was tested by β-galactosidase assay, using the same growth conditions as in previous experiments. With the exception of *csgD* (see above), none of these deletions had an impact on Cpx inhibition by *rprA* overexpression (Figure 4.10).

4.6 Cpx pathway inhibition by RprA is independent of CpxA and CpxP, but dependent on CpxR

Because our first attempts to identify the gene through which RprA exerts its regulatory effect on the Cpx pathway were unsuccessful, we next sought to determine the cellular location of the inhibitory signal generated by RprA in order to begin to define the mechanism. Most sources of signal input for the Cpx pathway are integrated through the sensor kinase CpxA rather than the response regulator CpxR, however it is well established that growth-dependent activation of the Cpx response is CpxR-dependent but does not require CpxA (10). Deletions of *cpxA, cpxP* and *cpxR* were generated in *E. coli* MC4100 and the effect of these mutations on Cpx pathway repression by RprA was tested by β-galactosidase assay using a chromosomal *PdegP::lacZ* transcriptional reporter (rather than *PcpxP::lacZ* – since activity of this reporter is shut off in a *cpxR* mutant). Deletion of *cpxP* or *cpxA* had no appreciable effect on repression by RprA, suggesting the mechanism does not involve the generation of an inhibitory signal that is sensed in the envelope by CpxA and/or CpxP (Figure 4.11). In a *cpxR::spec* mutant however, overexpression of *rprA* caused a much smaller, but still statistically significant decrease in

PdegP::lacZ activity (Figure 4.11), which suggests that *rprA* overexpression in some way impacts activity of the response regulator but not its abundance as indicated by Western blot experiments in Figure 4.5.

4.7 Cpx pathway inhibition by RprA is unaffected by acetyl-phosphate, pH, or growth phase

We next asked whether RprA inhibition of the Cpx response interacted with other known Cpx inducing signals, especially those known to impact CpxR specifically. The most well characterized CpxR-dependent inducing cue for the Cpx response is growth-dependent activation, which has been hypothesized to involve increasing levels of the small molecular weight phospho-donor acetyl-phosphate as the bacteria approach and enter stationary phase (15). Acetyl-phosphate is produced by the *ackA-pta* pathway, and therefore a deletion of these genes should result in depleted acetyl-phosphate. To determine whether or not Cpx repression by RprA involves changes in acetyl-phosphate levels, deletions of *pta* and *ackA* were generated in *E. coli* MC4100 bearing a chromosomal *PcpxP::lacZ* transcriptional reporter, and the effect that these mutations had on Cpx repression by RprA was tested using a β-galactosidase assay on subcultures grown in the same way as previous experiments. It was found that *rprA* overexpression inhibits the *PcpxP::lacZ* reporter regardless of a functional copy of *ackA* or *pta* (Figure 4.12), making it unlikely that RprA acts on the Cpx pathway through one of these genes. Furthermore, *rprA* overexpression in these strains inhibited the reporter in both pH 7 media and pH 5.8 media (in which activation by acetyl-phosphate would normally happen more readily (15)) (Figure 4.13). Additionally, deletion of *rprA* did not prevent growth-dependent activation of the Cpx response (Figure 4.14), which is thought to be due to rising acetyl-phosphate levels throughout growth (15). Finally, repression of Cpx pathway activity by RprA does not itself

depend on phase of growth, as its overexpression causes repression of the *PcpxP::lacZ* reporter from early exponential phase into stationary phase (Figure 4.15), although repression was most profound at later stages of growth. Cumulatively, these data demonstrate that RprA inhibition of the response is not altered by known growth-related activation cues of the Cpx response.

4.8 Cpx inhibition by RprA is partially GDAR-dependent

Recent work done by Bak *et al.* (16) showed that RNA levels of RprA, along with two other sRNAs (DsrA and ArcZ) increase upon extreme acid challenge, and that deletion of all three of these genes decreases cell survival upon acid challenge. This interaction was dependent on *gadX* of the glutamate-dependent acid resistance pathway (GDAR) and was posited to involve up-regulation of *rpoS* by these genes, and subsequent increased expression of the GadX transcriptional activator. Since acidic pH has long been known to cause repression of the Cpx pathway through an unknown mechanism (17), we reasoned that RprA might exert its repression on CpxR through regulation of some member of the GDAR pathway, particularly *gadX*. Deletions were made in *gadA* and *gadC*, encoding one half of the heterodimer decarboxylase GadAB and the antiporter GadC respectively, and *gadE, gadW,* and *gadX*, encoding transcriptional regulators of the pathway in *E. coli* MC4100 harboring a chromosomal *PcpxP::lacZ* transcriptional reporter. β-galactosidase assays revealed that deletion of *gadA* or *gadE* partially abolished Cpx pathway repression caused by overexpression of *rprA*, although neither deletion returned *PcpxP::lacZ* activity fully to vector control levels (Figure 4.16). Because of this we suspected that RprA might directly regulate *gadE* which is known to activate *gadA* transcription, which might explain why deletion of either gene is sufficient to partially abolish inhibition by RprA.

In order to determine the direction in which RprA might regulate the GDAR pathway, a *PgadE::lux* transcriptional fusion was constructed in the pJW15 plasmid, which was then transformed into *E. coli* MC4100 bearing either the pBR-*rprA* plasmid or the pBR322 empty vector. The GDAR pathway is auto-regulated by GadE (18), and thus changes in activity of the *PgadE::lux* reporter would reflect changes in activity of the pathway as a whole. These strains were subcultured 1:50 from overnight cultures into LB medium containing antibiotics selective for each plasmid and were grown for 4 hours, at which point activity of the luminescent reporter was determined with the plate reader. Overexpression of *rprA* was found to increase activity of this fusion more than 20-fold (Figure 4.17). Whether or not this activation is dependent on the previously established link between σ^S and *gadX* is still to be determined.

4.9 Conclusions and future directions

Taken together, these experiments show that RprA has a role as a feedback inhibitor of the Cpx envelope stress response. What makes RprA unique in this category of regulators is that it appears to repress the activity of its own regulatory not through direct translational inhibition or transcript destabilization but rather through impacting its activity indirectly. In the more narrow context of the Cpx regulon, RprA is also unique in its mechanism of inhibition. Until now there were few sources of signal input for the Cpx pathway which operated by impacting CpxR activity rather than CpxA activity, the most well-characterized being growth-dependent activation of the Cpx pathway (19). This latter signal involves increased phosphorylation of CpxR by the phosphodonor acetyl phosphate (15), however RprA's effect on CpxR does not appear to involve this mechanism. At this point, our data suggest that RprA upregulation of the glutamate-dependent acid resistance pathway (GDAR) in some way causes repression of CpxR activity, although importantly we do not yet know in what capacity increase GDAR pathway

activity causes inhibition of CpxR at the post-translational level. The next, most important step will be to determine whether or not RprA's activation of GDAR activity is dependent on *rpoS*, which has previously been proposed as the sole source of GDAR regulation by the sRNAs RprA, DsrA, and ArcZ (16). If RprA's effect is not *rpoS*-dependent, then the critical experiment that must next be done is to construct translation *gfp* or *lacZ* fusions of *gadE* to determine if RprA can directly influence translation of this gene, and if this is the case, mutational analyses must be done to determine the important basepairs in the putative RprA-*gadE* interaction. As previously stated, our assumption at this point is that RprA in some way reduces the activity of CpxR, but not its protein abundance. Critically, at this point we have no biochemical data which proves that CpxR is phosphorylated to a reduced level *in vivo* upon overexpression of *rprA*. In order to prove this, whole-cell lysates of *E. coli* overexpressing *rprA* or a vector control could be collected, and a Phos-Tag SDS-PAGE gel done to discriminate between phosphorylated and unphosphorylated CpxR using a Western blot, as described elsewhere (20). By this method we should be able to determine whether or not overexpression of *rprA* indeed alters the phosphorylation state of CpxR, an important piece of data in characterizing this mechanism of inhibition. Finally and probably most importantly, it remains to be determined how increased GDAR activity inhibits the phosphorylation of CpxR. The first step in this last goal is to determine which component of the GDAR pathway is responsible for the reduced CpxR activity, and from there biochemical assays can be done to elucidate the mechanism.

4.10: Tables and figures

Gene	Function
yrfG	Purine nucleotidase
ymgC	Biofilm formation (predicted)
sulA	Stress response protein
purH	Purine biosynthesis
ddpX	Cell wall biosynthesis
csgD	Curli regulator
ygeD	Lysophospholipid flippase
livK	Leucine ABC transporter
yggC	Unknown function
sufI	Stress-induced cell division protein

Table 4.1: List of genes predicted by sTarPicker to be targets of the sRNA RprA. We used the bioinformatic sRNA target prediction program sTarPicker (13) to predict target mRNAs of RprA. The program utilizes a two-step model for sRNA::mRNA hybridization, and predicted a long list of potential RprA targets; only the top ten predicted RprA targets are shown here.

Gene	p-value	Annotation	mRNA position
fliZ	2.96E-05	RpoS antagonist; putative regulator of FliA activity	246-263
aroE	2.97E-05	dehydroshikimate reductase NAD(P)-binding	230-259
rpoS	4.26E-05	RNA polymerase sigma S (sigma 38) factor	67-107
glnS	6.14E-05	glutamyl-tRNA synthetase	155-198
cysD	0.00015014	sulfate adenylyltransferase subunit 2	103-131
phoU	0.000494413	negative regulator of PhoR/PhoB two-component regulator	157-192
sulA	0.00068234	SOS cell division inhibitor	212-245
csgA	0.000843941	curlin subunit amyloid curli fibers cryptic	82-99
pal	0.000909008	peptidoglycan-associated outer membrane lipoprotein	30-75
yhjJ	0.001352229	putative periplasmic M16 family chaperone	153-204

Table 4.2: List of genes predicted by copraRNA to be targets of RprA. We used the bioinformatic sRNA target prediction program copraRNA (13) to predict target mRNAs of RprA. The program utilizes both conventional sRNA::mRNA target prediction as well as bioinformatic prediction of interaction conservation between orgamisnms, and predicted a long list of potential RprA targets; only the top ten predicted RprA targets are shown here.

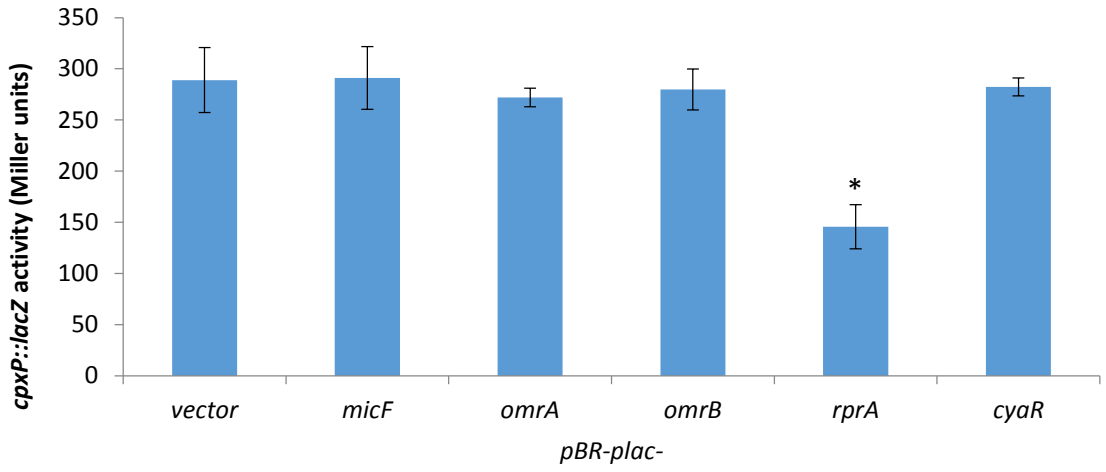

Figure 4.1: Overexpression of the small RNA gene *rprA* represses Cpx pathway activity. Strains carrying the pBR322 vector overexpressing *rprA*, *cyaR*, or three indirectly Cpx-regulated sRNAs *micF*, *omrA* and *omrB* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium, grown for 5 hours at 37^0C while shaking, and induced with 0.1 mM IPTG after 3 hours of growth. Cells were resuspended in 1X Z-buffer and activity of the *PcpxP::lacZ* reporter was recorded by measuring A_{420} with a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between said replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference from the vector control strain ($P<0.05$, one-way ANOVA test).

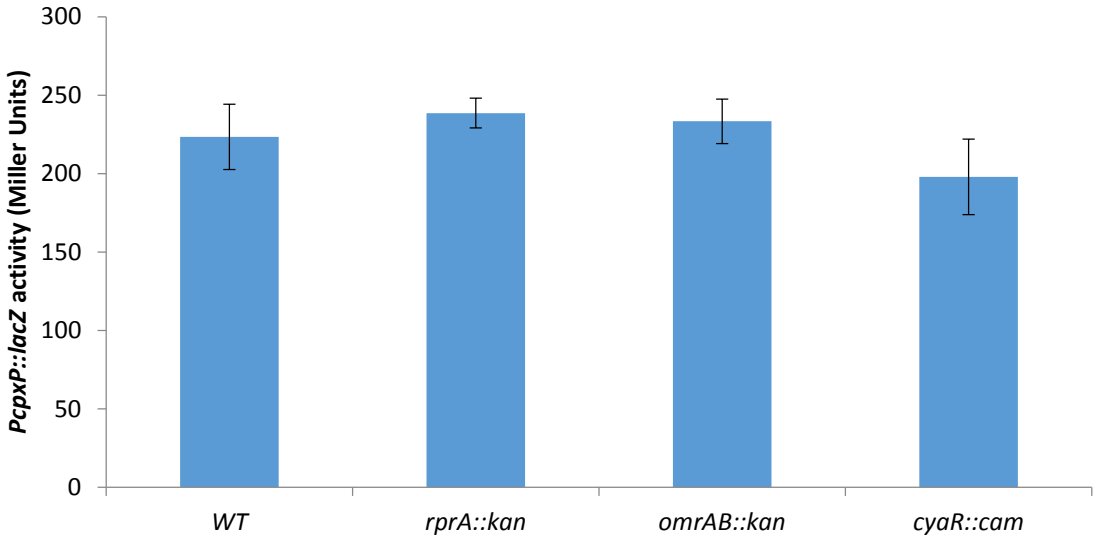

Figure 4.2: Deletion of *rprA* has no effect on Cpx pathway activity in the absence of prior induction of *rprA* expression. Strains bearing deletions of *rprA, omrA/B,* or *cyaR* and a transcriptional *PcpxP::lacZ* reporter were subcultured 1:50 from overnight culture into fresh LB medium and grown for 5 hours at 37^0C while shaking, and were induced after 3 hours of growth. Subsequently the cells were resuspended in 1X Z-buffer and *PcpxP::lacZ* activity was determined with the plate reader as A_{420}. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. None of these mutants were statistically significant from WT ($P<0.05$, one-way ANOVA tests).

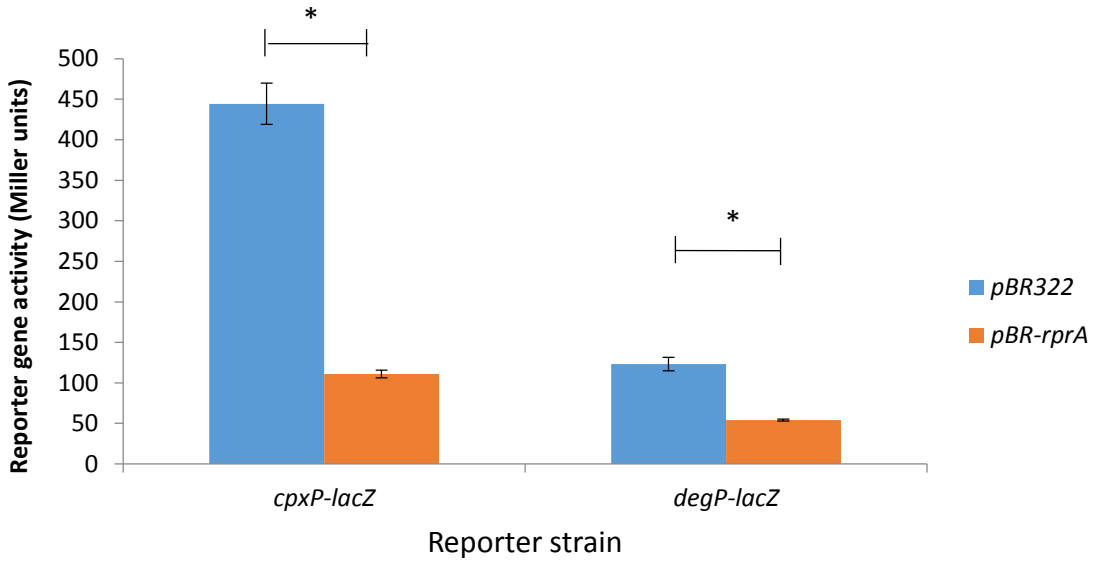

Figure 4.3: Overexpression of *rprA* inhibits two Cpx-regulated reporter genes. Strains bearing either *PcpxP::lacZ* or *PdegP::lacZ* transcriptional reporter genes and overexpressing *rprA* or the pBR322 vector control were subcultured 1:50 from overnight culture into fresh LB medium and grown for 5 hours at 37^{0}C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cells were resuspended in 1X Z-buffer and reporter gene activity was measured as A_{420} with a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, one-way ANOVA tests).

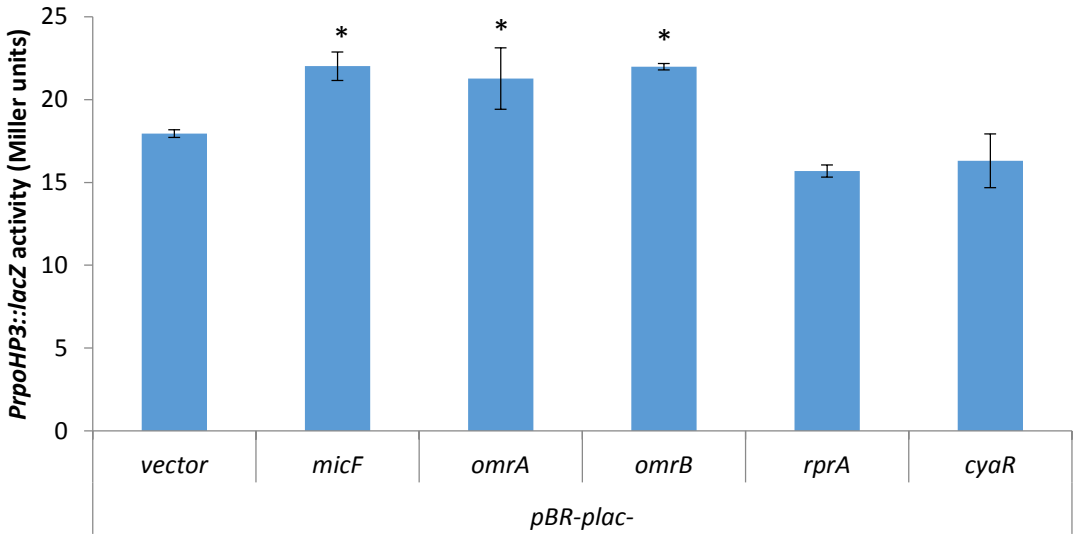

Figure 4.4: Overexpression of the small RNA gene *rprA* does not affect *rpoHP3::lacZ* activity. Strains overexpressing *rprA*, *cyaR*, or three indirectly Cpx-regulated sRNAs *rprA*, *omrA* and *omrB* or a pBR322 vector control and bearing an *rpoHP3::lacZ* transcriptional reporter were subcultured 1:50 from overnight culture into fresh LB medium, and grown for 5 hours at 37^0C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently, cells were resuspended in 1X Z-buffer and activity of the *rpoHP3::lacZ* reporter was measured using a plate reader as A_{420}. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference from the vector control strain ($P<0.05$, one-way ANOVA test).

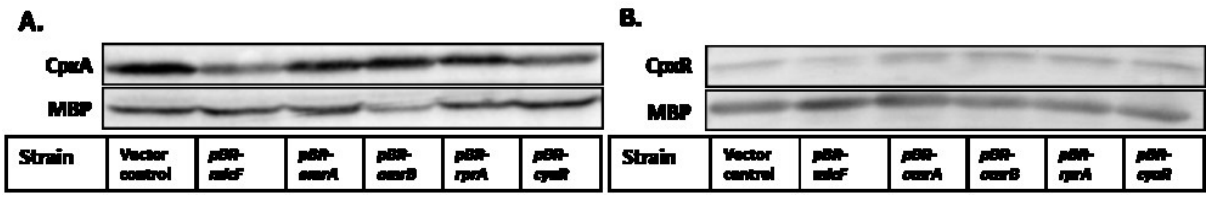

Figure 4.5: Overexpression of *rprA* does not affect CpxA or CpxR protein levels. Strains overexpressing *micF, omrA, omrB, rprA, cyaR* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium and were grown for 5 hours at 37^0C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cells were resuspended in 100 mL 2X Sample buffer, boiled for 5 minutes, then loaded into a 10% acrylamide gel (29:1 BIS). Following electrophoresis, protein was electrotransfered overnight to a nitrocellulose membrane and blotted with anti-CpxA-MBP or anti-CpxR-MBP, washed, then blots were exposed to a BioRad ChemiStar chemiluminescence kit (BioRad) and visualized with a chemiluminescent imager (BioRad). Results are representative of two independent experiments.

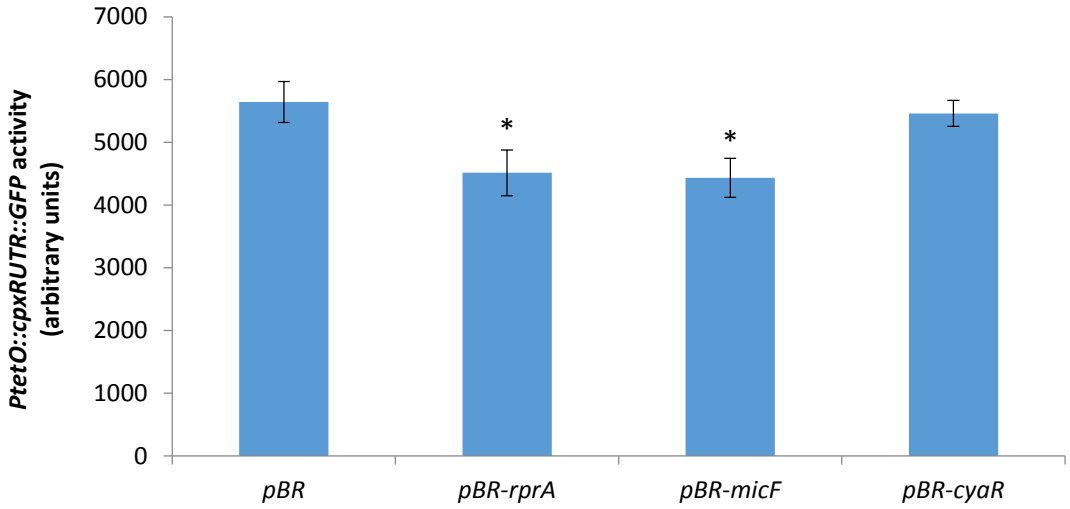

Figure 4.6: Effect of overexpressing *rprA* on *cpxR::GFP* translational fusion activity. Strains bearing a pXG10-*cpxR::GFP* translation fusion and overexpressing *micF, rprA, cyaR* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium in a 96-well plate and were grown for 5 hours at 37^0C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently *cpxR::GFP* translational fusion activity was measured as A_{509} using the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference from the vector control strain ($P<0.05$, one-way ANOVA test).

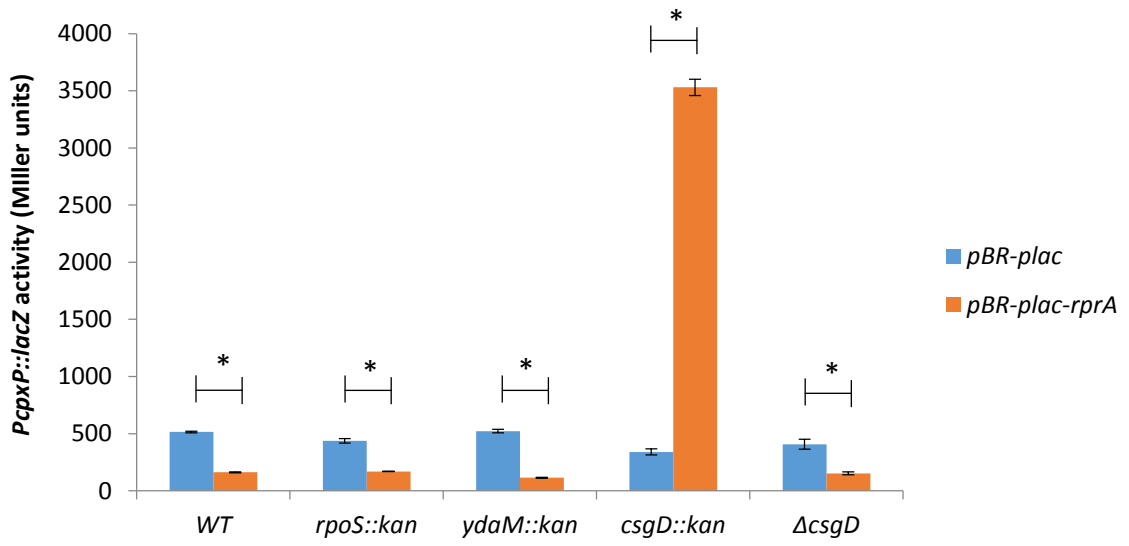

Figure 4.7: Cpx pathway inhibition by RprA is not dependent on its known target genes *rpoS, csgD,* or *ydaM*. Strains bearing a *PcpxP::lacZ* transcriptional reporter and mutations in these genes and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight culture into fresh LB medium and grown at 37^0C while shaking for 5 hours, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cells were resuspended in 1X Z-buffer, and *PcpxP::lacZ* activity was measured with a plate reader as A_{420}. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, one-way ANOVA tests).

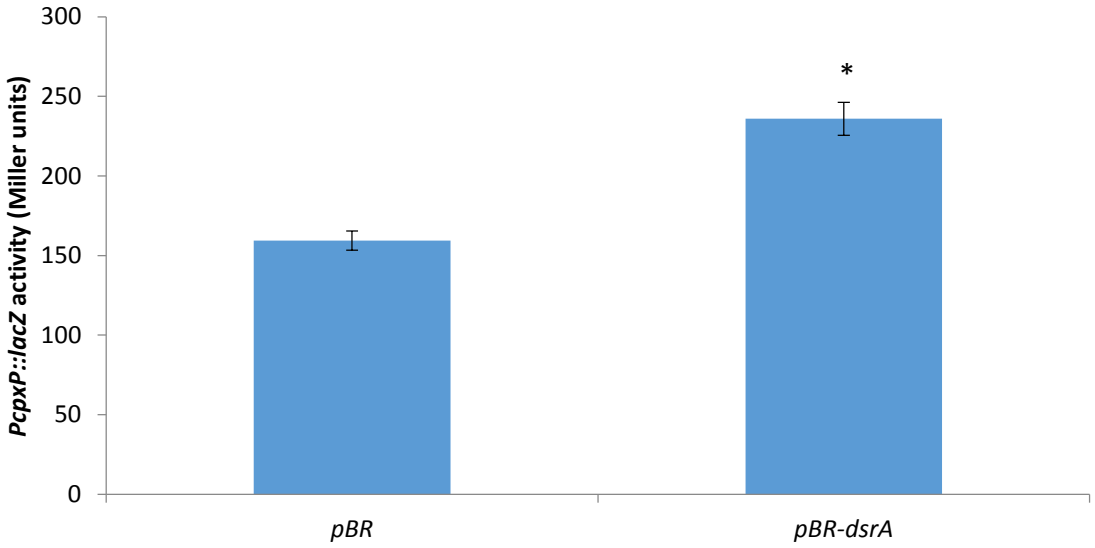

Figure 4.8: Overexpression of the small RNA gene *dsrA* activates the Cpx pathway activity. Strains bearing a *PcpxP::lacZ* transcriptional reporter and overexpressing *dsrA* or a pBR322 vector control were subcultured 1:50 from overnight culture into fresh LB medium, and grown for 5 hours at 37^0C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently, cells were resuspended in 1X Z-buffer and activity of the *PcpxP::lacZ* reporter was measured as A_{420} using a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference from the vector control strain ($P<0.0001$, unpaired t-test).

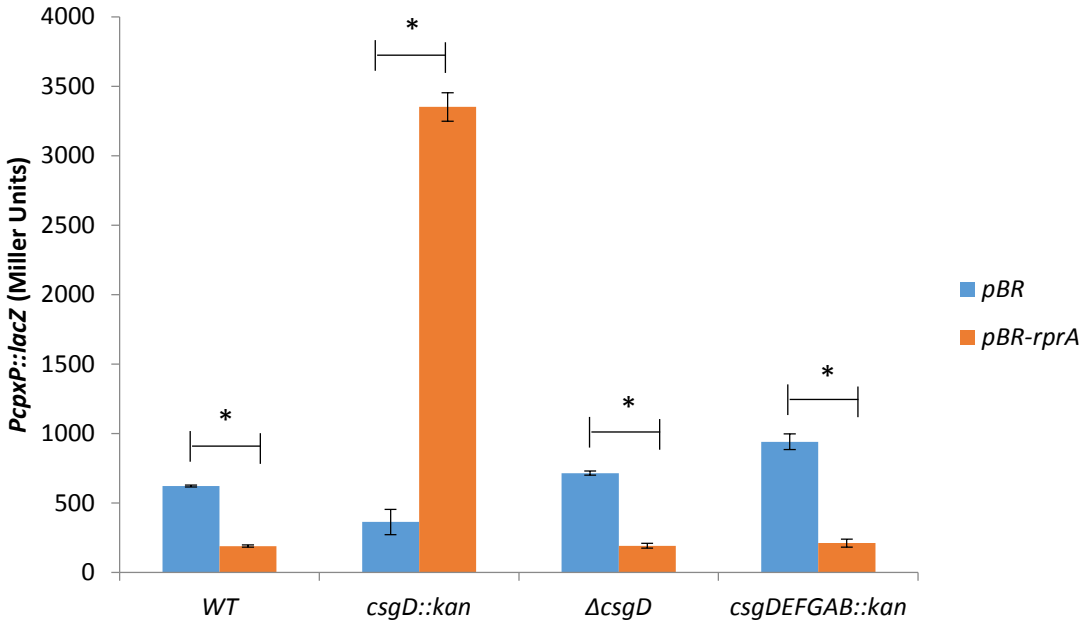

Figure 4.9: Effect of *csgD::kan* mutation on RprA inhibition is not due to disruption of downstream *csg* operon. Strains bearing a *PcpxP::lacZ* transcriptional reporter and an insertional mutation of *csgD,* a clean deletion of *csgD,* or a deletion of the *csg* operon and overexpressing pBR-*rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium and were grown for 5 hours at 37^0C while shaking, and cultures were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cultures were resuspended in 1X Z-buffer and activity of the *PcpxP::lacZ* reporter was measured as A_{420} using the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

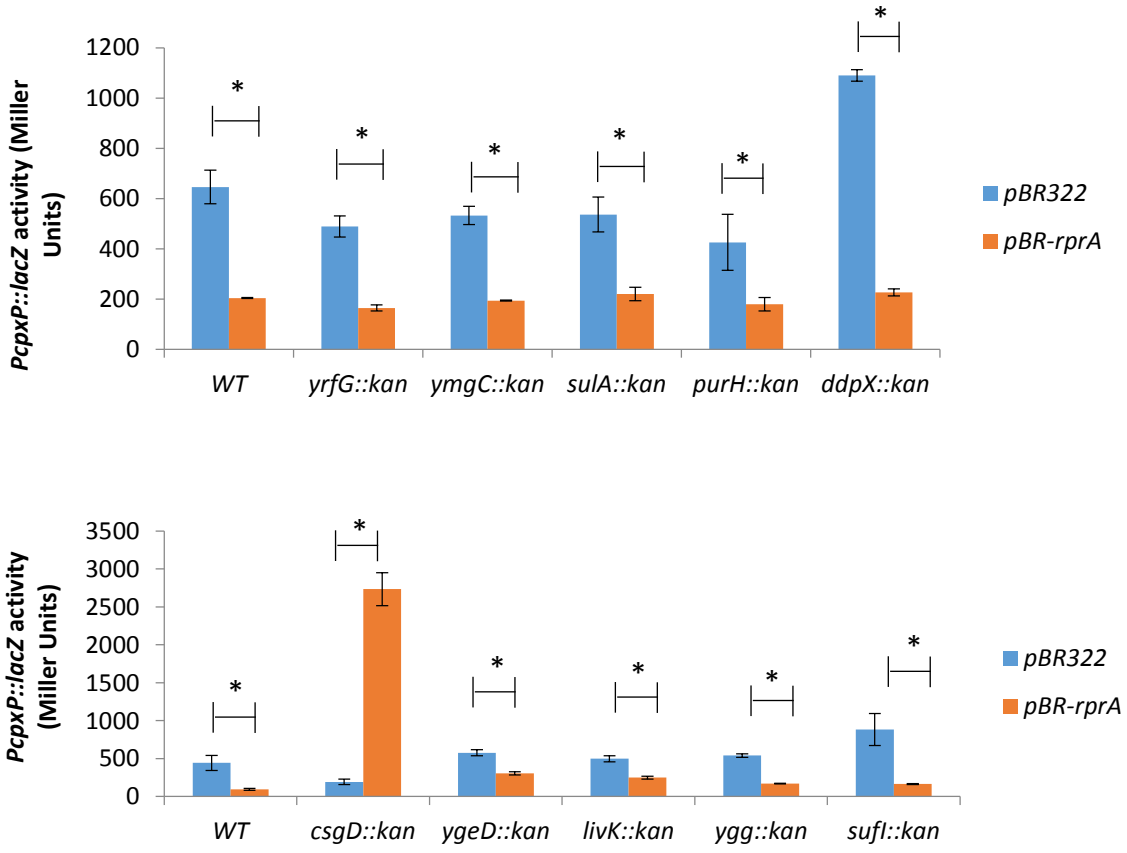

Figure 4.10: Cpx pathway inhibition by RprA is independent of several predicted target genes. Strains bearing mutations in genes predicted by target prediction alghorithm "STaRPicker" to bind RprA and overexpressing pBR-*rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 5 hours at 37^{0}C while shaking, and were induced with 0.1 mM IPTG after 3 hours. Cultures were then resuspended in 1X Z-buffer and *PcpxP::lacZ* activity was measured as A_{420} using a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

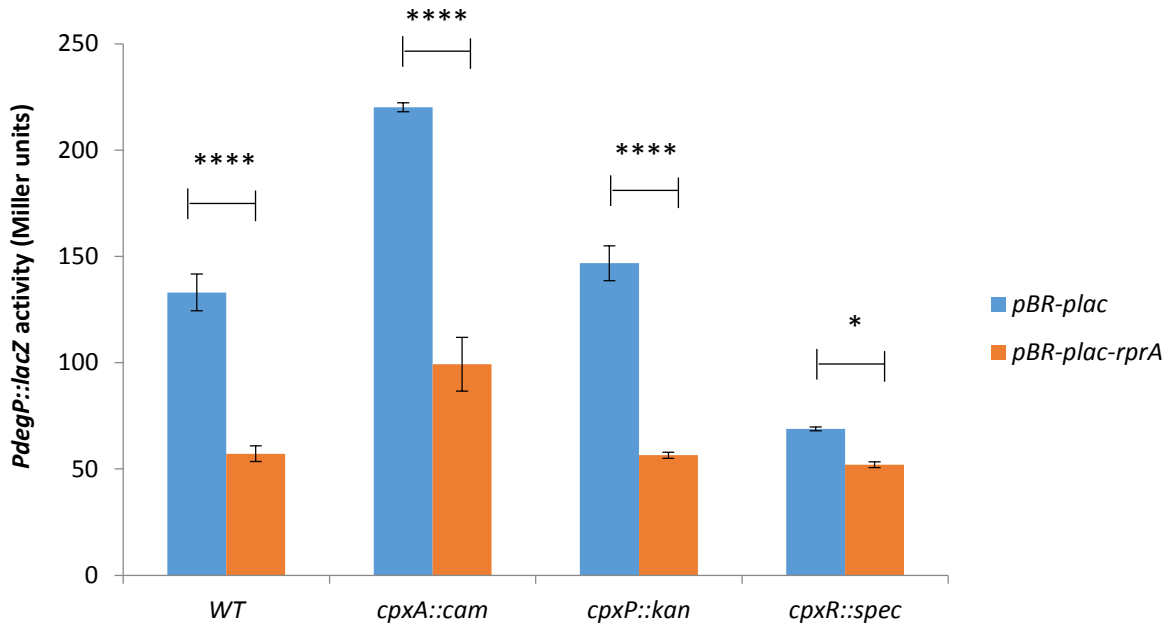

Figure 4.11: Cpx pathway inhibition by RprA is independent of CpxA and CpxP but dependent on CpxR. Strains bearing a *PdegP::lacZ* transcriptional reporter and mutations in *cpxA, cpxP,* or *cpxR* and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 5 hours at 37^{0}C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cultures were resuspended in 1X Z-buffer and *PdegP::lacZ* activity was measured with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

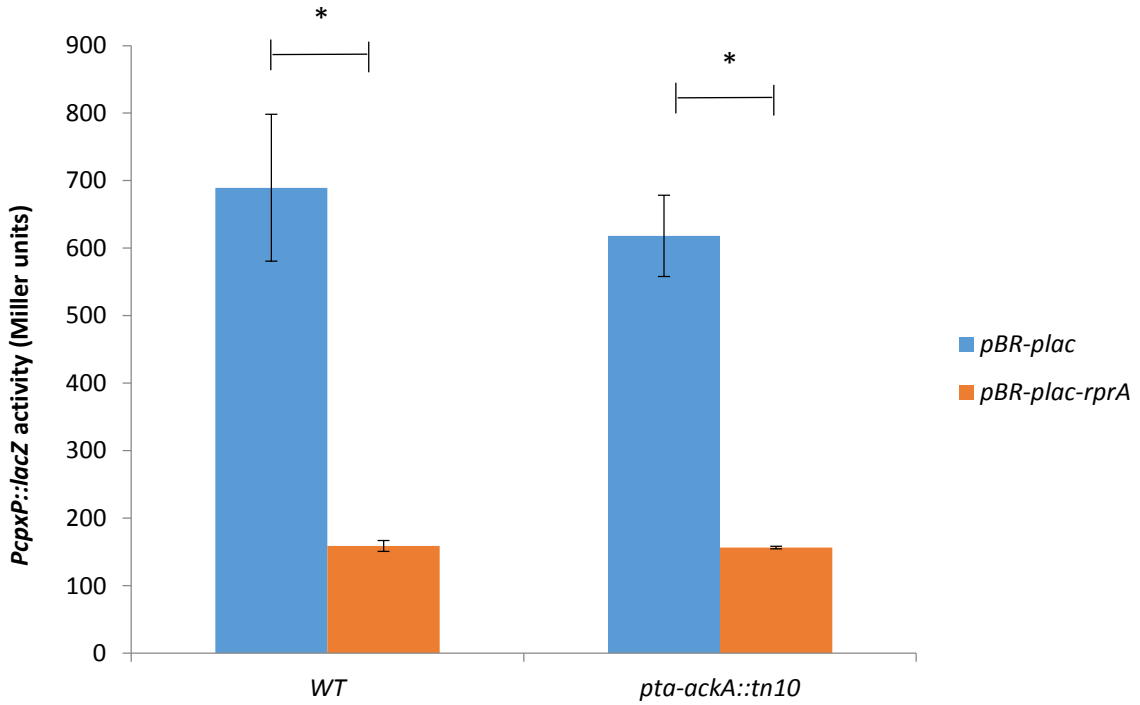

Figure 4.12: Cpx pathway inhibition by RprA is independent of the small molecular weight phosphodonor acetyl phosphate. A strain bearing a deletion of the *pta* and *ackA* operons and a *PcpxP::lacZ* transcriptional fusion and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 5 hours at 37^{0}C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cultures were resuspended in 1X Z-buffer and *PcpxP::lacZ* activity was measured as A_{420} with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

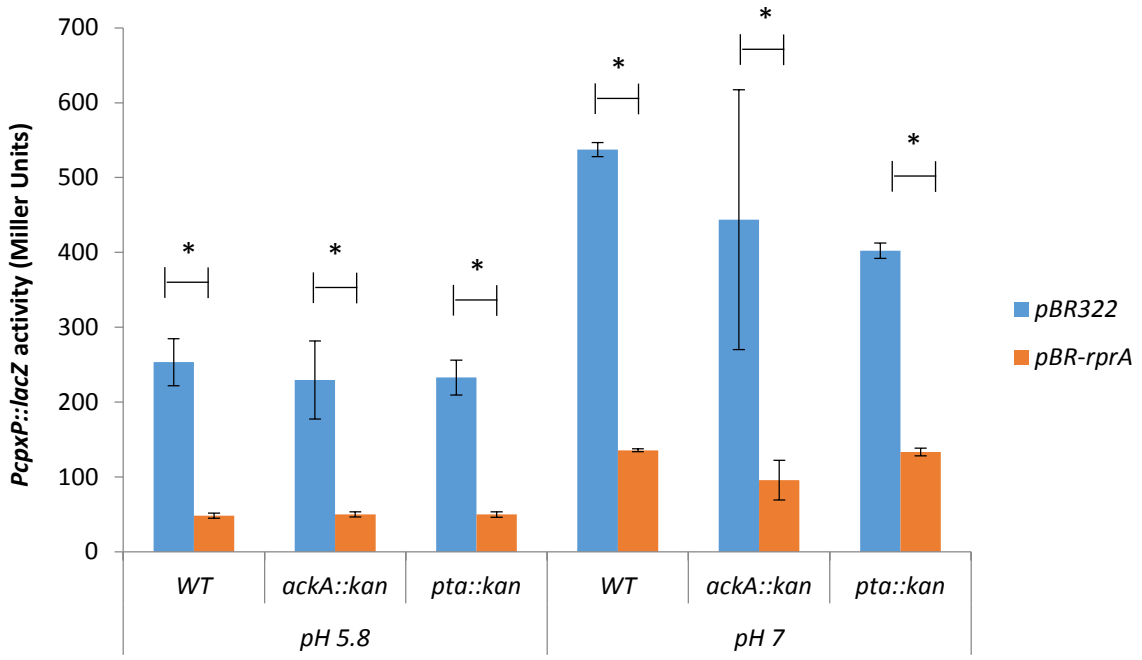

Figure 4.13: Cpx pathway inhibition by RprA is independent of the small molecular weight phosphodonor acetyl phosphate at both pH 5.8 and pH 7. A strain bearing a deletion of the *pta* and *ackA* operons and a *PcpxP::lacZ* transcriptional reporter and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium equilibrated to pH 5.8 or pH 7 with sodium phosphate buffers and grown for 5 hours at 37^{0}C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cultures were resuspended in 1X Z-buffer and *PcpxP::lacZ* activity was measured as A_{420} with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

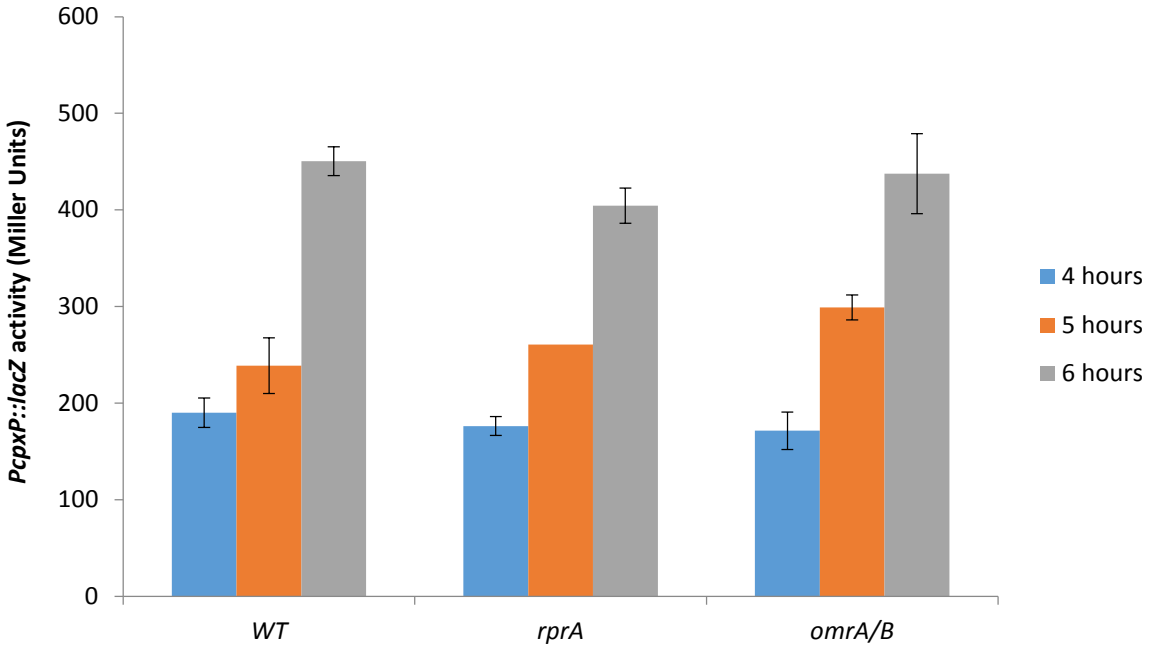

Figure 4.14: Deletion of *rprA* does not affect growth-dependent Cpx pathway activation. Strains bearing a *PcpxP::lacZ* transcriptional reporter and deletions of *rprA* or *omrA/B* were subcultured 1:50 from overnight culture into fresh LB medium and grown for 4-6 hours at 37^{0}C while shaking, and were induced hours prior to resuspension in all cases. Subsequently the cells were resuspended in 1X Z-buffer and *PcpxP::lacZ* activity was measured as A_{420} with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. There were no statistically significant differences between strains at any time points ($P<0.05$, one-way ANOVA tests).

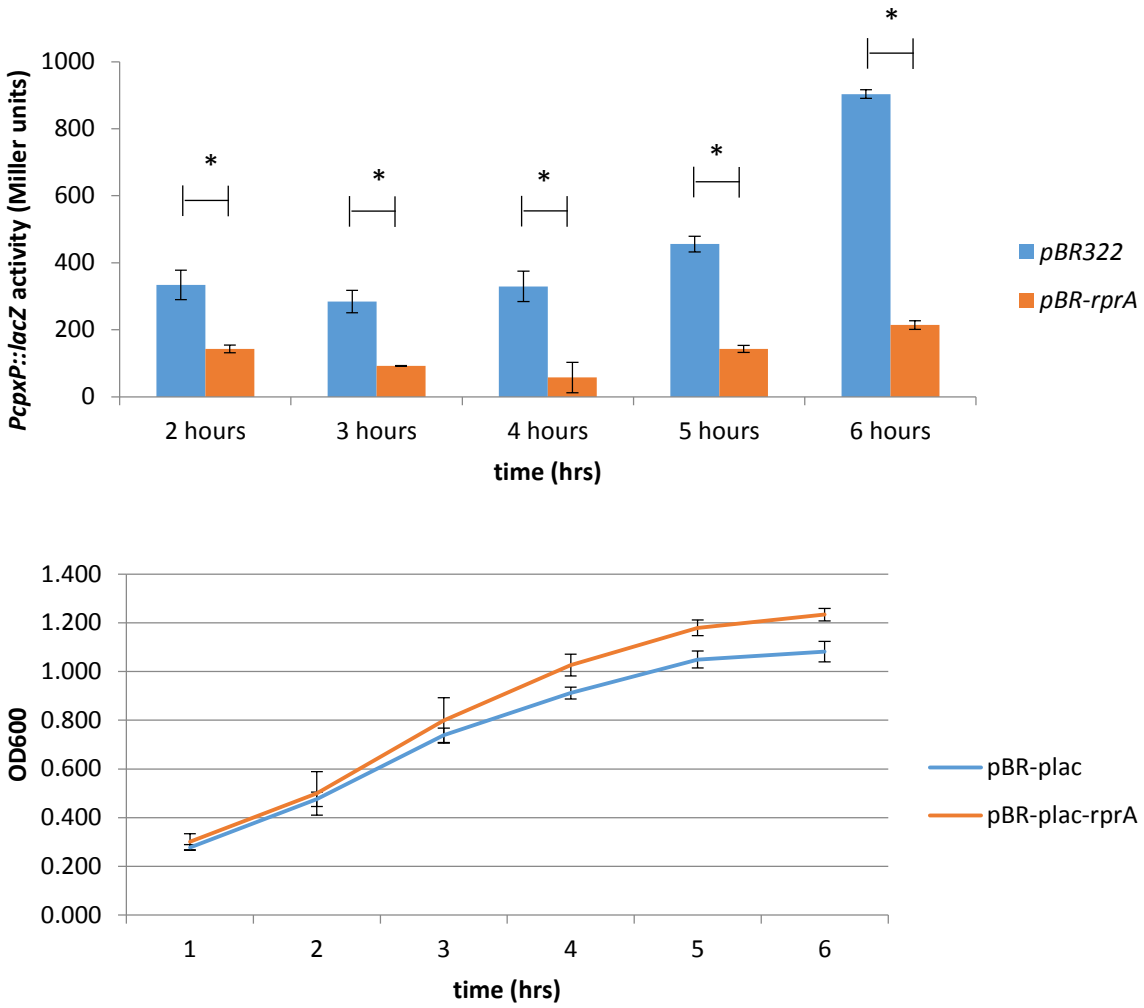

Figure 4.15: RprA inhibits the Cpx pathway in both exponential and stationary phase. *E. coli* MC4100 bearing a *PcpxP::lacZ* transcriptional reporter and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 6 hours. Cultures were resuspended in 1X Z-buffer in one hour intervals starting at 2 hours, and were induced with 0.1 mM IPTG two hours before resuspension in all cases. Subsequently *PcpxP::lacZ* activity and OD600 was measured as A_{420} with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

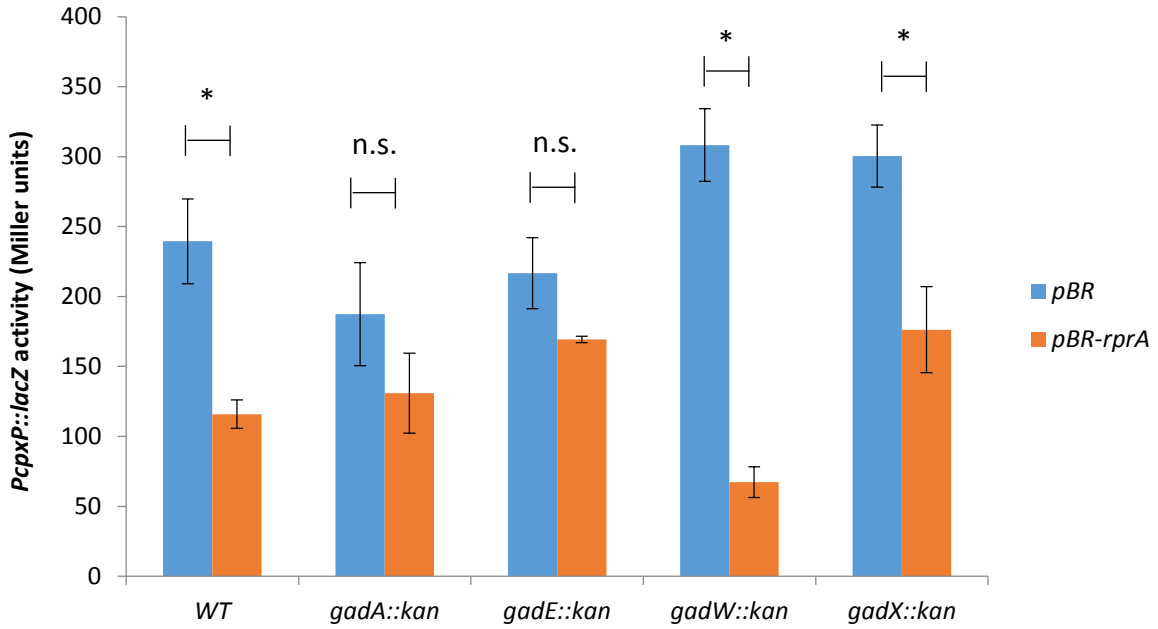

Figure 4.16: Cpx pathway inhibition by RprA is partially dependent on the GadA and GadE of the GDAR acid stress resistance pathway. Strains bearing a *PcpxP::lacZ* transcriptional reporter and mutations in *gadA, gadE, gadW* or *gadX* and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight culture into fresh LB medium and grown at 37^0C while shaking for 5 hours, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently cells were resuspended in 1X Z-buffer, and *PcpxP::lacZ* activity was measured as A_{420} with a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA tests).

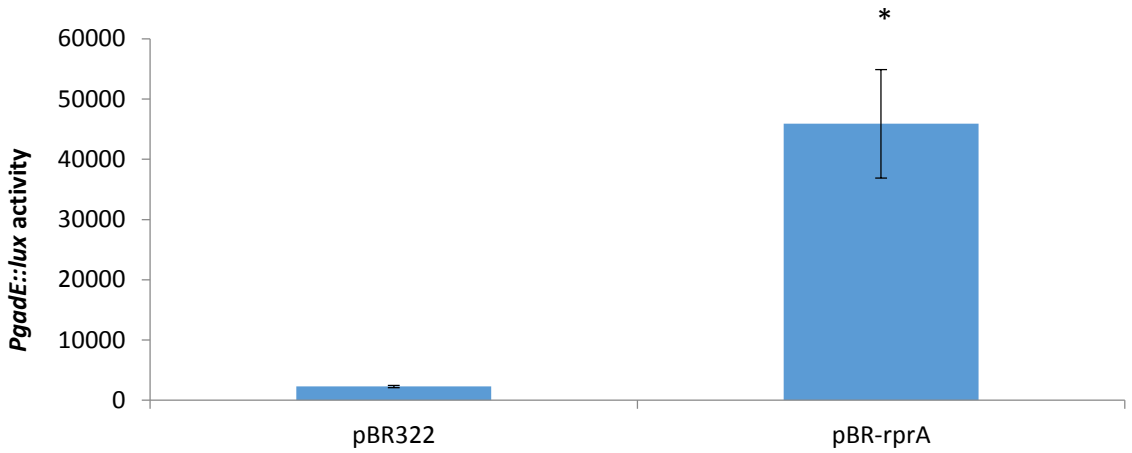

Figure 4.17: RprA is an activator of GDAR pathway activity. Strains overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium in a 96-well plate and grown at 37^0C while shaking for 5 hours. *rprA* overexpression was induced by addition of 0.1 mM IPTG upon subculturing. Subsequently *PgadE::lux* activity was measured for each strain by recording CPS absorbance values and adjusting for cell density. Bars represent average values between three replicates, and error bars represent standard deviation between said replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference compared to the vector control strain ($P<0.05$, two-way ANOVA tests).

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CHAPTER 5

THE CPX RESPONSE AND RPRA REGULATE COMMON PHENOTYPES

5.1 Introduction

In the previous chapter we demonstrated that RprA has a role as a feedback inhibitor of the Cpx response by inhibiting the activity of the response regulator CpxR. However, it also interested us to explore whether or not RprA contributed to any known phenotypes associated with the Cpx response. Here we show that RprA promotes two phenotypes associated with activation of the Cpx response, reduced motility and increased amikacin resistance, demonstrating that RprA acts both as a feedback inhibitor of the Cpx pathway as well as a regulator of cellular processes that are regulated by the Cpx response itself. We also demonstrate that RprA does not act as regulatory agent of the Rcs phoshorelay to repress Cpx pathway activity, as several Rcs pathway inducing cues do not repress Cpx pathway activity, regardless of the presence of a functional copy of *rprA*.

5.2 RprA does not mediate Cpx repression by the Rcs phosphorelay

rprA transcription is mediated primarily by activation by RcsB of the Rcs phosphorelay (9), and therefore we reasoned that activation of the Rcs response might lead to inhibition of the Cpx pathway through RprA. Previously Evans *et al.* demonstrated a potential connection between the Cpx pathway and Rcs pathways: deletion of several penicillin binding proteins (PBPs) induced both pathways, although Rcs pathway induction was dependent on a functional copy of CpxR (4). To test whether Rcs activation of RprA might represent a second link between the Cpx and Rcs responses, we overexpressed the small outer membrane lipoprotein protein RcsF, which is an inducer of the Rcs phosphorelay (6), from an IPTG-inducible pCA24N plasmid and assayed for changes in activity of the Cpx-responsive *PcpxP::lacZ* transcriptional fusion using a β-galactosidase assay. Surprisingly, *rcsF* overexpression caused a mild increase in Cpx pathway activity (Figure 5.1). In addition to this, two other Rcs inducing cues, high

osmolarity and high salinity did not repress Cpx pathway activity but rather resulted in mild induction, as has previously been described (8) (Figure 5.2). Thus, these findings do not support the hypothesis that RprA acts to repress the Cpx pathway upon Rcs phosphorelay induction.

5.3 RprA and the Cpx response both affect amikacin resistance

Mutational induction of the Cpx pathway has long been known to endow *E. coli* resistance to the aminoglycoside antibiotic amikacin (2), although the mechanism of this resistance has remained elusive. Since aminoglycoside resistance can be caused by depletion of the cell's proton motive force (PMF) (1), we thought it possible that *rprA* overexpression might actually decrease amikacin resistance through upregulation of GDAR activity, since GadAB decarboxylase activity has been linked to increased PMF in bacteria (5). *rprA* was overexpressed from the inducible pBR322 plasmid and this strain was grown in LB broth containing varying concentrations of amikacin for 8 hours at 37°C. Surprisingly, in LB medium containing 1.5 μg/mL amikacin, *rprA* overexpression increased survival dramatically, while wild-type bacteria did not grow whatsoever (Figure 5.3). To determine whether this effect was dependent on putative RprA regulation of the GDAR pathway, we next tested whether or not overexpression of *rprA* continued to cause this phenotype in *E. coli* MC4100 *gadA* or *gadE* mutants at the same concentration of amikacin. Deletions of either *gadA* or *gadE* did not prevent amikacin resistance caused by *rprA* overexpression, although deletion of these genes alone did increase amikacin resistance on its own (Figure 5.3), which is concurrent with previous findings that GadAB decarboxylase activity increases cellular PMF. Therefore these data suggest that the effect RprA has on amikacin resistance appears to be independent of its proposed role in upregulating the GDAR acid stress response, and more work must be done to determine the mechanism of RprA's amikacin resistance phenotype.

5.4 RprA and the Cpx response both affect swimming motility

If *rprA* overexpression causes depletion of the cell's proton motive force (PMF) as is suggested by the increased amikacin resistance upon its overexpression, then it follows that PMF-dependent processes might be affected by its overexpression as well. Swimming motility is a process which is known to be dependent on cellular PMF, as rotation of the flagellum dependent on a proton gradient (7). To test whether swimming motility is affected upon overexpression of *rprA,* overnight cultures of strains bearing pBR-*rprA* or a vector control were inoculated into 0.3% agar plates, and the next day swim diameters were measured to determine the effect of RprA on swimming motility. These assays showed that overexpression of *rprA* indeed decreases swimming motility nearly as much as overexpression of *omrA*, which is known to repress motility (3) (Figure 5.4). Whether or not this phenotype is a direct result of decreased PMF or perhaps repression of flagellum biogenesis cannot, however, be gleaned from these results, and deserves further investigation.

5.5 RprA and the Cpx response both affect adaptation to acid pH

The observation that RprA positively regulates the GDAR response but negatively regulates the Cpx response suggests that Cpx pathway activity might be detrimental at extreme acid pH. To test this hypothesis, strains bearing *cpxR::spec* and constitutively active *cpxA24* mutations were grown for 4 hours, challenged for an hour at pH 2.9, then spotted onto non-selective media. Compared to wild-type *E. coli*, the constitutively active *cpxA24* strain had significantly lower survival when exposed to acid challenge (Figure 5.5), which suggests Cpx pathway activation is indeed deleterious under acid challenge conditions. The *cpxR* mutant did not, however, exhibit improved growth after acid stress, which might simply mean that basal Cpx activity is not deleterious in acid stress conditions.

If Cpx induction is disadvantageous under acid stress conditions, then it follows that in absence of mutational Cpx induction the pathway should be repressed under acid stress conditions. To determine if this is true, *PcpxP::lacZ* transcriptional reporter activity was assayed immediately after acid challenge. Activity of this reporter and thus of the Cpx pathway was shut off almost entirely after acid challenge (Figure 5.6), suggesting that repression of Cpx pathway activity is beneficial during extreme acid stress. Since we showed that RprA inhibition of the Cpx response is partially dependent on the GDAR pathway, we performed the same experiment in GDAR defective mutants (*gadA, gadE*) to see if the GDAR pathway is responsible for the Cpx pathway shutoff seen in acid challenge conditions. We found, however, that acid shut-off of the Cpx response was not dependent on the GDAR pathway, as mutants in *gadA* or *gadE* still exhibit a severe decrease in *PcpxP::lacZ* activity following acid challenge (Figure 5.6).

5.6 Conclusions and future directions

These experiments have demonstrated that *rprA* overexpression causes two phenotypes known to be associated with the Cpx response: amikacin resistance and motility. Amikacin resistance has long been known to be endowed by mutational induction of the Cpx response (2), and motility has recently been shown to be directly Cpx regulated through inhibition of *flhDC* (10). One thing that is important to note is that these experiments do not demonstrate that RprA is *responsible* for these Cpx phenotypes, but rather that RprA activation and Cpx activation cause common phenotypes. In fact, work by Erin MacKinnon, an undergraduate in the Raivio lab, has shown that the amikacin resistance phenotype caused by mutational induction of the Cpx response by *cpxA24* is not dependent on a functional copy of *rprA* (data not shown). Thus it seems that either RprA and CpxR~P act independently to increase amikacin resistance, or RprA is redundant in this role, and there are other Cpx-regulated genes that also cause this phenotype.

It's also not known at this time how RprA is inducing amikacin resistance. Our results argue against a role for GDAR in this effect, because RprA upregulates GDAR and deletion of GDAR genes actually increases amikacin resistance. It seems entirely likely that RprA upregulation of *rpoS,* which encodes the general stress sigma factor, might be the cause of this increased resistance, and this remains to be tested by mutational analyses. In order to determine whether or not the repression of motility by the Cpx pathway is dependent on *rprA,* experiments must be done to test whether or not mutational induction of the Cpx response by *cpxA24* still represses motility in the absence of a functional copy of *rprA*. Virtual footprinting (http://www.prodoric.de/vfp) of the *flhD* promoter (whose product was putatively downregulated in our transcriptomic study) failed to locate a potential CpxR binding site, thus it is appealing to hypothesize that RprA might be responsible for the observed motility repression by the Cpx pathway. Taken together though, these experiments suggest that RprA has a role in the Cpx regulon not only to act in feedback inhibition of the response, but also to promote two known Cpx-related phenotypes.

Our data suggest that Cpx pathway induction is indeed deleterious under acid challenge conditions, and concurrent with this, Cpx pathway activity appears to be entirely repressed in acid challenge conditions. Unfortunately, this effect was found not to be dependent on the GDAR response or RprA itself, ruling out what made an attractive model of a regulatory loop. One possible explanation for this phenotype, then, is that perhaps the GDAR acid stress response is not the only acid stress response which impacts Cpx pathway activity under acidic conditions. While GDAR is thought to be the most important system for overcoming extreme acidic conditions in *E. coli*, several other AR systems exist, such as the lysine- and arginine-dependent acid stress response (LDAR and ADAR) (7). Therefore it is plausible that one or more of these

other acid stress responses impacts Cpx pathway activity in acidic conditions, but this of course remains to be tested. Finally, much work must be done to demonstrate why Cpx pathway activity is actually deleterious under acidic conditions; if Cpx pathway activity is under such severe repression under acidic pH, activity of the Cpx pathway must interfere with survival in some egregious way.

5.7: Figures

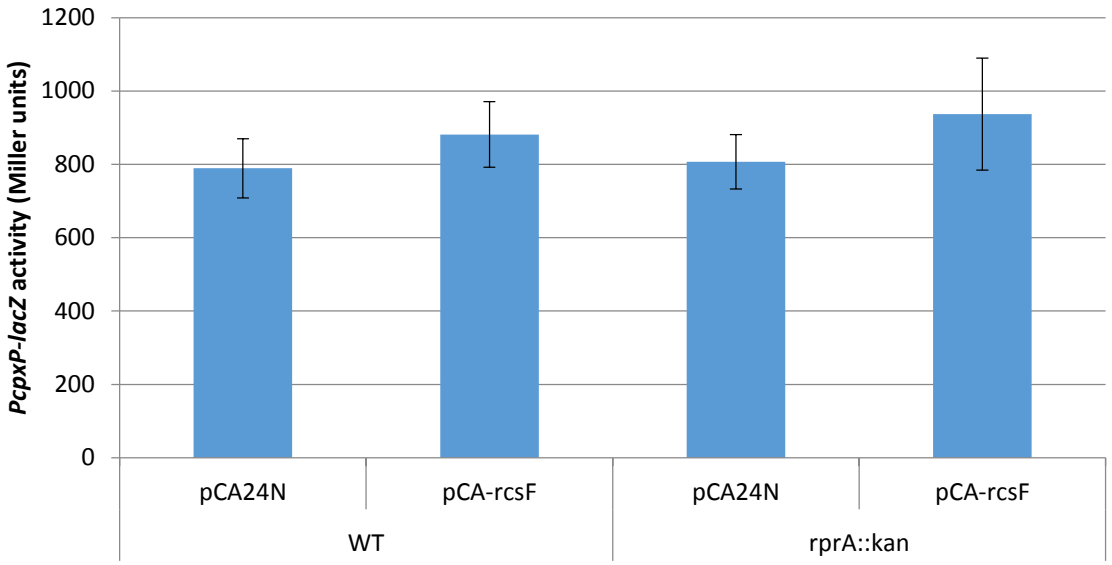

Figure 5.1: Overexpression of Rcs pathway inducer *rcsF* does not substantially affect Cpx pathway activity. Wildtype and *rprA::kan* mutant strains bearing a *PcpxP::lacZ* transcriptional fusion and the pCA-*rcsF* overexpression vector or a pCA24N vector control were subcultured 1:50 from overnight culture into fresh LB medium, and grown for 5 hours at 37^0C while shaking, and were induced with 0.1 mM IPTG upon subculturing. Subsequently, cells were resuspended in 1X Z-buffer and activity of the *PcpxP::lacZ* reporter was measured as A_{420} using a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. There was no statistically significant difference in reporter gene activity upon *rcsF* overexpression in either strains ($P<0.05$, two-way ANOVA tests).

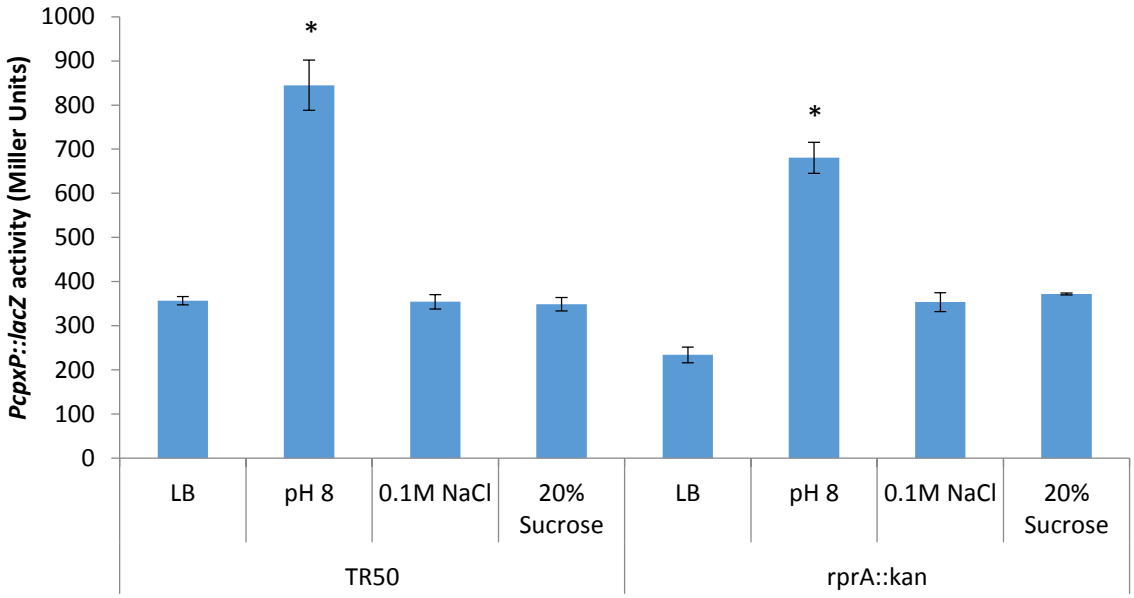

Figure 5.2: High osmolarity does not substantially affect Cpx pathway activity. Strains bearing a *PcpxP::lacZ* transcriptional reporter and an *rprA::kan* mutation were subcultured 1:50 from overnight culture into fresh LB medium equilibrated to pH 7 or 8 or containing 0.1M NaCl or 20% sucrose, and were grown for 5 hours at 37^0C while shaking. Subsequently, cells were resuspended in 1X Z-buffer and activity of the *PcpxP::lacZ* reporter was measured as A_{420} using a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference from the LB control ($P<0.05$, two-way ANOVA test).

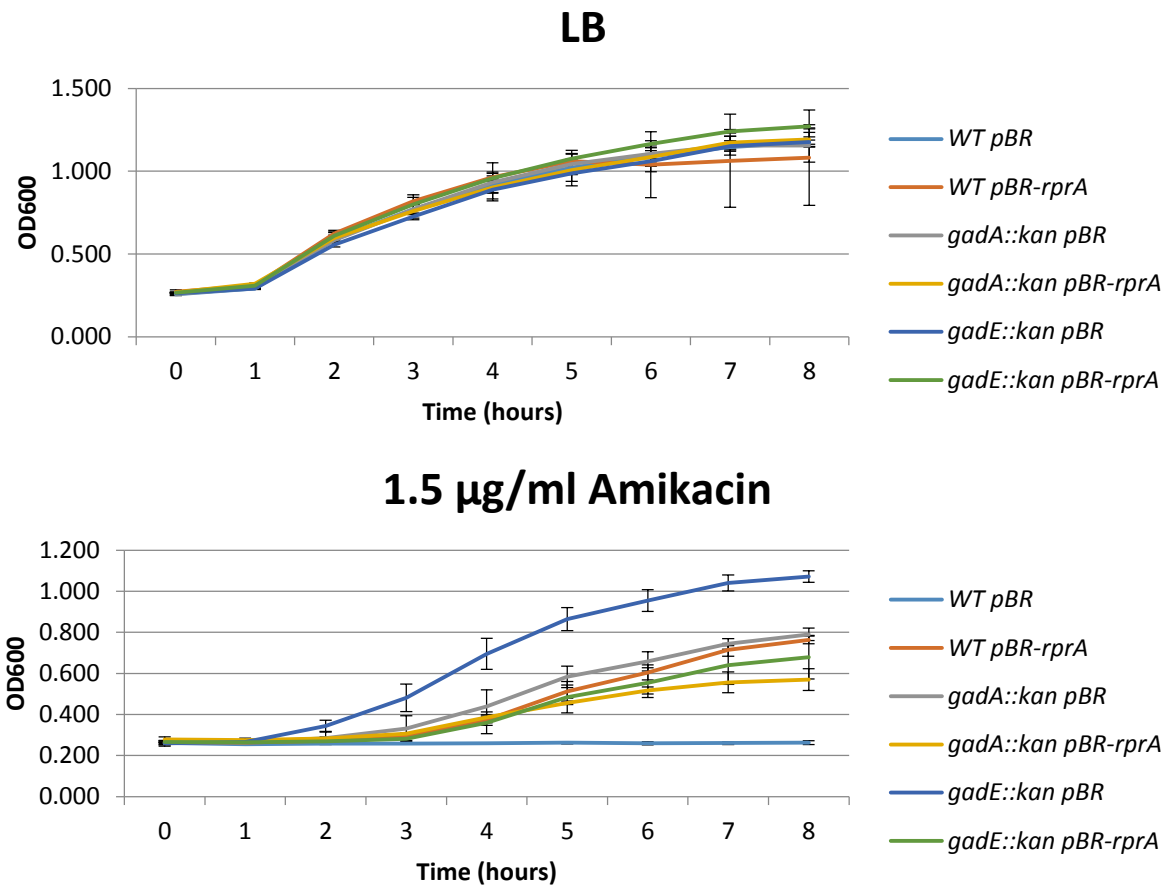

Figure 5.3: RprA-induced amikacin resistance is not *gad*-dependent. Strains bearing mutations in *gadA* or *gadE* and overexpressing *rprA* or a pBR322 vector control were subcultured 1:50 from overnight cultures into fresh LB medium containing 1.5 or 0 µg/ml amikacin, 100 µg/ml ampicillin and 0.1 mM IPTG, and were grown for 8 hours at 37^{0}C while shaking. OD_{600} was measured every hour with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments.

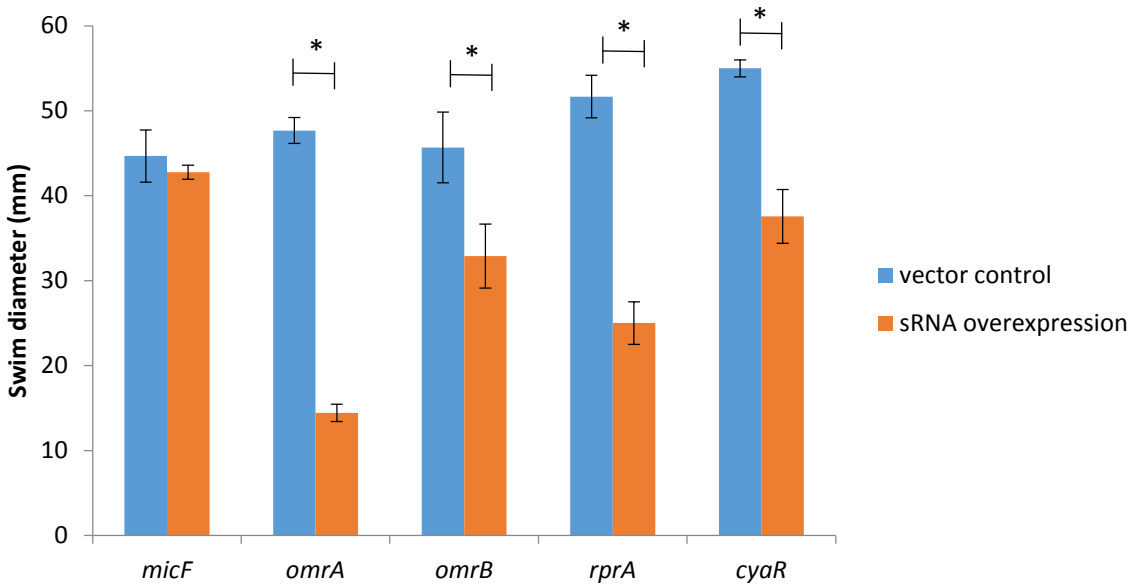

Figure 5.4: Overexpression of *rprA* decreases motility in *E. coli.* 10 µl of overnight cultures of *E. coli* MC4100 overexpressing *micF, omrA, omrB, rprA, cyaR* or a pBR322 vector control were stabbed into 1.5% agar plates containing 100 µg/ml ampicillin and allowed to grow for 14 hours at 37^{0}C. The next day the swim diameter of each strain was measured. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA test).

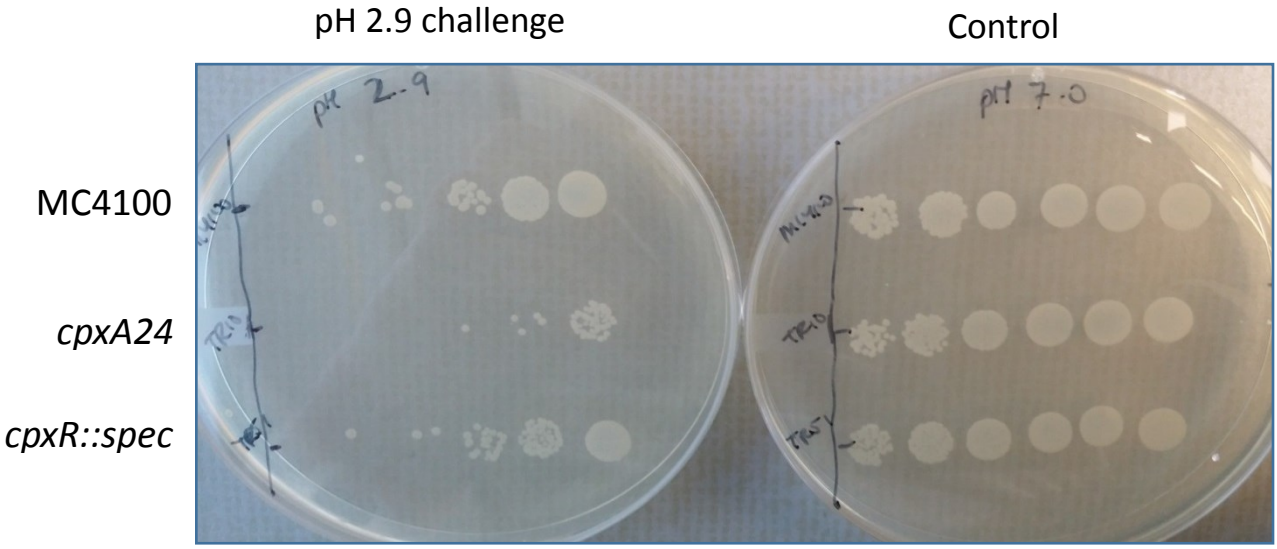

Figure 5.5: Cpx pathway induction decreases survival in acid challenge. Strains bearing *cpxA24* or *cpxR::spec* mutations were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 5 hours at 30^0C while shaking, were resuspended in LB equilibrated to pH 2.9 and grown for an additional hour at 30^0C while shaking. The cells were pelleted and resuspended in pH 7 LB, then were serially diluted in LB and 10 µl of each dilution was spotted onto non-selective medium. Results are representative of two independent experiments.

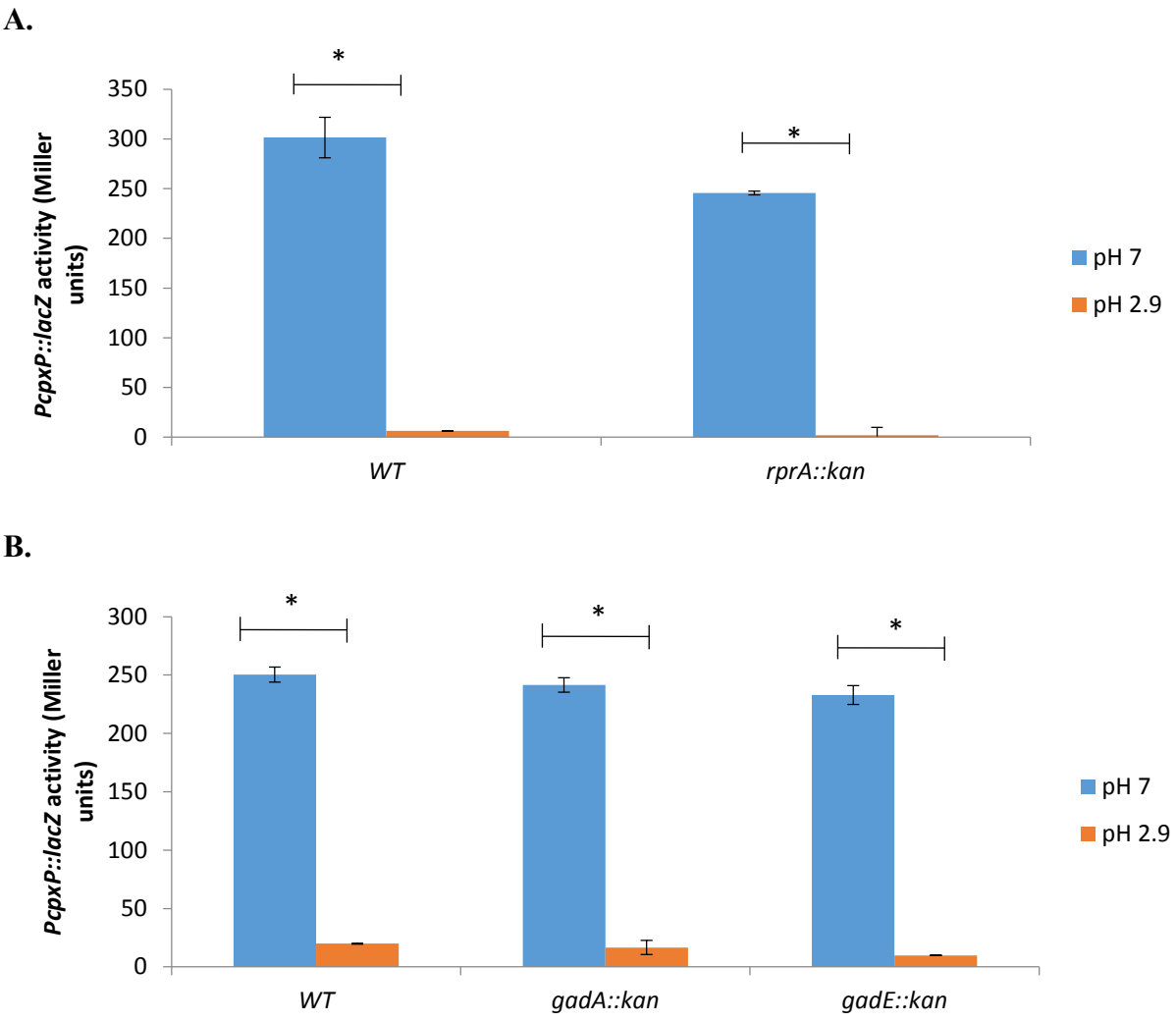

Figure 5.6: Acid challenge shuts off Cpx pathway activity in an *rprA*-independent manner. A) *E.coli* MC4100 wild-type and *rprA::kan* strains bearing a *PcpxP::lacZ* transcriptional reporter were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 5 hours at 37^{0}C while shaking. B) *E.coli* MC4100 wild-type, *gadA::kan*, and *gadE::kan* strains bearing a *PcpxP::lacZ* transcriptional reporter were subcultured 1:50 from overnight cultures into fresh LB medium and grown for 5 hours at 37^{0}C while shaking. Cells were pelleted, resuspended in LB equilibrated to pH 2.9 and grown for an additional hour at 37^{0}C while shaking. Subsequently cells were resuspended in pH 7 LB, then were resuspended in 1X Z-buffer, then were transferred to a 96-well plate and *PcpxP::lacZ* activity was measured as A_{420} with the plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. Results are representative of two independent experiments. Asterisks denote a statistically significant difference between strains ($P<0.05$, two-way ANOVA test).

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CHAPTER 6

DISCUSSION

6.1 Regulation of CyaR and RprA by the Cpx response

Here we have confirmed the results of our recent microarray (1) which suggest the Cpx envelope stress response regulates expression of the sRNA genes *cyaR* and *rprA*. We found that *cyaR* transcription is repressed roughly 2-fold in a *cpxA24* mutant in which the Cpx pathway is constitutively activated (Figure 3.1). Strangely, deletion of *cpxR*, which normally has the opposite effect as the *cpxA24* mutation on Cpx-regulated genes, caused a comparable decrease in *PcyaR::lux* expression. This prompted the hypothesis that the Cpx pathway might regulate *cyaR* transcription both directly and indirectly. Direct regulation would come in the form of promoter binding by and transcriptional upregulation by CpxR, which is supported by electrophoretic mobility shift assays (EMSAs) done by Randi Guest (2). On the other hand, indirect regulation of *cyaR* by the Cpx pathway is hypothesized to be caused by reduction of CRP-cAMP activity via downregulation of two glucose transporters, *uhpT* and *ptsG,* as indicated by results of the microarray (1). If this were the case, deletion of *cpxR* could lead to reduce *cyaR* transcription due to de-repression of these transporters resulting in increased glucose uptake, decreased cAMP levels, and thus lower levels of active CRP:cAMP, which is a transcriptional activator of *cyaR*. To test this theory, we made single *uhpT* and *ptsG* deletions as well as double *cpxR uhpT* and *cpxR ptsG* mutants to determine whether or not the *cyaR* repression observed in the *cpxR* mutant depended on these genes. Deletion of *ptsG* itself caused repression of the reporter comparable to that of the *cpxR* mutant, although deletion of *ptsG* did not abolish the repression in the *cpxR ptsG* double mutant (Figure 3.2). Thus it appears that the effect seen in the *cpxR* mutant might not be due to increase activity of the *ptsG* transporter. Still, it could be that de-repression of *both* of the Cpx-regulated transporter genes is required to relieve this phenotype, and this remains to be tested. In terms of what CyaR contributes to the Cpx envelope stress response, one possibility is

that it acts in a coherent feedforward loop in the regulation of the hypothetical inner membrane protein gene *yqaE*, which has been established as being transcriptionally upregulated by the Cpx pathway (1, 2). CyaR has been shown to directly bind and repress *yqaE* translation and stability, and thus it is conceivable that Cpx repression of *cyaR* exists to more quickly de-repress its expression. The usefulness of YqaE in the amelioration of envelope stress is yet to be determined, as its function is still unknown.

Interestingly, the microarray experiment revealed that Cpx pathway induction by overexpression of *nlpE* causes an 8-fold upregulation of *rprA* in enteropathogenic *E. coli* but not in our K12 background, MC4100. Indeed, when we attempted to determine the extent of *rprA* regulation by the Cpx pathway in MC4100 using a *PrprA::lux* reporter gene, we could not detect any meaningful increase in its expression in a wild-type MC4100 background (data not shown). We did, however, find that if the signal kinase *rcsC* was first deleted, a significant increase in *rprA* transcription could be measured in a constitutive Cpx pathway mutant *cpxA24* compared to wild-type (data not shown). The *rcsC* mutant is expected to exhibit higher Rcs pathway activity because in its uninduced state the signal kinase exerts phosphatase activity towards the response regulator RcsB, keeping the pathway inactive. Thus in this mutant we expect there to be higher basal expression of *rprA,* which appears to be necessary to see Cpx upregulation of the sRNA in this genetic background. The experiments shown here were, however, conducted using another strain of K12 *E. coli* W3110, in which *rprA* has a higher basal expression level. In *E. coli* W3110, deletion of *rcsC* is not necessary to observe upregulation of *rprA* expression after Cpx induction, which incidentally matches the extent of *rprA* upregulation found in *E. coli* EPEC in the microarray (roughly ~8-fold) (Figure 3.3). Whether or not this key difference in regulation is in fact a result of the higher basal level of *rprA* expression in W3110 is not certain; it may reflect

a regulatory connection between the Cpx and Rcs signal transduction pathways that exists in *E. coli* MC4100 but not W3110. Another possibility is that CpxR activates *rprA* transcription together with RcsB as a heterodimer, and the Rcs pathway is inherently less active in MC4100. Recent work has suggested such a link between the two pathways in *E. coli*: deletion of four penicillin-binding proteins (PBPs) induces both pathways, but induction of Rcs activity was found to be contingent on a functional copy of *cpxR* (3), setting a precedent for the possibility of direct transcriptional control of some component of the Rcs pathway by CpxR. This might explain why an *rcsC* mutation (which makes the Rcs pathway signal blind) is required to see upregulation of *rprA* by the Cpx pathway; perhaps CpxR both directly activates *rprA* transcription but indirectly represses its activity through some form of regulation of the Rcs phosphorelay. These speculations, of course, require much development before this elaborate regulatory loop can be claimed to be real. Altogether though these results, when paired with the electrophoretic mobility shift assays (EMSAs) done by Randi Guest (2), strongly suggest that CpxR directly binds the promoter of *rprA* to promote its transcription, thereby increasing its expression.

6.2 RprA represses the Cpx envelope stress response

There are numerous examples of small RNAs which regulate the activity of their own transcriptional activator. In *Vibrio sp.* the Qrr sRNAs act in a feedback inhibition loop to repress their own regulator, HapR (4, 5) thereby keeping its expression in check while at the same time contributing to the quorum sensing signal cascade which results in virulence activation. Examples of feedback inhibition by sRNAs in *E. coli* include the well-studied MicF-Lrp (6) and OmrA/OmrB-OmpR (7); in both cases these sRNAs function to fine-tune the expression of their own regulator in order to achieve an appropriate but not overreaching regulatory effect, while at

the same time contributing to the regulatory goals of the signal cascade by up/down-regulating target genes of the regulon. Here we have shown that RprA, too, is an sRNA which participates in feedback regulation of its own activator CpxR. Like MicF and OmrA/B, overexpression of *rprA* causes repression of the pathway which activates its transcription (Figure 4.1), and like OmrA/B, RprA feedback inhibits its own regulatory pathway through the response regulator rather than the signal kinase (7) (Figure 4.11). Deletion of *rprA* on the other hand had no impact on Cpx pathway activity, which could mean (broadly) two things: 1) either basal expression levels of *rprA* in *E. coli* MC4100 are not sufficient to have an effect on the Cpx pathway, and stimulation of *rprA* transcription by some inducer is required to have an effect, or 2) RprA is acting redundantly with some other negative regulator to influence Cpx pathway activity. Our evidence suggests that option 1) is more likely, as we found that activity of a *PrprA::lux* transcriptional reporter in MC4100 has almost no activity in the absence of an Rcs pathway inducer (data not shown), and fairly low activity in the absence of a Cpx pathway inducer (Figure 3.3). Interestingly, unlike OmrA/B or MicF, our evidence suggests that RprA does not inhibit activity of CpxR through direct translational repression, as abundance of the CpxR protein is unaffected upon overexpression of *rprA* (Figure 4.5). This finding is simultaneously exciting and confounding: very few sources of signal input, positive or negative have been found which modify Cpx pathway activity through the response regulator CpxR, yet the effect of RprA is not dependent on the only known mechanism of CpxA-independent CpxR phosphorylation, acetyl phosphate (Figure 4.12). The latter finding was surprising; it made an appealing model that RprA might modulate the activity of the Cpx pathway by decreasing activity of the acetyl-phosphate biosynthesis pathway (the *ackA ptA* pathway), but as we discovered, Cpx pathway inhibition by RprA was not contingent on functional copies of these genes. We also determined

that addition of exogenous glucose (which increases acetyl phosphate production (8)) did not interfere with Cpx pathway repression by RprA in a wild-type genetic background (data not shown), and pathway repression could happen at either pH 7 or pH 5.8 (at which acetyl-phosphate production is stimulated) (Figure 4.13). Finally it was determined that deletion of *rprA* did not interfere with growth-dependent activation of the Cpx response, which is a signal that is, while poorly understood, thought to occur via the increase in acetyl-phosphate generation as the cell progresses into stationary phase (Figure 4.14). Therefore we concluded that RprA must be inhibiting CpxR activity (and presumably its phosphorylation) but not through modulating the production of acetyl-phosphate. From here we considered broadly two possibilities in terms of how RprA might be altering the activity of the CpxR response regulator: RprA might behave as one of the rare sRNAs which physically bind to and modulate activity of a protein, or RprA might modulate the activity of some unknown target gene whose gene product acts to affect CpxR phosphorylation status. The former possibility, that RprA might physically bind to and affect activity of CpxR, has not been investigated whatsoever at this time. The only sRNAs known to behave in this manner are the CsrB-family sRNAs all of which bind to and antagonize activity of the carbon storage regulator CsrA, which is an RNA-binding protein itself (9). These sRNAs have multiple characteristic GGA repeat motifs which are required for protein binding (10) which RprA lacks. However, recent work has identified the sRNA McaS, with which RprA shares 63% sequence identity, as another CsrA-regulator (11), although McaS does have multiple GGA repeats while again RprA does not. We cannot exclude the possibility that RprA might physically interact with CpxR at this time, however, and experiments could to be done in order to test this theory. One approach is the co-immunoprecipitation of CpxR to determine if RprA physically associates with it *in vivo.* This approach has had much success in identifying sRNAs

which interact with the RNA chaperon Hfq (12), and thus could conceivably work with CpxR. The crystal structure of CpxR has yet to be solved, but once this is done the structure might give some indication as to whether or not CpxR has any putative RNA-binding domains. For now however we must only consider to this to be an unexplored option which remains to be investigated. What we consider to be the more likely model for RprA inhibition of CpxR activity is that RprA regulates the activity of some target gene which modulates the phosphorylation status of CpxR.

At first, our default hypothesis was that RprA indirectly represses CpxR activity through one of its published target genes: *rpoS, csgD,* or *ydaM* (13, 14). It was reasonable to think that RprA could have some regulatory effect on the Cpx pathway through its upregulation of *rpoS*, as RpoS regulates roughly 10% of the genome in *E. coli* (15) and both the Cpx response and RpoS are most highly active during stationary phase. In the case of *csgD* and *ydaM,* however, there is not an immediately obvious reason why changed expression of either of these genes would result in altered CpxR activity. CsgD is the master curli regulator, and while it has previously been established that Cpx pathway induction causes repression of curli genes (16), there is no evidence to suggest the CsgD transcription factor influences expression of any Cpx genes. The same goes for *ydaM,* which encodes a di-guanylate cyclase enzyme whose expression is required for CsgD activity (17). In any case, with all three of these genes, we found that their deletion bore no impact on RprA's ability to repress Cpx pathway activity (Figure 4.7), and thus we concluded that whatever the mechanism of indirect inhibition of Cpx pathway activity by RprA, it must be through an previously uncharacterized target gene. Interestingly, while a clean deletion of *csgD* did not have any impact on RprA's ability to inhibit the pathway, the insertional *csgD::kan* mutant exhibited a striking increase in Cpx pathway activity upon RprA

overexpression (Figure 4.7). This was an exciting finding at first, and we hypothesized that the insertion interfered with translation of downstream genes *csgE, csgF,* or *csgG* all of which are encoded in an operon with *csgD*. We reasoned that overexpression of RprA in the absence of expression of one of these genes was causing significant signaling to the Cpx pathway, although this theory was ruled out because deletion of these genes had no effect on Cpx pathway activity in the presence or absence of *rprA* overexpression (Figure 4.9). At this time we don't know why *rprA* overexpression in the *csgD::kan* mutant caused such a large increase in Cpx pathway activity; one hypothesis is that the *csgD::kan* transcript which contains part of the 5' untranslated region (UTR) of *csgD* may still bind *rprA* and thus overexpression of *rprA* causes this aberrant transcript not to produce the kanamycin resistance enzyme maximally, resulting in toxicity and stress, although this theory has not been tested.

In an early attempt to determine the identity of a putative RprA target gene affecting the Cpx response, we made use of the bioinformatic sRNA target prediction tool sTarPicker (18) to predict potential RprA targets based on the estimated energetics of hybridization. Mutations were made in the top ten predicted target genes (ranked by predicted ΔG of binding): *yrfG, ymgC, sulA, purH, ddpX, csgD, ygeD, livK, ygg* and *sufI.* These genes encode proteins of a variety of functions ranging from purine biogenesis to cell division, and notably include the confirmed RprA target gene *csgD*. We found that deletion of none of these genes had any significant effect on RprA's ability to inhibit the Cpx pathway (with the exception of the insertional *csgD::kan* mutation as previously stated). It's worth mentioning that this target prediction tool predicted many more targets for RprA, but only the top 10 predicted targets were assayed, and therefore this approach was not exhaustive. It's also worth mentioning that since these experiments were conducted, a promising new bioinformatic sRNA target prediction program has been developed

called CopraRNA (19), and we have yet to test experimentally some of the top predicted RprA targets from this program. This new program is likely considerably more powerful that previous iterations of sRNA target prediction programs; its main draw is the fact that it not only predicts sRNA::mRNA hybridization kinetics but it also predicts how well conserved these interactions are likely to be in closely related bacteria (19). In fact, this program claims to generate less false-positives than a tiling microarray when predicting targets for a given sRNA.

Serendipitously, a clue about a potential mechanism of Cpx inhibition by RprA came recently when Bak *et al.* discovered two things: 1) RprA RNA levels are increased in conditions of extreme acidity, and 2) overexpression of *rprA* causes resistance to extreme acid stress (20). These authors determined that this phenotype was dependent in the *gadX* gene which encodes a transcriptional activator of the glutamate-dependent acid resistance pathway (GDAR), and they hypothesized that RprA acted on *gadX* through upregulation of *rpoS*, which is required for *gadX* transcription (20). While alone this finding didn't necessarily provide a model for RprA's inhibition, it has also been long known that the Cpx pathway is repressed in more acidic pH (21), although the reason or the mechanism for this are not known. Thus we hypothesized that perhaps RprA might repress the Cpx pathway upon stimulation by acid stress, and that this might involve modulating activity of the GDAR acid resistance pathway. This hypothesis was strengthened by our finding that constitutive induction of the Cpx pathway results in reduced survival to extreme acid challenge (Figure 5.5), and that activity of a *PcpxP::lacZ* transcriptional reporter is reduced to almost nothing in extreme acid challenge (Figure 5.6) – and as a reminder this was the condition that Bak *et al.* found stimulated RprA transcription. Indeed, we found that *E. coli* lacking a functional *gadE* or *gadA* gene exhibit less Cpx pathway repression by RprA (Figure 4.16), suggesting that RprA's role in acid resistance may in fact interact with Cpx pathway

activity. We also confirmed that RprA stimulates activity of the GDAR pathway: overexpression of *rprA* increased activity of a transcriptional *PgadE::lux* reporter more than 20-fold (Figure 4.17) – signifying that RprA increases activity of this pathway as a whole since GadE both autoregulates and activates transcription of every gene in the pathway. While this finding alone suggests that RprA indeed increases activity of the GDAR pathway, more experiments need to be done in order to determine whether or not RprA indeed directly affects translation and/or stability transcripts of the GDAR pathway. First and foremost, epistasis analysis must be done in order to determine whether or not this observed activation of GDAR pathway activity by RprA is dependent on *rpoS* as Bak *et al.* hypothesize, or rather through direct basepairing by RprA to the *gadE* or *gadA* transcript. Translational *gfp* or *lacZ* reporters need to be constructed with the 5' UTRs of *gadE* and *gadA* to determine if this interaction involves direct regulation of these transcripts, as has been described elsewhere (6). For now, however, we can only conclude that our data seem to suggest a role for the GDAR pathway in Cpx repression by RprA, although the specific target gene is as of yet unknown.

If RprA indeed represses Cpx pathway activity through upregulation of the glutamate dependent acid resistance pathway (GDAR), the looming question is how increased activity of the GDAR pathway would repress Cpx pathway activity – specifically, repress CpxR activity but not expression. This question is not an easy one to answer; there is no obvious mechanism by which altered expression of either the GadAB glutamate decarboxylase or the GadC glutamate/GABA antiporter could change the phosphorylation state of CpxR. One way to examine the problem is to ask whether or not this effect depends on increased *activity* of GadAB/C or rather increased expression of the GadE transcription factor (in the latter case GadE would influence CpxR activity indirectly through transcriptional control of some unknown

factor). We can address this problem by determining whether or not RprA inhibition of the Cpx pathway occurs in the absence of glutamate, since glutamate is absolutely required for activity of the GadAB decarboxylase but not for transcriptional activation of the GDAR genes (22). In this way we could at the very least determine whether or not the perceived effect of increased GDAR activity on Cpx pathway activity is dependent on the *function* of the GadAB decarboxylase. If this is the case, then there are theoretically two aspects of GadAB activity that must be considered: 1) activity of the decarboxylase increases intracellular pH by its net shuttling of protons out of the cell through GadC (22) and 2) activity of the decarboxylase generates intracellular CO_2 as a by-product of the conversion of glutamate to γ-butyric acid (23). We consider it unlikely that the latter consequence has any influence on CpxR phosphorylation, as CO_2 is a fairly unreactive molecule in the cell. Increased intracellular pH, too, seems unlikely to interfere with phosphorylation of CpxR, as the aspartate residue on OmpR-like transcription factors such as CpxR is un-protonated at higher pH, making it amenable to phosphorylation (24). At any rate, our next step in elucidating the mechanism by which increased expression of the GDAR pathway represses Cpx pathway activity is to determine whether or not this effect is indeed dependent on the enzymatic activity of the decarboxylase by determining the dependence of this effect on exogenous glutamate.

6.3 The Cpx response and RprA regulate common phenotypes

One of our first thoughts when we determined that overexpression of *rprA* caused inhibition of the Cpx pathway was whether or not this inhibition might come in response to induction of the RcsBCD phosphorelay, since *rprA* transcription is dependent on RcsB (13). Not only would this provide a physiological role for the inhibition of Cpx pathway activity by RprA, but it would lend biological relevance to the phenotype, since sRNA overexpression experiments

can be fraught with artificial phenotypes. This is because at high levels of overexpression, sRNAs may bind and affect mRNA targets non-specifically or less specifically, and overexpression of an sRNA can cause fluctuations in the amount Hfq chaperones available to other sRNAs (25). Thus determining a physiological cue for which RprA inhibits the Cpx response is crucial. Unfortunately, attempts to elucidate such a signal have as of yet been unsuccessful, as three inducers of the Rcs phosphorelay were not found to cause repression of the Cpx response, let alone cause Cpx repression in an *rprA*-dependent manner. These inducers were overexpression of *rcsF,* which has been shown to activate RcsC in a manner non unlike NlpE activation of CpxA (26), as well as high osmolarity via exogenous sucrose or salt (Figures 5.1 and 5.2). The latter two inducers were not expected with much confidence to repress the Cpx pathway as previous results have suggested high osmolarity is a mild activator of the pathway rather than a repressor (27), but we found that neither of these conditions was capable of any significant increase or decrease in activity of the *cpxP* reporter gene (Figure 5.2). These data therefore do not support the hypothesis that RprA acts to repress the Cpx pathway upon induction of the Rcs phosphorelay.

Another physiological condition which stimulates the expression of *rprA* is acidic pH (20, 28). The mechanism of this upregulation has not yet been established, although it does not seem entirely unlikely that the transcription factor GadE might directly upregulate *rprA* transcription via promoter binding, because co-activation of acid-related genes by RcsB and GadE has previously been reported (29). Not only this, but overexpression of *rprA* has been shown to be sufficient to endow *E. coli* with increased resistance to extreme acid conditions (20). Because of this, in conjunction with our findings that RprA increases activity of a *PgadE::lux* transcriptional reporter and that RprA inhibition of Cpx pathway activity is partially *gadE*-

dependent, we asked whether acidic pH might repress the Cpx pathway in an *rprA*-dependent manner. What made this an even more appealing hypothesis is the long established finding that lower pH reduces Cpx pathway activity through an unknown mechanism (21). Interestingly, in an extreme acid challenge experiment, activity of a *PcpxP::lacZ* reporter was diminished almost entirely, although this was not determined to be dependent on either *rprA* or *gadE* (Figure 5.6). Additionally, constitutive activation of the Cpx pathway by a *cpxA24* mutation caused a roughly 10-fold decrease in survival after the same acid challenge experiment (Figure 5.5). Thus, while it appears that Cpx pathway activation is disadvantageous in extreme acid conditions and the pathway is repressed entirely, we cannot surmise that RprA or the GadE transcription factor is responsible for this effect. It's important to note, however, that RprA is not the only sRNA which has been connected to acid resistance and the glutamate-dependent acid resistance pathway (GDAR). Two other sRNAs, DsrA and ArcZ increase acid resistance of *E. coli* significantly when overexpressed (20), and as with RprA this mechanism is thought to involve upregulation of the GDAR transcription factor gene *gadX* through their shared upregulation of the stationary phase sigma factor gene *rpoS*. Thus it could be expected that if RprA inhibition of the Cpx pathway comes in response to acidic conditions, deletion of only *rprA* might not be sufficient to prevent Cpx pathway repression by acidic pH (if we assume that perhaps these other two sRNAs might share this role of Cpx repression). An immediate problem with this theory is that that overexpression of *dsrA* does not cause repression of the *PcpxP::lacZ* reporter, but rather causes a small but significant increase in its activity (Figure 4.8), although we have not assayed for an effect of *arcZ* on the reporter. Another possibility is that RprA is simply redundant with some other unknown regulator in the repression of Cpx pathway activity upon acid stress, although this line of thought has yet to be tested. More troubling, of course, is the finding that neither *gadA* or

gadE is necessary for the Cpx pathway shut-off observed in extreme acidic conditions (Figure 5.6). This suggests that RprA regulation of the GDAR pathway has no bearing on this Cpx repression phenotype, although since deletions in *gadB, gadC, gadX* and *gadW* have not been tested we cannot yet rule this out entirely.

If RprA regulates activity of the GDAR acid resistance pathway, it stands to reason that a side effect of this would be an effect on the cell's proton motive force (PMF) since activity of glutamate decarboxylase enzymes is associated with an increase in the $\Delta\Psi$ component of the PMF (30). This effect is caused by the net shuttling of protons out of the cytoplasm in the conversion of glutamate to γ-butyric acid (which is transported out of the cell through GadC). One way to measure changes in the cell's PMF is to assay for changes in resistance to aminoyglycoside antibiotics such as amikacin, since resistance to aminoglycosides can be mediated by either depletion of the cell's PMF or decreased protein translation and secretion through the Sec translocon (31). We found that overexpression of *rprA* in fact caused a marked increase in resistance to amikacin at a concentration of 1.5 μg/mL (Figure 5.3), which is actually contrary to what we expect if RprA activates activity of the GadAB decarboxylase (whose activity is associated with increased PMF). Furthermore, deletion of either *gadA* or *gadE* caused an increase in amikacin resistance (which we expect), and overexpression of *rprA* in these mutants actually reduced amikacin resistance (Figure 5.3). Given this, we must consider that the effect of RprA has on amikacin resistance may not be due to its putative upregulation of *gadE,* but rather due to upregulation of the general stress sigma factor gene *rpoS*, whose expression might conceivably increase resistance to amikacin. In fact, we have yet to directly demonstrate that overexpression of *rprA* actually leads to a change in the cell's PMF, since again, amikacin resistance is not only promoted by changes in PMF. In order to demonstrate that RprA directly

alters the cell's PMF, we must next conduct a proton gradient determination assay which employs a dye that requires an active PMF to enter the cell, to show that overexpression of *rprA* alters the rate of entry of the colored dye in to the cell (32). Finally, it's worth noting that the dependence of this amikacin resistance phenotype on RprA's published targets (*rpoS*, *csgD*, and *ydaM*) is yet to be determined. Ignoring for a moment our hypothesis that RprA is depleting the cell's proton motive force, the fact that RprA overexpression causes amikacin resistance is in and of itself a very interesting finding, given that Cpx pathway induction has long been known to cause resistance to this antibiotic (33). The effect of Cpx induction on resistance to amikacin has not been clearly illustrated, although it has long been presumed to be due to the repression of various envelope-localized proteins which require translation through the Sec translocon – since killing by amikacin involves jamming of the Sec translocon (31). Importantly, work done by an undergraduate student in our lab, Erin MacKinnon, showed that deletion of *rprA* does not abolish the amikacin resistance of the constitutively active *cpxA24* mutant (data not shown), so unfortunately we cannot attribute this resistance phenotype to Cpx regulation of *rprA*. Still, it is satisfying to observe that RprA is capable of endowing the cell with amikacin resistance as part of the greater Cpx-mediated resistance to this antibiotic even if the mechanism of the resistance at both the level of RprA and of the Cpx pathway in general is not clear.

We also show here that overexpression of *rprA* leads to a decrease in motility (Figure 5.4). We initially hypothesized that this might be due to the effect that RprA putatively has on the cell's PMF (as indicated by the amikacin resistance experiments), as rotation of *E. coli*'s flagella is known to require an active PMF (34). Although as stated above, we have yet to conduct direct biochemical experiments to demonstrate that RprA depletes the cell's proton motive force, it was appealing to attempt to connect this perceived reduction in proton motive force to a tangible

effect on cell behaviour. Overexpression of *rprA,* in addition to *omrA* which is known to repress flagella biosynthesis (35), caused roughly a 2-fold decrease in swim diameter in a conventional soft agar motility assay (Figure 5.4). We attempted to show that this decrease in motility was not due to a decrease in flagella elaboration by extracting and quantifying flagella from cells overexpressing *rprA*, but had difficulty detecting the flagella and thus cannot yet conclude that this motility phenotype is not due to regulatory inhibition of flagella biosynthesis by RprA (data not shown). Indeed, there is some reason to suspect that *rprA* may regulate flagella biosynthesis, it shares 63% sequence identity with the sRNA McaS which represses translation of the flagella regulatory gene *flhD (36)*. Thus, more experiments must still be done to determine the mechanism of repression of swimming motility by RprA; foremost a translational *flhD::lacZ* or *flhD::GFP* fusion should be assayed for changes in activity upon overexpression before we can rule out direct repression of flagella akin to that of McaS. As with amikacin resistance, it's worth noting that in its repression of motility, RprA is in line with the greater regulatory scheme of the Cpx pathway, given that our microarray revealed significant reduction in expression of the aforementioned *flhDC* transcript upon induction of the Cpx response. It may be worth testing the dependence of this regulatory effect on a functional copy of *rprA*; although there is some likelihood that CpxR might indirectly repress the *flhDC* operon through its upregulation of EnvZ/OmpR and therefore *omrA (37)*.

6.4 Final model and concluding remarks

While many questions still remain, the experiments detailed here demonstrate that the Cpx envelope stress response upregulates the transcription of the small RNA RprA, likely in concert with the response regulator RcsB of the Rcs phosphorelay (Figure 6.1). Once expressed, RprA acts to increase translation of the general stress response regulator and stationary phase

sigma factor RpoS, and in doing so aiding the response to envelope stress by inducing a global shift to stationary phase. RprA also represses the translation of *csgD*, thereby inhibiting the biosynthesis of curli at the post-transcriptional level and thus limiting traffic in the envelope which also aids in the response to envelope stress. RprA also enhances resistance to the aminoglycoside amikacin which is known to cause envelope stress by jamming the Sec translocon, and represses motility, both of which are phenotypes associated with activation of the Cpx envelope stress response. Furthermore, our data suggest that once expressed, RprA acts to limit Cpx pathway activity through its role in upregulation of the glutamate-dependent acid resistance pathway (GDAR), whose increased activity in some way impacts the phosphorylation status of CpxR. Given that Cpx pathway activation seems to be deleterious in acidic conditions, we propose that RprA may act to repress Cpx pathway activity in the acidic conditions associated with stationary phase, and this may occur in part to counteract growth-dependent activation of the Cpx response. We suspect that if this is the case, RprA might in fact be redundant in this role with some other regulator, since deletion of *rprA* did not abolish the sever Cpx pathway repression we observed in extreme acid conditions. The dual-modal function of RprA in the Cpx regulon is therefore remarkable; RprA acts to further the goals of the Cpx envelope stress response through its published target genes and by promoting two Cpx-related phenotypes, while simultaneously preventing the Cpx response from reaching levels of activity that might be deleterious to the cell in conditions of high acidity.

6.5 Figures

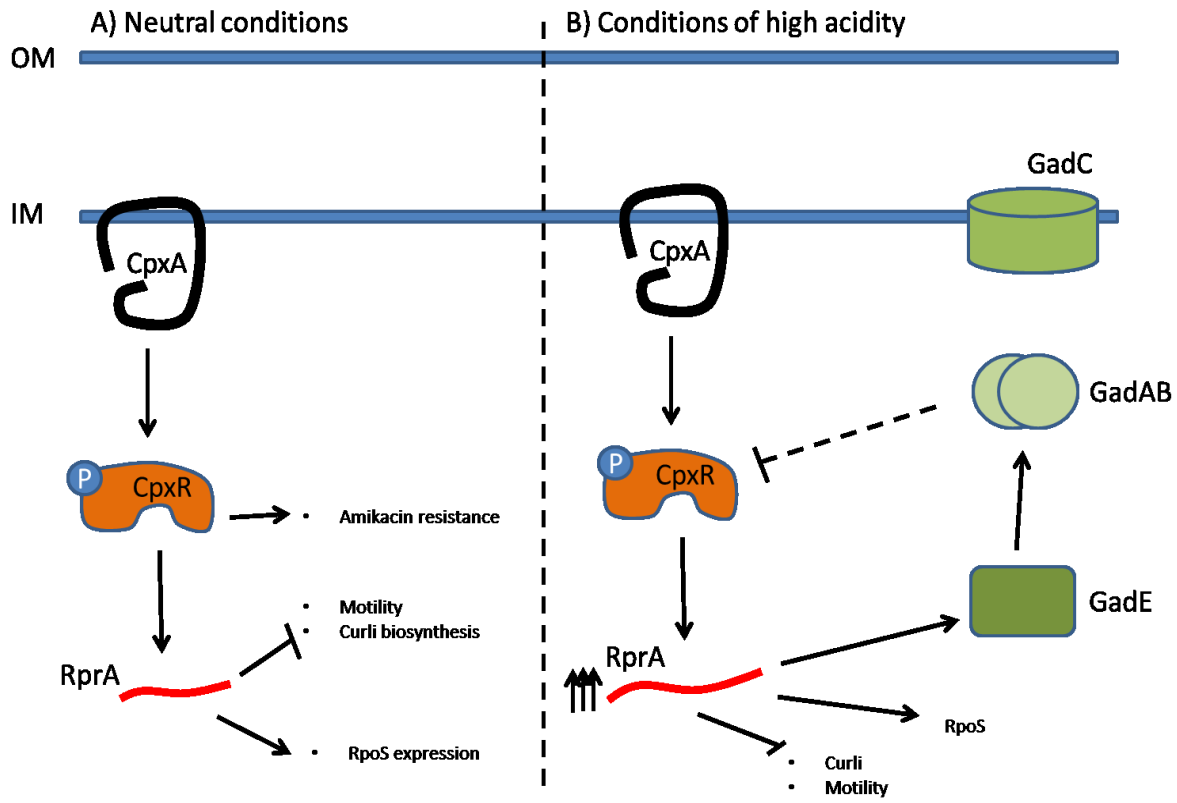

Figure 6.1: Model for the role of RprA in the Cpx regulon. In neutral pH, and when the Cpx pathway is at basal to moderate activity levels, *rprA* is upregulated and promotes Cpx-related phenotypes of curli repression, motility repression, and amikacin resistance. In conditions of high acidity, *rprA* expression is increased by an unknown mechanism and represses the Cpx pathway via upregulation of the glutamate-dependent acid resistance pathway (GDAR).

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APPENDICES

APPENDIX I – Stnc870

Recent work done by Chao *et al.* (1) demonstrated that in *Salmonella typhimurium*, the 3' untranslated region (UTR) of *cpxP* encodes an sRNA which results from the processing of the full length *cpxP* transcript, which they have recently termed CpxQ (2). In order to determine whether 3' UTR of *cpxP* encodes such an sRNA species in *E. coli*, Northern blot assays were done to detect it in the presence and absence of Cpx pathway activation. DIG-U-labelled RNA probes overlapping either the entirety of the full length *cpxP* transcript or just its 3' UTR were generated by *in-vitro* transcription and their corresponding Northern blots were compared. These blots did not reveal any substantial difference in the abundance of the full length *cpxP* transcript versus the *stnc870* moiety upon Cpx induction, although both RNA species were decreased in abundance in a *cpxR::spec* mutant and increased in abundance in the *cpxA24* constitutive Cpx pathway mutant (Figure S1).

cpxP overexpression is known to cause repression of Cpx pathway activity (3), although previous *cpxP* overexpression vectors contained a *cpxP* gene not including its 3' UTR. Thus we asked the question if the *stnc870* species might itself be capable of inhibition of the Cpx pathway. Vectors overexpressing full-length *cpxP* (including *stnc870*), truncated *cpxP* (excluding *stnc870*), or only *stnc870* were transformed into a strain bearing the *PcpxP::lacZ* transcriptional reporter and the effect of these constructs on Cpx pathway activity was measured by a β-galactosidase assay. Overexpression of truncated *cpxP* but not full-length *cpxP* or *stnc870* was capable of very mildy inhibiting the reporter (Figure S2). These data suggest that this sRNA species does not contribute to the inhibition of Cpx pathway activity associated with the *cpxP* locus.

APPENDIX II – Putative Cpx-regulated sRNAs in *E. coli*'s IGRs

The microarray recently done in our lab (4) not only suggested that *rprA* and *cyaR* are regulated by the Cpx pathway, but also that 36 intergenic loci of *E. coli*'s genome produce RNA species whose abundance is affected upon induction of the Cpx response. We posited that while these intergenic regions (IGRs) might encode un-annotated small proteins or might simply be artefacts of the microarray, they might also encode small RNAs yet to be characterized in *E. coli*. To narrow down the list of IGRs to just those which are most likely to encode sRNAs, preliminary bioinformatics analyses were done to predict the presence of: ρ-independent terminator sequences, σ^{70} promoter sequences, and CpxR binding sites. We also looked for proximity to other Cpx-regulated genes, as these IGRs might simply represent uncharacterized untranslated regions of Cpx-regulated genes. Table S1 shows the results of these bioinformatics analyses, and allows us to narrow down the list of IGRs to 5 loci which are deemed most likely to encode biologically relevant sRNA species.

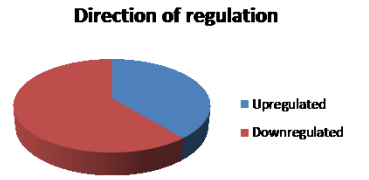
APPENDIX III – A Screen for RprA's target

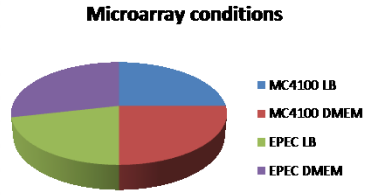
At the same time as directed efforts to determine the mechanism of Cpx repression by RprA were taking place, a random mutagenesis screen was conducted to search for mutations which abolished RprA's inhibitory activity. *E. coli* overexpressing *rprA* were mutagenized by Tn5::tp^R transposons and were then screened for absence of inhibition of a *cpxP::lux* reporter gene (Figure S3). 9 mutants which prevented Cpx inhibition by RprA were sequenced, and independent mutations in 7 of these genes were generated by transduction of WT *E. coli* with Keio library mutations in these genes. Unfortunately, the independent mutations were unable to replicate the abolishment of Cpx pathway repression found in the screen, and thus these candidates likely represent false-positives. More candidate positives from the screen must still be sequenced, and the screen has not in fact been conducted to saturation, and thus the screen might

still potentially yield important information about RprA's mechanism in the event that directed approaches to do not succeed.

Appendix tables and figures

IGR	Upstream gene	Downstream gene	Rho-independent terminator?	CpxR box?	Notes
IG_3198607_3198847-f	N/A	ygiM	0	No	antisense to ygiF
IG_1269684_1269971-r	chaA	ldrC	1	No	
IG_1048986_1049249-r	cspH	gfcA	1	Yes	
IG_3598415_3598658-f	N/A	N/A	0	No	antisense to rpoH
IG_3598415_3598658-r	N/A	N/A	0	Yes	
IG_980010_980269-f	mukB	ycbB	0	No	likely ycbB 5' UTR
IG_986206_986807-f	N/A	N/A	2	Yes	antisense to ompF 5' UTR
IG_986206_986807-r	asnS	ompF	2	Yes	
IG_3571136_3571407-f	N/A	N/A	0	No	antisense to glgB
IG_2404662_2405580-r	N/A	lrhA	0	No	antisense to putative alaA 5' UTR
IG_2099291_2099916-r	insH-7	gnd	0	No	
IG_1640092_1640512-r	ydfT	csbB	1	No	
IG_1640092_1640512-f	cspF	N/A	1	No	
IG_2085091_2085350-r	yeeY	plaP	1	No	
IG_2238369_2238647-r	galS	mglB	0	No	
IG_2238369_2238647-f	N/A	N/A	0	No	antisense to mglB 5' UTR
IG_1732126_1732458-r	N/A	grxD	0	No	antisense to putative ydhO 5' UTR
IG_2529254_2529482-r	N/A	zipA	0	Yes	antisense to cysZ 5' UTR
IG_1903284_1903657-f	yobD	mntP	0	No	
IG_237008_237334-f	aspV tRNA gene	yafT	1	No	
IG_2111084_2111455-r	galF	rfbB	0	No	
IG_688237_688565-f	N/A	N/A	0	No	antisense to predicted insH-3 5' UTR
IG_2347493_2347706-r	glpQ	inaA	0	No	
IG_2234521_2234762-r	mglC	N/A	1	No	
IG_3245069_3245412-f	exuT	yqjA	0	No	
IG_3475057_3475276-r	slyD	yheO	1	No	
IG_1341353_1341620-r	yciT	osmB	0	No	
IG_3154533_3154753-f	yqhD	dkgA	0	No	
IG_1702701_1702972-f	blr	cnu	0	No	
IG_3530078_3530455-f	N/A	pck	1	No	antisense to yhgE
IG_889043_889311-f	N/A	ybjM	0	No	antisense to predicted ybjL 5' UTR
IG_1741267_1741480-r	N/A	ribC	0	No	
IG_330721_331594-f	betT	yahA	1	No	
IG_2925695_2926250-f	ygdH	sdaC	1	No	
IG_613163_613379-r	N/A	N/A	0	No	antisense to ybdZ
IG_953690_954094-r	ycaO	focA	0	Yes	





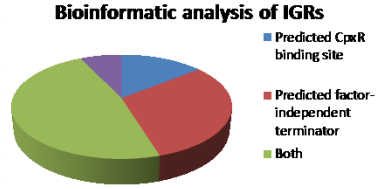


Table S1: Bioinformatic analyses of putatively Cpx-regulated IGRs. Intergenic regions enriched in the microarray were analyzed. We looked for upstream or downstream genes which are known to be Cpx-regulated and did bioinformatic analyses to find putative CpxR binding sites and factor-independent terminator sequences within 500 bp of each IGR sequence.

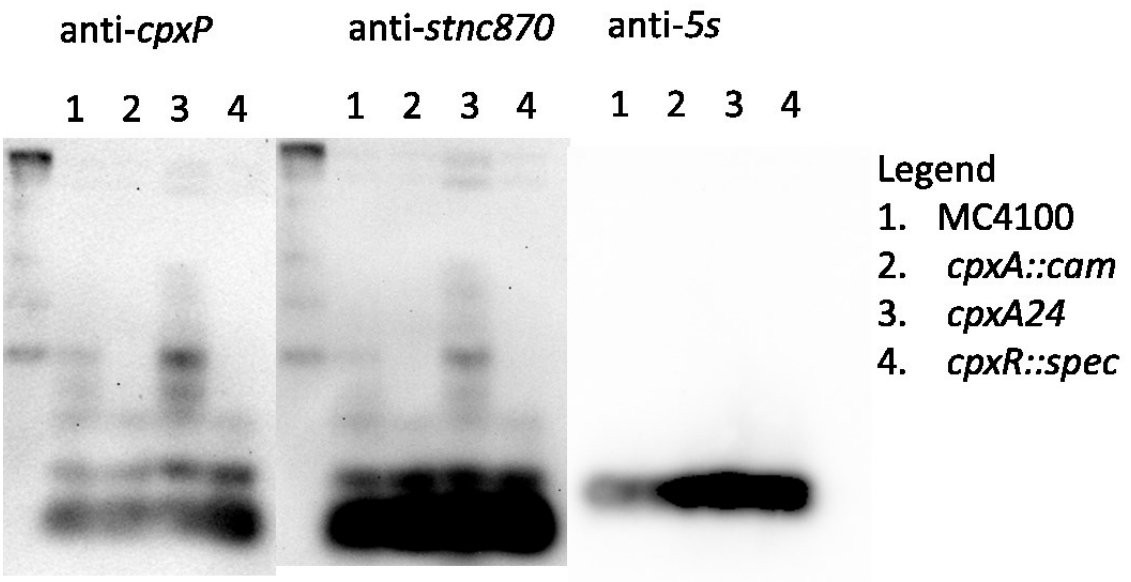


Figure S1: Detection of the RNA species Stnc870 upon Cpx induction. Strains bearing *cpxA::cam, cpxA24,* or *cpxR::spec* mutations or wild-type were subcultured 1:50 from overnight cultures into fresh LB medium and were grown for 6 hours, at which point total RNA was collected from cultures. Northern blot experiments were conducted using probes specific to Stnc870 or the full length *cpxP* transcript, or an anti-5S RNA control probe.

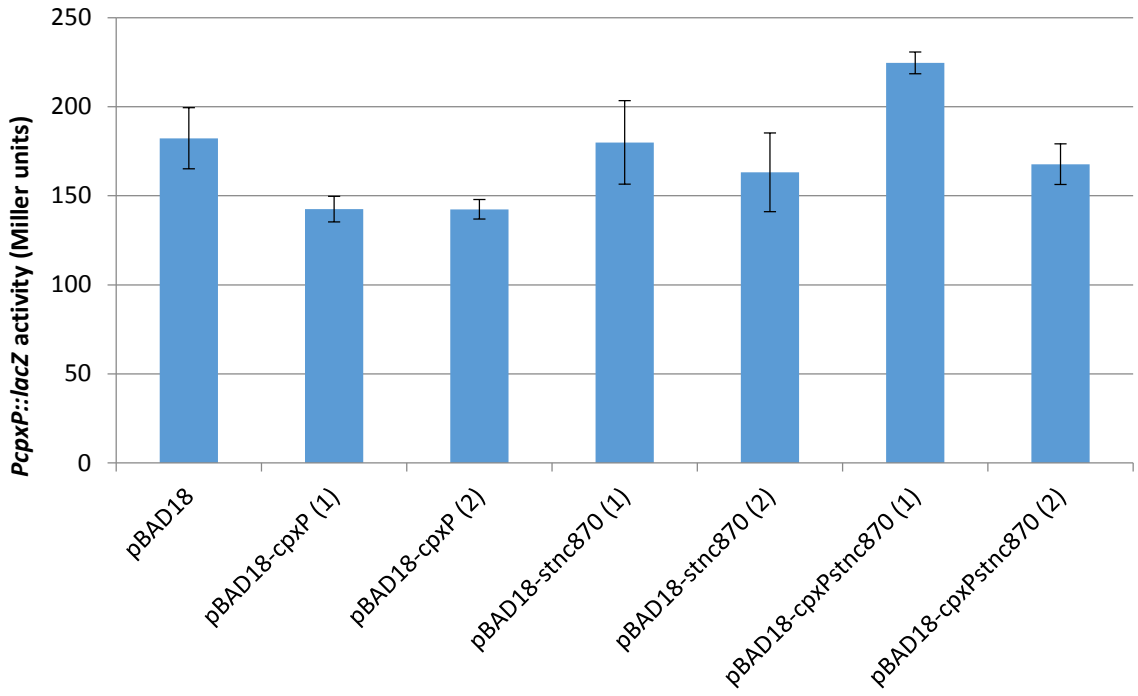


Figure S2: Overexpression of *stnc870* constructs does not affect Cpx pathway activity. Strains overexpressing various pBAD18-*stnc870* constructs were subcultured 1:50 from overnight culture into fresh LB medium, and grown for 5 hours at 37^0C while shaking, and were induced with 0.1 mM IPTG after 3 hours of growth. Subsequently, cells were resuspended in 1X Z-buffer and activity of the *PcpxP::lacZ* reporter was inferred using a plate reader. Bars represent average values between three replicates, and error bars represent standard deviation between those replicates. These are representative results of two independent experiments.

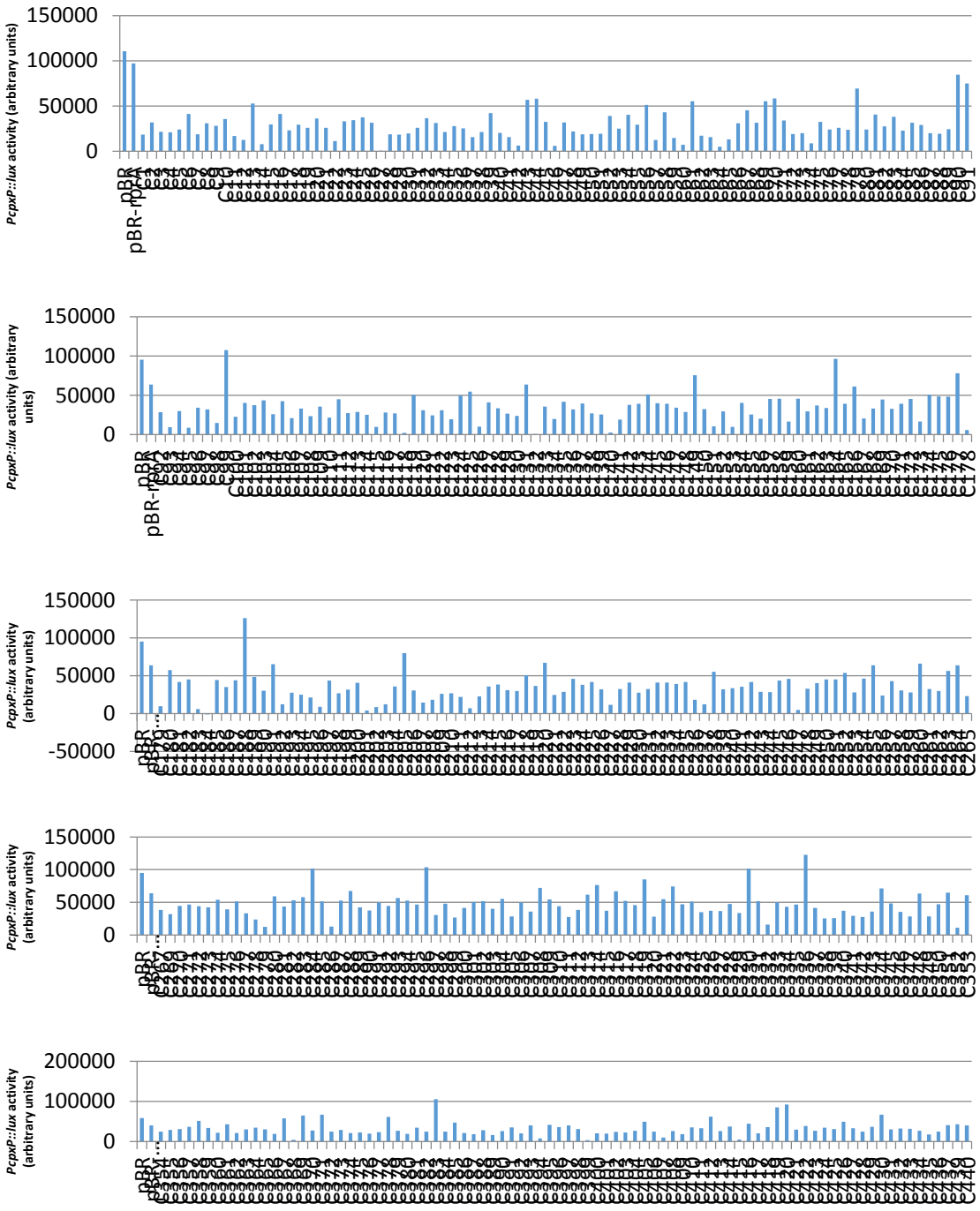


Figure S3: A screen for transposon mutants which abolish Cpx inhibition by RprA. *E. coli* MC4100 bearing the *PcpxP::lacZ* fusion and overexpressing *rprA* was transduced with the Epicenter EzTn transposon mutagenesis kit (Epicenter). Trimethroprim resistant colonies were then inoculated into a 96-well plate containing selective LB medium and 0.1 mM IPTG, and were grown for 5 hours at 37^{0}C while shaking. Luciferase activity was then measured with the plate reader, and cultures were then mixed 1:2 with 20% glycerol in a new 96 well plate to make frozen stocks

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