The Cpx and Rcs responses signal envelope stress through independent mechanisms But are coordinated through the sRNA RprA in the cytoplasm

By

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Abstract

Bacteria have several mechanisms which play a major role in adaptation to environmental stresses. One method involves the use of signal sensing pathways that activate transcription of specific genes encoding proteins to alleviate bacterial stress. Upon sensing an inducing cue, an inner membrane-bound histidine kinase will autophosphorylate on a conserved histidine residue and transfer said phosphate to a conserved aspartate residue of its cognate response regulator in the cytoplasm. This phosphorylated response regulator is then able to control transcription of its regulon members. Notably, the Cpx (conjugative pilus expression) two-component system is important in many Gram-negative bacteria to maintain envelope homeostasis by the upregulation of protein folding and degrading factors, ultimately alleviating stress in the periplasm. In enteric bacteria, the Cpx two-component system has also been found to play a role across many Gram-negative species for proper colonization of hosts and expression of virulence factors. A second phosphorelay, more recently characterized, is the Rcs (regulator of capsule synthesis) phosphorelay, originally discovered for its ability to regulate colanic acid capsular polysaccharide production that protects the cell by promoting biofilm development. The Rcs phosphorelay is an atypical two-component system that has an added level of complexity with an intermediate inner membrane phosphotransfer intermediate, RcsD, that transfers a phosphate from the hisitidine kinase, RcsC, to the response regulator of this system, RcsB. There are also a greater number of auxiliary proteins at the outer membrane and periplasm that generate a signal sensed by RcsC to activate the pathway. The response regulator of the Rcs signal transduction system, RcsB, controls the positive regulation of the small RNA, RprA. Interestingly, the Cpx system has also been recently implicated in regulation of RprA, yet the mechanism behind this interaction is yet to be defined. Several similar inducing signals and toxic molecules have also been shown to activate both the Cpx and Rcs stress responses, such as polymyxin antibiotics and the metal chelator, EDTA. Activation of these responses has also conferred resistance to some of these toxic molecules. The purpose of this study was to test common and potential inducers of each pathway to identify any relationships between these pathways and the regulation of the small RNA, RprA. We identified a link between the microbial

response to fluctuations in pH and the regulation of RprA by the noncognate Cpx and Rcs envelope stress responses. Additionally, we discovered that although both stress responses were induced by the same molecules, each pathway was activated independently of members of the noncognate pathways, and by members of each pathway at different cellular locations at both mid- and late-logarithmic growth. Both the Cpx and Rcs signaling pathways have implications in regulating genes that encode both virulence factors and antibiotic resistance mechanisms in Gram-negative pathogens. As the rise of multi-drug resistant bacteria persists, designing novel therapeutics that can efficiently kill bacteria without activating key stress responses involved in transcription of virulence-associated genes or antibiotic resistance genes will be an important feature to prevent further resistance to antimicrobial drugs. To identify whether one stress response implicated in virulence and antibiotic resistance, the Cpx response, can become activated by toxins known to kill *Escherichia coli,* we tested its ability to sense toxic molecules and bacterial structures that exist in nature but kill *E. coli* efficiently. These toxins were type B colicins, T4 bacteriophage, and the type VI secretion system of *Vibrio cholerae*. Each toxin was chosen because of its ability to interact and damage the cell envelope. The Cpx response did not appear to sense exposure to T4 phage or type B colicins, nor did overactivation or repression of the system confer resistance or susceptibility. Overactivation and repression of the Cpx response also did not alter survival during attack by type VI secretion systems of *Vibrio cholerae*, however it could have potentially activated the Cpx response. Overall, there is evidence that at least type B colicins or T4 bacteriophage could pose as potential antimicrobials that evade at least one important stress response in Gram-negative bacteria while mediating cell killing.

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1.1 *Escherichia coli* as a model organism

In 1884, Theodore Escherich discovered a bacterial strain capable of growing rapidly in the intestines of humans (Blount, 2015). This strain, now known as *E. coli,* has been widely associated with studies of Gram-negative bacteria for its ability to grow rapidly (doubling time of 20 minutes) and with minimal cost. Additionally, its genome was sequenced in 1997, allowing researchers to further characterize this bacterium (Barr, 2017). In addition to working with these bacteria directly, the bacteriophages that use *E. coli* as a host for replication could also be studied. *E. coli* research has uncovered many growth-related aspects of bacterial physiology and evolution, and the study of conjugation helped in the understanding of the exchange of genetic information. *E. coli* has been invaluable in characterizing biochemical pathways, gene regulation and many other key factors in the biology and proliferation of bacteria. *E. coli* is a facultative anaerobe meaning it can grow anaerobically but in the presence of oxygen it can adapt and switch to an aerobic lifestyle, making it easy to work with in laboratory environments (Unden et al., 1994). Strains of *E. coli* are vastly abundant and although it is known that *E. coli* lives as a commensal in the intestine of mammals, several pathogenic serotypes have emerged. Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroadherent aggregative *E. coli* (EAggEC), verotoxigenic *E. coli* (VTEC) and enterohemorragenic *E. coli* (EHEC) can cause a wide range of intestinal damage (Alteri & Mobley, 2012). Additionally, extraintestinal pathotypes of *E. coli* (ExPEC) can cause septicemia, meningitis and other diseases outside the intestinal tract.

1.2 Cell envelope of Gram-negative bacteria

The site of all extracellular stress a bacterium will encounter begins at the cell envelope; a heterogeneous multi-layer complex with proteins that sense and respond to environmental

signals to ensure survival and proliferation (Glauert and Thornley, 1969; Silhavy et al., 2010). These extracellular signals include surface contact, and damaging conditions such as pH, temperature fluctuations and antimicrobial stress. Gram-negative bacteria, such as *Escherichia coli*, have a cell envelope composed of an outer membrane, a periplasmic space and an inner membrane that encloses the molecular constituents of the cell (Glauert and Thornley, 1969). The outer membrane serves as an extra layer of protection, which result in many cases of Gram-negative bacteria being more inherently resistant to antibiotics and antimicrobial agents than their Gram-positive counterparts. Both Gram-positive and Gram-negative bacteria have inner membranes, separating the cytoplasm from the exterior of the cell. The combination of these three layers maintain a balance of energy and osmolarity while controlling both the import of molecules essential for cell function and export of waste products and toxins (Silhavy et al., 2010).

1.2.1 Outer membrane, periplasm and inner membrane

At the outermost layer of the cell envelope, the outer membrane is the first point of contact for Gram-negative bacteria with the extracellular environment. This barrier is an asymmetric lipid bilayer composed of beta-barrel proteins (some as porins), phospholipids on the inner leaflet, roughly 100 types of lipoproteins and glycolipids (predominantly lipopolysaccharides, LPS) (T. J. Silhavy et al., 2010). Beta-barrel proteins are important in the selective import and export of molecules across the outer membrane. Most are porins that vary in size to allow specificity in molecule transport. Lipoproteins are sent to either the inner or outer membrane to perform a variety of functions. When lipoproteins are transported to the outer membrane through the Lol pathway, some are threaded through beta-barrel proteins by the Bam complex (Narita and Tokuda, 2010). The LPS, on the extracellular side of the outer membrane is comprised of lipid A, a core oligosaccharide and a repeated O-antigen, which can differentiate *E. coli* strains due to its immunogenic properties. The LPS is an important component of an efficient outer membrane

barrier and its negative charge provides stability to this outer layer through interactions with divalent cations in the environment and hydrocarbon chain packing (Snyder and McIntosh, 2000).

Between the outer and inner membranes of Gram-negative bacteria, the periplasmic space houses many important intermediates that are modified for final integration in the periplasm, outer membrane or extracellular appendages. Chaperone proteins that facilitate proper folding and proteases that degrade misfolded proteins are important in the periplasm to ensure damage is not caused by unfolded and misfolded proteins (Goemans et al., 2014). Molecules that require periplasmic modifications include flagella, pili, LPS, lipoproteins and porins (that localize in the outer membrane). Types of periplasmic modifications include the introduction of disulfide bonds by protein disulfide isomerases (such as DegP) and the introduction of *cis-trans* isomerization of peptidyl bonds by peptidyl-prolyl isomerases (for example, PpiD) (Miot and Betton, 2004). When proteins are mislocalized due to this misfolding, several stress response systems have components that sense these changes and activate a regulon containing genes encoding protein folding and degrading factors that can alleviate the stress. Two-component systems have histidine kinases threaded through the inner membrane with periplasmic sensing domains which can sense perturbations in periplasmic pH, osmolarity, peptidoglycan damage and misfolded proteins to name a few (Evans et al., 2013; Raivio, 2014). The ability of stress responses to sense damage to the peptidoglycan layer in the periplasm is an important defence mechanism to ensure structural integrity of the cell envelope (Evans et al., 2013; Laubacher and Ades, 2008). The peptidoglycan layer is a structural matrix composed of glycan strands linked to peptides that allows flexibility, variable pore sizes and thickness. When damaged it can be lethal to the cell if not repaired, indicating its essential function in envelope structure.

The inner membrane is a barrier between the cytoplasm and the periplasm containing important proteins for signal transduction, transport of molecules, energy generation, cell envelope biogenesis and efflux of molecules (Allen et al., 2009; Silhavy et al., 2010). All cell envelope components are generated in the inner membrane or cytoplasm (some as precursors) which then pass through the inner membrane before transport to their final destination in the periplasm or outer membrane (Silhavy et al., 2010). The inner membrane is a phospholipid

bilayer that facilitates protein secretion in three major ways; using the signal recognition particle (SRP), the Sec translocon and the Tat pathway. These transport complexes facilitate movement of folded and unfolded proteins into the inner membrane and periplasm.

Lipoproteins are altered by the Lol system, first by a protein complex in the inner membrane, LolCDE and subsequently by a protein in the periplasm, LolA which transports it to a final protein anchored to the periplasmic side of the outer membrane, LolB (Narita and Tokuda, 2010). Precursors for lipoproteins that have been transported through the inner membrane to its outer leaflet by the Sec translocon then interact with the ABC transporter LolCDE if they are destined for integration into the outer membrane (Hayashi and Wu, 1990). Once LolCDE recognizes the lipoprotein by its sorting signal on its N-terminus, the lipoprotein undergoes aminoacylation of the N-terminal cysteine by LolCDE and is released into the periplasm. Another component of the Lol machinery, LolA forms a complex with the lipoprotein and transports it to the outer membrane (Taniguchi et al., 2005). The last component of this pathway, LolB, which forms a stronger interaction with the transported lipoprotein than LolA, ensures the lipoprotein doesn't travel back to LolCDE and instead becomes inserted into the inner leaflet of the outer membrane (Okuda and Tokuda, 2009).

It is still not well understood how some lipoproteins adopt a surface-exposed region through the outer membrane and into the extracellular space. It was originally thought that this phenomenon was a rare occurrence but evidence continues to emerge that insinuates otherwise (Hayashi and Wu, 1990; Wilson and Bernstein, 2016). One notable discovery identified a surface exposed lipoprotein RcsF exists in a complex with the outer membrane proteins like OmpA (Konovalova et al., 2014). RcsF has a transmembrane conformation that can become threaded through several different outer membrane beta-barrel proteins, allowing its N-terminal end to be exposed to the extracellular environment and the C-terminal domain resting in the periplasm. This ability to pass through the lumen of these outer membrane proteins involves an interaction of RcsF with BamA from the Bam machinery. The beta-barrel assembly machinery (Bam) complex is important in the assembly of beta-barrel proteins at the outer membrane after they utilize the Sec translocon to initiate this interaction at the inner membrane (Bakelar et al., 2016).

Subsequently, proteins such as Skp or SurA chaperone the beta-barrel proteins to the Bam complex (Hagan et al., 2011; Ricci and Silhavy, 2012) . Four lipoprotein components (BamB, BamC, BamD and BamE) and one outer membrane protein (BamA) make up this machinery. The evidence presented from Konovalova et al. (2014) shows that RcsF and BamA interact to ensure RcsF is inserted through outer membrane proteins such as OmpA. This interaction allows for RcsF to sense signals in the extracellular environment.

In addition to outer membrane lipoproteins, transmission of stress signals to the cytoplasm requires inner membrane proteins called histidine kinases with periplasmic sensing domains and cytoplasmic phosphotransfer domains. For specificity of activation of these stress responses, the kinases must be properly inserted into the inner membrane.

1.3 Two-component signal transduction systems

Two-component systems have a role in relieving any stress or damage incurred at the cell envelope (Raivio and Silhavy, 1999). *Escherichia coli* have approximately 30 histidine kinases and 30 cytoplasmic response regulators, transferring an external stimulus through the cell envelope to the cytoplasm to activate a regulon, encoding proteins to relieve envelope stress. Signal transduction systems typically contain an inner membrane histidine kinase, sometimes with dual phosphatase activity. Additionally, in the cytoplasm a response regulator protein that can sometimes form hetero- or homodimers to activate transcription of its associated regulon. Additional members of stress response systems include intermediary phosphodonors, periplasmic proteins, inner membrane proteins, cytoplasmic proteins and outer membrane anchored lipoproteins (Gerken et al., 2009; Laloux and Collet, 2017; Raivio and Silhavy, 1997; Snyder et al., 1995).

1.3.1 The Cpx two-component signal transduction pathway

The Cpx signal transduction system senses and responds to cell envelope perturbations caused by changes in the outside environment and/or an accumulation of misfolded proteins in the periplasm. The original discovery of the Cpx response was focused on the histidine kinase, CpxA*,* and the reduction in conjugal transfer of the F plasmid because of a non-functional F pilus when the gene encoding it, *cpxA,* was deleted (McEwan and Silverman, 1980). Effects of this deletion of *cpxA* included sensitivity to temperature, altered membrane protein profiles and other phenotypes associated with a compromised cell membrane (Raivio and Silhavy, 2001). Shortly after CpxA was identified as the two-component system sensor protein (Nixon *et al.,* 1986). The gene *cpxR* was found to encode the cognate response regulator, upstream of the *cpxA* gene (Dong et al., 1993; Raivio and Silhavy, 1997).

The Cpx response can become activated by the autophosphorylation of the histidine kinase, CpxA in the inner membrane and phosphotransfer to CpxR, the response regulator in the cytosol (Weber and Silverman, 1988; Dong *et al*., 1993). Phosphorylated CpxR will then upregulate genes containing a CpxR binding site in their promoter region to initiate transcription of the encoded proteins that will alleviate envelope stress such as protein folding and degrading factors (Price & Raivio, 2009). The original Cpx regulon consisted of genes encoding for four main categories: protein folding factors, protein degrading factors, motility proteins, and components of the Cpx pathway (*cpxRA* and *cpxP*) (Raivio *et al*., 2000; Price and Raivio, 2009). Aside from these main categories, scored genomic sites with a CpxR-P weight matrix was used to predict affinity for CpxR binding sites on potential regulon members (DeWulf *et al*., 2002).

Another important Cpx signalling protein is NlpE, a lipoprotein attached to the periplasmic side of the outer membrane where it can sense events at the cell surface and relay this information to CpxA, which is predicted to be a direct interaction (Otto and Silhavy, 2002). Later studies suggest that NlpE may directly interact with CpxA through one of two proposed mechanisms (Hirano *et al.,* 2007). Hirano and colleagues (2007) suggested that either the unstable N-terminal domain unfolds to allow the C-terminus to directly interact with the inner membrane where CpxA is located, or when an excess of periplasmic protein folding is necessary, NlpE may become misfolded and mislocalized to the inner membrane to induce the Cpx pathway.

Further studies will clarify whether NlpE is required to be misfolded in the inner membrane or if it is folded properly and retains its N-terminal domain anchoring it to the outer membrane when inducing the Cpx response. Although NlpE has been shown to activate the Cpx two-component system when cells are adhered to a surface, it is not involved in other Cpx inducing events such as changes in pH (DiGuiseppe and Silhavy, 2003). NlpE is required to activate the Cpx response when adhered to a surface as an *nlpE* deletion strain no longer induces the Cpx response in adhered cells (Otto and Silhavy, 2002). Further studies of NlpE may help identify where else it is required in CpxA autophosphorylation, such as sensing the presence of metals, or other extracellular molecules that interact with the outer membrane. A Cpx-mutant screen provided preliminary evidence that NlpE may be essential in sensing copper, therefore there may be many other roles yet to be discovered for this lipoprotein (Gupta *et al.,* 1995).

1.3.1.1 Cpx regulon members

The abundance of studies that proceeded the discovery of the Cpx two component system summarized a role for this stress response in cell envelope homeostasis when activated by misfolded proteins in the periplasm (Raivio, 2014). A number of regulon members of the Cpx stress response were subsequently discovered with roles in protein folding and degradation in the periplasm (Cosma et al., 1995; Danese and Silhavy, 1997; Danese et al., 1995; Mileykovskaya and Dowhan, 1997). Important Cpx regulon members for envelope maintenance include the genes *degP*, *spy*, *dsbA*, *ppiA and cpxP*. DegP, a periplasmic protease, facilitates degradation of misfolded or excess proteins in the periplasm under Cpx-inducing conditions (Danese *et al.,* 1995). Genes encoding similar proteins, namely DsbA and PpiA are upregulated as well when CpxR becomes phosphorylated, that also function to reduce envelope associated stresses. DsbA is a disulfide bond oxidoreductase protein, which was found to be induced by the Cpx response. It introduces disulfide bonds in many extracytoplasmic proteins in *E. coli* (Danese and Silhavy, 1997). PpiA is a peptidyl-prolyl isomerase which facilitates protein folding which is necessary to reduce misfolded protein accumulation in the periplasm (Pogliano *et al.,* 1997). CpxP is a small

periplasmic chaperone and inhibitor of CpxA under nonstressed conditions (Danese and Silhavy, 1997; Raivio et al., 1999). There is evidence that inhibition of CpxA is through interactions of CpxP in the periplasm and under conditions of stress it will dissociate allowing CpxA autophosphorylation (DiGiuseppe and Silhavy, 2003; Fleischer et al., 2007; Isaac et al., 2005; Thede et al., 2011; Zhou et al., 2011). Evidence of CpxP as a chaperone indicate a role in bringing misfolded proteins to the protease DegP, where both CpxP and the misfolded protein become degraded (Isaac et al., 2005). Although there are many Cpx-regulated genes, those that alleviate stress within the cell envelope have some of the highest expression levels of all regulon members identified, indicating their importance in cell survival (Price and Raivio, 2009).

More recent studies have expanded our knowledge of Cpx-regulated gene targets for their roles beyond general envelope stress and into other cellular processes such as translocation (*secA*), virulence in uropathogenic *E. coli* (*mviM*), metabolism of DNA (*ung*) and accumulating and storing iron (*ftnB* and *efeU*), respiration and pathogenesis (Cao et al., 2007; De Wulf et al., 2002; Ogasawara et al., 2004; Yamamoto and Ishihama, 2006; Raivio et al., 2013). From transcriptomic studies it has been observed that large respiratory complexes are downregulated strongly by activation of the Cpx stress response (Raivio et al., 2013). Further investigation has shown that stability and overall function of these inner membrane respiratory complexes is affected by Cpx regulon members (Guest et al., 2017). Most notably are the effects on pathogenesis of Gram-negative bacteria which must extend structures through the envelope for a variety of functions including attachment to hosts. A properly functioning Cpx stress response is needed to properly express the type IV bundle forming pili of enteropathogenic *E. coli*, for subsequent anchoring to epithelial cells (Nevesinjac & Raivio, 2005). Interestingly, the Cpx signal transduction pathway plays a negative role in expression of type 3 secretion substrates located primarily on the locus of enterocyte effacement (LEE) pathogenicity island (Macritchie et al., 2008). Activation of the Cpx stress response downregulates several of the LEE operons required for causing attaching and effacing lesions during infection in hosts which destroy microvilli on the outer surface of intestinal cells, and attach the bacteria to its outer membrane (Franzin & Sircili, 2015). Furthermore, in mouse and zebrafish infection models, uropathogenic *E. coli* (UPEC)

requires a functional Cpx transduction pathway for virulence (Debnath et al., 2013). This study highlighted the importance of this two-component system as deletion of the *cpxAR* operon diminished colonization in mouse bladders and reduced virulence significantly in zebrafish embryos in localized and systemic infections with UPEC, however as deletion of *cpxA* leads to higher pathway activity, it is likely the *cpxR* gene deletion that caused reduced virulence. It has become evident that the Cpx regulon contains a diverse number of genes that benefit cell survival and alter expression of virulence determinants. Exploring the known and unknown genes under Cpx pathway regulation will benefit the characterization of the Cpx regulon further.

1.3.1.2 CpxA and CpxR components of the Cpx system

CpxA is a histidine kinase with two hydrophobic transmembrane domains, one in the periplasm and one in the cytoplasm to facilitate activation of its regulon (Weber and Silverman, 1988). In the periplasmic region a domain was identified as the component that responds directly to envelope stress (Raivio and Silhavy, 1997), where modifications can affect its kinase and phosphatase functions. This protein has both phosphatase and autokinase activity. CpxA can act as a phosphatase to prevent phosphorylation of CpxR which will repress gene transcription of the Cpx regulon (Fig. 1.1). On the other hand, CpxA also has autokinase activity to quickly autophosphorylate and activate CpxR under envelope stress conditions. In either case, its ability to regulate the pathway allows rapid amplification or inhibition of transcription of Cpx regulon genes.

CpxR, the transcriptional activator of the Cpx response, becomes activated when CpxA transfers a phosphate group to CpxR at a conserved aspartate at residue 51, identified by mutagenesis of *cpxR* (Hoch and Silhavy, 1995). This phosphotransfer then allows CpxR to regulate expression of genes encoding for proteins to relieve envelope stress. On the contrary, under non-stressed conditions CpxA can use its phosphatase action to inhibit CpxR phosphorylation in the absence of an activating cue (Raivio and Silhavy, 1997). Mutations in

either CpxA or CpxR activity can adversely affect the way *Escherichia coli* respond to envelope perturbations (Bontemps-Gallo et al., 2015; Mahoney and Silhavy, 2013; Vogt and Raivio, 2012)

Mutations to the Cpx system can have both positive and negative effects on the expression of Cpx regulated genes. In *cpxR* deletion mutants there is no transcriptional activator for the pathway to be turned on, so essentially the pathway is inactivated when CpxR is not present (Raivio and Silhavy, 1997). This can be observed by looking at expression of genes that are solely regulated by CpxR, such as CpxP. In *Escherichia coli* harbouring a *cpxR* deletion *cpxP* transcription is abolished (Danese and Silhavy, 1998).

Interestingly, with a CpxA deletion mutant the Cpx regulated gene transcripts such as *cpxP* and *degP* become upregulated with or without stress-induced cues (Danese *et al.,* 1995). This result is due to CpxR activity no longer being repressed by the phosphatase action of CpxA, and phosphorylation occurring from other donor molecules (Fig 1.1). It was discovered that one donor molecule is acetyl phosphate, which is able to phosphorylate CpxR with or without membrane stress, but the phosphate cannot be removed by the phosphatase activity of CpxA. To confirm this observation, under low acetyl phosphate conditions it was shown that CpxR is unable to activate transcription of its target genes in the absence of CpxA (Danese *et al.,* 1995).

When both CpxA and CpxR are intact and the Cpx pathway is induced, the *cpxRA* operon and *cpxP* gene are transcribed, suggesting the response can control further expression of its own gene transcripts (Pogliano *et al*., 1997). Also, in a gain of function CpxA* mutant or when *nlpE* is overexpressed the Cpx regulon transcript levels are increased (Raivio *et al.,* 1999). This information suggests that the Cpx response creates a positive feedback loop under stress inducing conditions.

1.3.2 The Rcs response

The atypical Rcs (Regulator of capsule synthesis) stress response system is an important signaling pathway in the regulation of colonic acid capsule, resulting in bacterial colonies with a mucoid phenotype (Gottesman et al., 1985). The primary components of the Rcs signaling

pathway include a lipoprotein tethered through the outer membrane, RcsF, two inner membrane proteins, RcsC and RcsD, and the cytoplasmic response regulator, RcsB (Castanié-Cornet et al., 2006; Majdalani and Gottesman, 2005). There are additional protein members that affect Rcs activity in both the periplasm and cytoplasm. IgaA was recently characterized as a repressor of the Rcs response in which it interacts with the histidine kinase, RcsC, to inhibit its autophosphorylation and subsequent phosphorylation of the response regulator RcsB (Domínguez-Bernal et al., 2004). Under inducing conditions RcsF facilitates an interaction with IgaA, dissociating it away from RcsC which can then autophosphorylate and transfer the phosphateto RcsB (Cho et al., 2014). An intermediate inner membrane protein, RcsD, is phosphorylated by RcsC as it lacks its own autokinase abilities. RcsB is then phosphorylated in the cytoplasm by RcsD which interacts with the phosphoreceiver domain of RcsB (Schmöe et al., 2011a). RcsB can then form a dimer with itself, or several other auxiliary proteins including RcsA, BglJ and GadE to effect changes in transcription of different subsets of genes.

1.3.2.1 Rcs regulon members

The Rcs regulon is involved with regulation of adaptation to osmolarity, pH, antibiotics and virulence attributes (Majdalani and Gottesman, 2005). Studies of the Rcs response are essential to understand how this pathway contributes to cell survival in pathogenic Gram-negative bacteria. The Rcs regulon can be broken down into two main categories: RcsB homodimer and RcsB-accessory protein regulon targets. When RcsB forms a homodimer with another RcsB protein, its regulon includes genes encoding the small RNA RprA, FtsZ, a cell division gene, and two osmolarity-regulators OsmB and OsmC (Majdalani and Gottesman, 2005; Pannen et al., 2016). RcsB has several notable accessory proteins that it can interact with as well. The RcsB-RcsA transcriptional targets include the well-known *cps* operon which regulates capsule synthesis, and *rcsA*, encoding the RcsA transcription factor, to increase expression of capsule synthesis (Ebel and Trempy, 1999). A RcsB-GadE complex was also identified which controls activation of the GAD-dependent acid stress response (GDAR) (Castanié-Cornet et al., 2010).

GadE is responsible for transcriptional activation of the *gadABC* operon through binding 63 bp upstream of the transcriptional start site of these genes (Ma et al., 2003) Basal activity of RcsB stimulates *gadABC* expression in the presence of GadE, whereas activation of the Rcs response and higher activity of RcsB represses the Gad-dependent acid stress response (Castanié-Cornet et al., 2010). A RcsB/GadE heterodimer and a GAD box is required for *gadA* transcription, whereas an RcsB box was found upstream of the *gadA* promoter to control repression of the operon. Under inducing conditions of acid stress, the current model for GAD-dependent acid resistance shows RcsB which forms a homodimer and upregulates the non-coding small RNA RprA (Bak et al., 2014). RprA overexpression increases *rpoS* transcription, then the encoded protein RpoS can then induce *gadX* expression, which leads to elevated expression of *gadE*. GadA and GadB are glutamate decarboxylase isoforms that convert glutamate into gamma-aminobutyrate (GABA) that results in consumption of an intracellular proton (Ma et al., 2003; Waterman and Small, 2003). This conversion maintains pH at roughly neutral when extracellular conditions are acidic. GadC is an integral membrane protein that brings in glutamate from the environment and exchanges it for the decarboxylated product GABA (Castanié-Cornet et al., 2010). As GadE is the transcriptional activator of *gadA* and *gadBC,* this relay of transcriptional activation is essential for transcribing members of the GAD acid resistance pathway. The input signals for GAD-dependent acid resistance are still unknown, and which members contribute to sensing this pH stress are yet to be determined.

1.3.2.2 Outer membrane, inner membrane and cytoplasmic components of the Rcs response

At the outer membrane, RcsF and OmpA are found in a complex where RcsF has a N-terminal surface exposed region, important in sensing LPS defects (Cho et al., 2014; Konovalova et al., 2014). This complex is created using the Bam complex, which inserts beta-barrel proteins and associated lipoproteins in the outer membrane. Under certain stress conditions the Bam complex is unable to funnel RcsF through OmpA, its primary associated beta-barrel protein in the

outer membrane so that it's N-terminal domain can be surface exposed (Cho et al., 2014). RcsF cannot be properly inserted into the outer membrane, leading to hyperactivation of the Rcs response due to misfolded RcsF accumulation at the inner membrane where it interacts with IgaA, the inhibitor of RcsC autophosphorylation. This results in continuous autophosphorylation of RcsC in the periplasm. OmpA is therefore essential in the specificity of RcsF-mediated signaling, and without this major interacting partner RcsF also accumulates at the inner membrane (Cho et al., 2014; Konovalova et al., 2014). Although RcsF can form a complex at the outer membrane with additional outer membrane proteins such as OmpC and OmpF, the removal of OmpA and subsequent hyperactivation of the Rcs through RcsF:IgaA interactions indicates it plays a majority role in maintaining RcsF tethered through the outer membrane, allowing IgaA to keep the Rcs pathway in an inactive state (Cho et al., 2014; Konovalova et al., 2014). Under inducing conditions RcsF somehow dissociates from its complex with OmpA and accumulates at the inner membrane where it will interact instead with IgaA (Farris et al., 2010). RcsF relieves the inhibition of RcsC autophosphorylation by IgaA, activating the pathway through autophosphorylation of RcsC.

RcsC is a transmembrane histidine kinase with phosphatase action. It can activate the Rcs pathway by transferring a phosphoryl group to the intermediate phosphotransfer protein in the inner membrane, RcsD. RcsD then transfers the phosphate to the receiver domain of RcsB in the cytoplasm, but lacks its own autophosphorylation ability (Majdalani and Gottesman, 2005). A deletion of *rcsD* was shown to be required for activation of the Rcs response when bacteria cells were co-incubated with amphipathic cationic antimicrobial peptides, indicating this intermediate phosphotransfer protein is important for Rcs pathway signaling. Due to RcsC's dual kinase/phosphatase roles, it can also remove the phosphoryl group from RcsB when the pathway needs to be inactive (Schmöe et al., 2011).

RcsB can dimerize with many proteins whether active or inactive (Pannen et al., 2016). It is the most important Rcs member, as a deletion of *rcsB* will completely shut off transcription of its regulon. In the presence or absence of RcsC, RcsB can be acylated by acetyl phosphate, and further deacylated by the protein deacetylase, CobB (Guo and Sun, 2017). Acetyl phosphate is

termed a global signal for *E. coli*, which seems to play a role in responding to an array of signals in the cell (Klein et al., 2007). This alternative to activation of stress responses by signal sensing is inexpensive to the cell metabolism and is short-lived, indicating it could play an important role in adaptation to sudden stressful conditions. Protein deacetylases would therefore work to control activation of stress responses by acetylation to ensure overactivation of the stress responses does not become lethal (Hu et al., 2013).

1.4 Common inducers of the Cpx and Rcs phosphorelays

Investigation of inducers of the Cpx and Rcs have uncovered many similar cues that include deficiencies in phospholipid biosynthesis (Mileykovskaya and Dowhan, 1997; Nagahama et al., 2006; Shiba et al., 2012; Shimohata et al., 2002), deletion of inner membrane localized, conserved DedA protein family members (important in cell division processes and maintenance of the proton motive force) (Sikdar et al., 2013), deletion of multiple PBPs (Evans et al., 2013), EDTA, polymyxin B and over-expression of the lipoprotein NlpE (Audrain et al., 2013; Bury-Moné et al., 2009; Snyder et al., 1995; Vogt et al., 2014). The Rcs and Cpx envelope stress responses have also demonstrated regulation of common genes. Both responses negatively regulate production of proteinaceous curli fibers on the cell surface (Dorel et al., 1999; Jubelin et al., 2005; Vianney, 2005) and impact the expression of envelope spanning protein complexes, including secretion systems of pathogenic species (Flores-Kim & Darwin, 2012; Raivio, 2014). Each pathway may act independent of one another to upregulate their respective downstream targets under similar inducing cues, each pathway with a specific role. However, studies of overlapping stress response systems identified interactions that occur through phosphorylation of noncognate response regulators, histidine kinases, and overlap in regulon members (Danese and Silhavy, 1997; Guckes et al., 2017; Howell et al., 2006). Whether the Cpx and Rcs co-regulate or respond to inducing cues independently will offer insights as to the specificity of signals from the extracellular environment.

1.4.1 EDTA

EDTA (Ethylenediamine tetraacetic acid) is commonly used against infections, as it is a metal chelator and breaks down biofilms (Banin et al., 2006; Finnegan and Percival, 2015). EDTA is often used in combination with antimicrobial agents, increasing their efficiency by interacting with cations to destabilize the cell envelope and allow entry of foreign molecules. It's use in medicine and veterinary medicine has proved important when infections with Gram-negative and Gram-positive bacteria arise. In Gram-negative bacteria, EDTA works by sequestering magnesium and calcium ions from the outer membrane, and it can rapidly reduce the amount of LPS by up to 50% in *Escherichia coli* (Leive, 1965). Magnesium and calcium ions are essential to the structural integrity of the LPS as they are abundantly found interacting with LPS to facilitate aggregation. Removal of these ions destabilizes the STM 7 ribbon-like structures of the LPS and allows the LPS to become water soluble (Shands and Chun, 1980).

1.4.2 Polymyxin B

Polymyxins are a class of last resort antibiotics typically used against persistent, multidrug resistant bacteria where other antibiotics fail. The polymyxins are polymyxin B and E (also known as colistin) which are derived from *Bacillus polymyxa* and are effective against many Gram-negative bacteria with the exception of the genera *Neisseria* and *Proteus* (Landman et al., 2008). Polymyxin B and E both have a cyclic heptapeptide loop structure with a tripeptide side chain and a fatty acid chain (Yu et al., 2015). They contain both hydrophilic and lipophilic groups which result in an amphipathic molecule. Polymyxins target first the lipid A of the LPS at the extracellular side of the outer membrane. It is hypothesized that polymyxins destabilize the interactions of adjacent LPS structures and displace divalent cations so that the hydrophobic fatty acyl chain can be inserted into the cell envelope (Yu et al., 2015). Once it has entered into the cell it will cause further damage at the inner membrane through a feature known as membrane thinning, which is an interaction with the head groups of fatty acyl chains that leads to lysis of the inner membrane and cell death. Polymyxins are a known inducer of both the Cpx and Rcs

response systems that monitor integrity of the cell envelope (Bury-Moné et al., 2009a). Characterizing how stress responses sense antibiotic damage such as the polymyxins will play a role in development of novel therapies to overcome resistance conferred by Gram-negative pathogens.

1.5 The Cpx response and antimicrobial resistance

Gram-negative bacteria pose a major threat when ingested through contaminated water and food products. Their dual membranes, secretion systems and efflux pumps have been used successfully in evasion of antibiotic and host immune system stresses to cause infection and allow them to grow in many diverse and polymicrobial environments. The outer membrane itself creates an additional layer of complexity that antibiotics must be able to penetrate through. Two modes of entry exist for antibiotics: hydrophilic antibiotics travel through general diffusion porins and hydrophobic antibiotics use a lipid-mediated pathway (Delcour, 2009). Stress response systems sense external dangers such as traditional antibiotic therapeutics and allow bacteria to survive by activating transcription of many regulons with roles that include deactivation, outer membrane alteration, or pumping out antibiotics from the interior of the cell. The Cpx two-component system has been linked to studies of general toxicity from bactericidal antibiotics as well as aminoglycoside and beta-lactam resistance (Bryan and Van Den Elzen, 1977; Cosma et al., 1995; Davies et al., 2009; Hirakawa et al., 2003; Kohanski et al., 2010; Kohanski et al., 2008; Plate et al., 1986; Srinivasan and Rajamohan, 2013; Srinivasan et al., 2012; Thorbjarnardottir et al., 1978). Genes controlled by the Cpx response include those encoding protein folding and degrading factors that are upregulated during antimicrobial stress such as DegP, CpxP and Spy. As Cpx response activation has been shown to confer resistance to some antimicrobials, it is likely that these proteins present misfolded and damaged proteins to proteases, act as proteases, or contribute to proper folding of those damaged by antimicrobials in the cell envelope.

Damage at the cell envelope is a consistent activator of the Cpx two-component system. The search for novel therapeutics continues to persist as an important issue in healthcare and

understanding how stress response systems such as the Cpx pathway generate resistance or multi-drug resistance calls for unique therapies. Hallmarks of an effective therapeutic would ideally: 1) kill cells not just inhibit growth, 2) bypass stress responses (i.e. they would not activate the responses), and 3) be economically sound for mass production. Three ways in which we know that *Escherichia coli* can be killed are through type VI secretion systems and release of their effector molecules by other bacteria in close proximity, T4 bacteriophage killing and type B (pore forming) colicins (Fig. 1.2; Bryan et al., 2016; Cascales et al., 2007; MacIntyre et al., 2010). Further studies would elucidate if any of the three toxic molecules/ machinery could serve as a potential novel therapeutic against similar Gram-negative bacteria, and whether the Cpx becomes active and can confer resistance in their presence.

1.5.1 Secretion systems, types 1-6

Gram-negative bacteria can produce six types of nanomachines with ranging functions, including to attack other bacteria and export virulence factors across cell membranes (Costa et al., 2015). These molecular machines, known as secretion systems, can span both the inner and outer membrane (types I, II, III, IV and VI), or solely the outer membrane (type V). The final location of the secreted substrates from these systems can be one of three possible outcomes: continued association with the outer membrane, release into the extracellular environment or association and injection into a targeted cell (Gerlach and Hensel, 2007). Although each secretion system is unique in structure and function, there are homologies between many of the proteins that compose these molecular machines. For example, both the type II and type III secretion systems have the ring-shaped secretin channel, although the type III has a needle-like complex and type II does not (Hodgkinson et al., 2009).

1.5.1.1 Type VI secretion systems

Type six secretion systems (T6SS) are one of seven secretion systems identified in Gram-negative bacteria used to export virulence factors and effector proteins across another microorganism's bacterial envelope (Costa et al., 2015). Type VI secretion systems are double membrane penetrating which allows transfer of these toxins directly into the cytoplasm of host cells or into the periplasm to affect cell wall integrity. For example, in *Vibrio cholerae* the secretion of four proteins: Hcp, VgrG-1, VgrG-2, and VgrG-3 is mediated by the *vas* gene cluster, conserved in many Gram-negatives (Das and Chaudhuri, 2003; Pukatzki et al., 2006). The cytotoxic effect of pathogenic bacteria such as *Vibrio cholerae* was lost when the T6SS *vas* genes were inactivated, which encode components of the T6SS (Pukatzki et al., 2007). This indicates the T6SS has an important role in virulence. Hcp forms a hollow tube-like tail, VgrG proteins form the cell-puncturing tip and two proteins, and TssB and TssC, form a sheath used to provide the translocation energy for the secretion of effectors and virulence factors into host/prey cells (Russell et al., 2014). Effector proteins, like colicins, are transcribed with their associated immunity proteins to protect the host cell. Once the bacterium's T6SS has punctured the membranes of its target cell it releases effector proteins and virulence factors into the cytosol or periplasm. The structure and function of effectors varies among genus and species of bacteria.

In *Vibrio cholerae*, three important effector proteins are released by the type VI secretion machinery that facilitate cell killing (Dong et al., 2013). Each individually is required to kill prey cells. Effector molecules are described as ligands that can bind with specificity to proteins and regulate their function. Vgr3, which has recently been identified as an effector protein, and recognized as a component of the T6SS puncturing tip, causes cell rounding and lysis in target cells (Sheahan et al., 2004). The second effector protein, TseL, has a lipase domain, therefore it is hypothesized it may target lipids associated with the cell membranes (Dong et al., 2013). The last effector, VasX, is a pore-forming colicin that interacts with phospholipids in the membrane (Joshi et al., 2017; Miyata et al., 2011). Aside from their toxic effect once secreted into target cells, *tseL* and *vasX* deletion mutants also remove the virulence capabilities of *V. cholerae,* suggesting they are important to gain environmental advantage when competing with other microbes for resources in nature (Dong et al., 2013).

1.5.2 Colicins

Colicins are specialized antimicrobial proteins created by *Escherichia coli* and are toxic to other *E. coli* strains and similar species that lack the corresponding immunity genes. It is assumed that production of colicins provide an environmental advantage to outcompete other bacteria in a given niche. Colicins are encoded on plasmids called pCol with their associated immunity proteins that will inactivate these proteins if they remain within their native bacteria (Cascales et al., 2007). There can be multiple colicins encoded in a pCol plasmid, therefore the bactericidal effect can vary.

Three important domains allow these toxins to interact with prey cells and allow their entry (Cascales et al., 2007). The first step of binding to an outer membrane receptor is an interaction with the central R domain of a colicin. Translocation into the cell is coordinated by the N-terminal T-domain, and parasitization of proteins and cellular energy. The respective toxic effect of each colicin is conserved in the C- terminal active domain, which can interact with different essential components of the prey cell such as the inner membrane where it becomes destabilized and creates pores, or having nuclease activity, cleaving chains of nucleic acids (Cascales et al., 2007; Lazdunski et al., 1998).

Subsequent entry of these toxins into prey *E. coli* cells is facilitated by binding to outer membrane porins and translocation through either the Tol or TonB protein pathways. Both proteins have homologous transmembrane anchors and are located in the periplasm, however the Tol pathway is important in membrane integrity, as mutations in this protein increase susceptibility to antibiotics and detergents (Levengood-Freyermuth et al., 1993). TonB is required to transport siderophores into the cell for iron acquisition (South et al., 2009). Both systems have two components: one for energy harvesting at the cytoplasmic membrane, and the second for transfer to the outer membrane (Cascales et al*.,* 2007). TonB has an energy harvesting complex called ExbB/ExbD, and the Tol system uses TolQ/TolR. TonB and TolA are inner membrane-anchored, periplasm spanning components that transfer this energy between inner and outer

membranes to facilitate energy-dependent translocation of molecules between membranes, as the periplasm does not contain ATP (Allen et al., 2009).

1.5.2.1 Group A and group B colicins

The group A colicins are A, E1-E9, K, L, N, S4, U and Y (Cascales et al., 2007). This classification is based on the use of the Tol system for entry into prey cells (Tol-dependent). Group A colicins are generally encoded on small plasmids and released in the extracellular environment. The group B colicins (Ia, B, D, Ib, M, V, 5 and 10) require the TonB pathway (Ton-B dependent) to translocate into vulnerable *E. coli* cells (Cascales et al., 2007). These are generally encoded on larger plasmids than group A and are not excreted into the outside environment. For our purposes, the focus of this study will be on colicins B and Ia, two of the pore-forming colicin types.

After initial outer membrane binding to either the Cir (colicin Ia) or the FepA (colicin B) outer membrane proteins these colicins rely on the energy-dependent uptake by TonB for entry (Lazdunski et al., 1998). The Cir outer membrane porin is an iron siderophore transporter which contains a semi-conserved N-terminal domain and a TonB box, where TonB interacts. Colicin Ia uses two Cir porins, one as a binding receptor, and a second for translocation. The receptor (R) domain of colicin Ia binds the first Cir porin, while the N-terminal and C-terminal domains bind to an adjacent Cir porin for entry into the periplasm (Jakes and Finkelstein, 2010). At high concentrations, colicin Ia undergoes receptor binding saturation, therefore a second Cir porin is unavailable for translocation into prey *E. coli* cells which can inhibit or slow their toxic effect (Jakes and Finkelstein, 2010). The FepA porin in *E. coli* is also an iron siderophore receptor with a TonB box, similar to the receptors for all TonB-dependent colicins (group B). Colicin B only needs one FepA porin and a functional TonB pathway to facilitate entry, therefore cell killing is not inhibited at high concentrations (Lazdunski et al., 1998). Colicins B and Ia can then form pores in the phospholipid bilayer and destabilize the membrane by depolarizing the phospholipid bilayer with their C-terminal domain, creating ionic pores.

The role of the TonB protein is to mediate any transfer of energy from the inner membrane to the receptor on the outer membrane, without which colicins would not be able to translocate into vulnerable cells. This is shown by mutations to *E. coli* cells that remove the TonB protein (Davies and Reeves, 1975). Group B colicins are unable to enter these mutants, just as a mutation in the Tol protein would inhibit group A colicin entry. Both the TonB and Tol proteins could potentially become mutated in bacteria that are constantly in proximity to *E. coli* cells that produce them, therefore the potential for colicins to be used as an antimicrobial will require further studies to determine if this occurs in nature, or if cells can afford mutations to these proteins.

1.5.3 Bacteriophage

Bacteriophages were first observed killing cultures of bacteria that cause cholera in 1896 by the bacteriologist Ernest Hankin (Wittebole et al., 2014). It was originally found that when Indian river water was filtered to remove bacteria and then mixed with a culture of bacteria, the culture of cells began to die. Shortly after, a microbiologist named Frederick Twort observed similar occurrences, and added that these mysterious agents were unable to grow in the absence of bacteria and could not be subcultured. Two years after this discovery, another researcher, Felix d'Herelle, identified these agents in patient studies with bacillary dysentery. This finding lead him to propose that bacteriophages could be used in clinical medicine, and led to a series of human trials across the world such as intravenous phage treatment for infections.

Our understanding of bacteriophage biology has expanded tremendously since these first observations, and over 6000 different types of bacteriophages have been identified thus far (Ackermann and Prangishvili, 2012). Although they contain a genome, these agents are classified as non-living. Phage can only reproduce and survive by acting as an intracellular parasite to specific bacteria where they hijack the cellular energy of their host cell to reproduce their viral genome and progeny phage particles (Hershey, 1952). The general structure of a phage contains a protein coat (capsid) which encloses the viral genome, a tail (96.3% of phages), and a few associated structures for docking to the host cell (Ackermann and Prangishvili, 2012).

Classification of phages can be based on several factors including structure, host range, environmental niches, their viral genome or their lifecycle (Wittebole et al., 2014).

1.5.3.1 Lytic versus lysogenic bacteriophage

One major difference between bacteriophages, and a common way to differentiate them, is their lifecycle. Regardless of type, the first step is attachment of the phage to outer membrane receptors of its host (Wittebole et al., 2014). This step is necessary for the successful transfer of the viral genome and confers specificity to the host-phage interaction. Once binding occurs between the phage tail fibers and the host cell, the phage can then induce pore formation and import phage early genes. A study by Pukatzki and colleagues (2007) revealed structural homologies between the type VI secretion system (T6SS) and the lysogenic T4 phage architecture. A notable similarity is their binding domain for peptidoglycan (PG). The tail spike used by the T4 phage also resembles an inverted T6SS, which is not surprising as both perform similar roles (injecting proteins/genetic material into a target cell) (Basler, 2015).

The main differences between lifecycles are that the lytic cycle results in cell death, whereas the lysogenic cycle involves incorporating phage DNA into the host genome where it is dormant until transcribed (Todar, 2012). When phage early genes are produced in the lytic cycle, the host's genome and protein synthesis machinery are reprogrammed to reproduce viral nucleic acids and proteins. New phages are assembled in the cytosol and phage late genes are expressed encoding lysins and murein synthesis inhibitors. These proteins weaken the bacterial cell membrane and release of the progeny result in host cell lysis.

The lysogenic life cycle phages are also known as temperate phages (Wittebole et al., 2014). Instead of reproducing immediately in the host cell, the viral genome (the prophage) is inserted into the host DNA chromosome. There is no self-replication, and this DNA can be passed on to progeny until the lytic cycle is induced. There can be expression of these genes under stress-inducing conditions such as UV irradiation and presence of the antibiotic

phagolessin, where the phage needs to replicate and evade its host cell’s death (Hall-asheshov and Asheshov, 1956).

1.5.3.2 T4 bacteriophage

Within the family Myoviridae, the bacteriophage T4 has become one of the most well-known bacteriophages that infect *Escherichia coli* (Schaechter, 2009). It belongs to the T-even group of bacteriophages that include T2 and T6, all of which are considered model organisms in phage evolution despite their complexity as viruses with about 160 genes. Since the discovery of T-even bacteriophages they now contain many members that infect bacteria other than *E. coli*. The T4 phage is characterized by its lytic lifecycle and its structure consists of an icosahedral head containing a dsDNA genome (roughly 170,000 bases), and a contractile tail used for insertion of viral DNA into the host. The end of the contractile sheath contains a hexagonal baseplate with six tail fibers attached to its exterior (Black and Rao, 2012). The T4 phage infection cycle begins with adsorption into a host cell, characterized as high affinity binding of the tail fibers to the *E. coli* outer membrane and contraction of the tail fibers to facilitate injection of viral DNA into the cell. Viral DNA is then capable of hijacking the host cell’s DNA replication machinery and synthesizes new T4 phage (Clokie et al., 2011). Ultimately, the replication of bacteriophage within the host cell leads to lysis, where the T4 phage can exit into the environment and search out future hosts for viral replication and new phage synthesis.

1.6 Hypothesis and thesis objectives

The overarching hypothesis of this study is that envelope stress responses are activated and subsequently protect bacteria from envelope stress caused by toxic molecules, environmental changes (adhesion to a surface) and agents of bacterial warfare (type VI secretion systems) through specific signal recognition proteins that coordinate discrete signaling pathways through phosphorylation of

transcriptional regulators. We began this study attempting to reconcile contradictory findings where it was observed that a Cpx-activating cue (NlpE overexpression) upregulated RprA transcription, while constitutive activation of the Cpx response by mutation of the periplasmic loop domain of CpxA (the mutation denoted as *cpxA24**) led to inhibition of *rprA* expression. We additionally wanted to further characterize the relationship between the Cpx and Rcs phosphorelays to identify if any further interactions between these pathways exist between known causes of activation of both pathways. The objectives of the second part of the thesis work described here were to identify if type B colicins, type VI secretion systems of *Vibrio cholerae*, or T4 bacteriophage that interact with and damage the cell envelope are sensed by the Cpx two-component system. The Cpx two-component system responds to damage caused to the cell envelope and utilizes a surface sensing signaling protein for at least one extracellular signal (surface adhesion), therefore we hypothesized that the Cpx response could be capable of sensing type VI secretion systems of *Vibrio cholerae*, type B colicins or T4 phage attack.

1.7 Figures

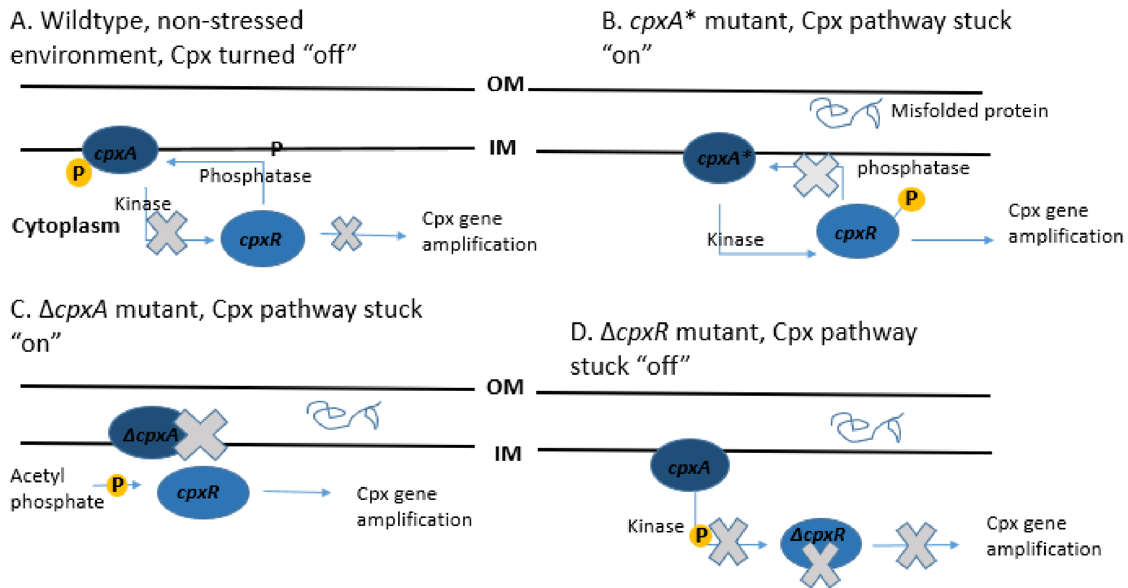

Figure 1.1 Defects in the Cpx response by mutations to CpxA and CpxR proteins under stressed (B, C, and D) and non-stressed (A) conditions. In the absence of inducing signals (A) or constructing a deletion mutant of CpxR (B) shuts down the Cpx signal transduction pathway. Contrastingly, when CpxA phosphatase activity is removed (B) or CpxA is removed entirely (C), gene targets of the Cpx regulon increase in expression. When CpxA is deleted, other small molecules such as Acetyl phosphate can donate a phosphate to CpxR for activation (C), and without the phosphatase activity of CpxA there is a high level of Cpx regulon target expression.

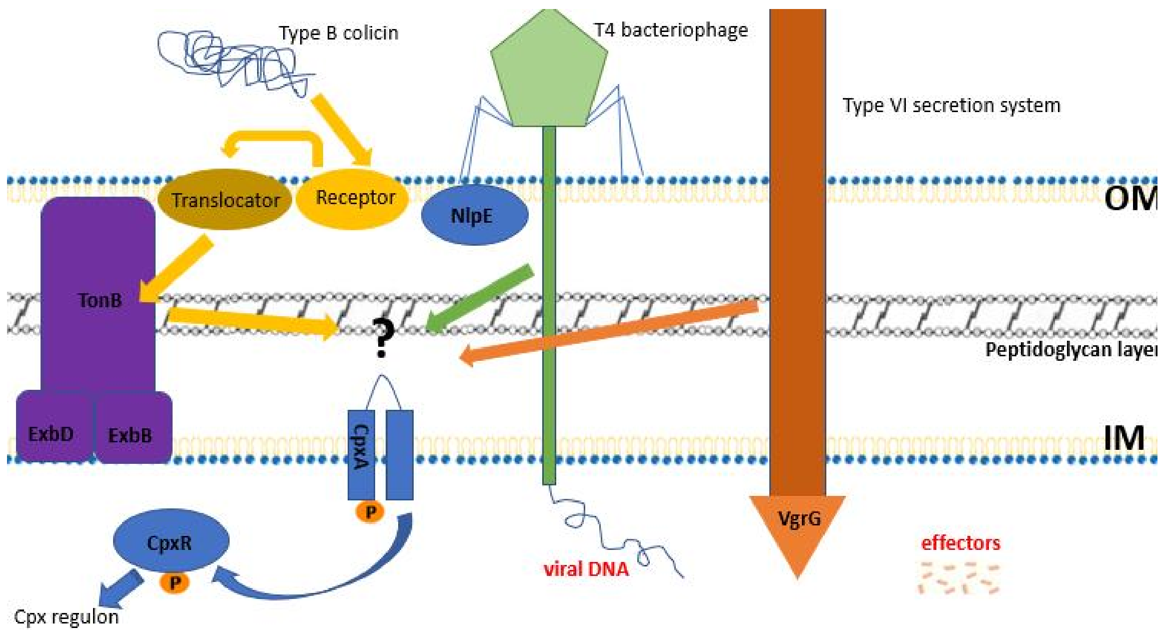

Figure 1.2 The Cpx response and mechanisms of toxicity. The first point of contact for toxins is at the outermost layer of the bacterial envelope. Colicins and bacteriophage must first bind to the outer membrane to facilitate entry into Gram-negative bacteria. Type VI secretion systems do not bind however they do interact with the outer membrane to sense an adjacent bacterium before puncturing the membrane with a hollow tube containing effector proteins that will be injected into the prey cell. Colicins and type six secretion systems cause damage to the membranes among other toxic activities leading to the death of targeted cells. Bacteriophages incorporate their viral DNA into the prey genome and lay dormant until an inducing cue stimulates a lytic lifecycle where viral DNA begin transcribing new bacteriophage until they burst from the cell by lysing the membranes and killing the cell. The Cpx response may be capable of sensing these toxins at different stages of entry as a cell envelope monitoring system through the inner membrane histidine kinase CpxA or the outer membrane lipoprotein sensor NlpE as they must first make contact at the outer membrane or through the entire cell envelope to exert their toxic effects inside the cell.

Chapter 2: Materials and Methods

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2.1 Growth conditions

The wildtype *Escherichia coli* K-12 strains used in this study were MG1655 and MC4100 (a complete strain list can be found in Table 2.1). All strains were incubated at 37°C in Luria Bertani (LB) broth with aeration (shaking) at 225 rotations per minute (rpm) or on LB plates grown statically on a standing incubator (0.5% Bacto yeast extract, 0.5% NaCl, 1% Bacto Tryptone and 1.5% Bacto agar for plates). Antibiotic concentrations used were as follows, when necessary: 50μg/mL kanamycin, 25μg/mL chloramphenicol, 100 μg/mL ampicillin, 50 μg/mL rifampicin, 100 μg/mL amikacin. *E. coli* strain MC4100 was used in studies when assessing Cpx activity during P1 bacteriophage and type B colicin attack as it is frequently prey for these bactericidal molecules. *E. coli* strain MG1655 was used for studies with type VI secretion attack by *V. cholerae*, as it was necessary to have a prey *E. coli* strain with different antibiotic resistance than *V. cholerae* for cell enumeration on antibiotic supplemented LB agar plates. MG1655 was also used for studies looking at both Rcs and Cpx response activity as the MC4100 strain has very low expression levels of the Rcs reporting gene *rprA*, as previously determined (Vogt et al., 2014).

2.2 Strain construction.

Escherichia coli MG1655 and MC4100 mutant strains were constructed by P1-*vir* transduction (for creation of single and double gene deletions). Luminescent gene reporter plasmids and overexpression plasmids were inserted into *E. coli* by transformation. All genes deleted in *E. coli* strains MG1655 and MC4100 in this study had kanamycin resistance cassettes inserted in their place by P1-virulent phage lysates. To create single and double gene knockout mutant strains of *Escherichia coli*, strains were constructed from kanamycin resistant deletion strains from the KEIO library (a transposon mutant library of *E. coli* K-12) by generalized P1 transduction to move the desired mutant alleles into *E. coli* strains MC4100 and MG1655 (Silhavy

et al., 1984). Strains had their open-reading frame replaced with a kanamycin resistance cassette from the corresponding KEIO collection strain (Baba et al., 2006). The gene deletions were confirmed with PCR by using primers flanking the genes of interest.

2.3 Preparation of P1-*vir* lysates

Overnight cultures of donor strains (from the KEIO collection harboring single gene deletions of interest) were made by inoculating 5 mL of LB broth into a 15 mL glass tube with single colonies of *E. coli* K-12 BW25113 donor bacteria (Baba et al., 2006). Cultures were incubated overnight at 37°C with aeration (shaking) at 225 rotations per minute (rpm). The next day, donor bacteria were subcultured 1:50 into 5 mL fresh LB medium containing 0.2% glucose and 5 mM $CaCl_2$ in a 15 mL glass tube and then were grown for 30 minutes at 37°C with aeration at 225 rpm. 100 µL of P1 lysate made from *E. coli* strain MC4100 was then added and tubes were incubated at 37°C with aeration at 225 rpm for an additional two hours. 100 µL of chloroform was then added to ensure lysis of cells and release of P1 phage. Cells were then centrifuged at 4,000 rpm and the supernatant was transferred to a 5 mL glass screw-capped tube. Another 100 µL of chloroform was added and P1 lysates were stored at 4°C.

2.4 P1-*vir* mediated transduction

Overnight cultures of recipient bacteria (either MC4100 or MG1655, receiving a kanamycin resistance cassette to replace a gene of interest) were made by inoculating 5 mL of LB broth into a 15 mL glass tube with single colonies of *E. coli* recipient strains. Cultures were incubated overnight at 37°C with aeration at 225 rotations per minute (rpm). The next day, overnight cultures were centrifuged at 4,000 rpm for 10 minutes and then resuspended in 2.5 mL distilled water (dH_2O), containing 5 mM $CaCl_2$ and 10 mM $MgSO_4$. 100 µL of the recipient cell solution was mixed with 100 µL of the donor P1-vir lysate in a 1.5 mL microcentrifuge tube and mixtures were then incubated at 30°C for 30 minutes grown statically in a standing incubator. 1

mL of fresh LB broth containing 10 mM citrate was added to each solution and then re-incubated for another 45 minutes at 37°C, grown statically. Solutions were then centrifuged at 4,000 rpm for 10 minutes and subsequently resuspended in 100 µL 1M citrate. From each solution, 100 µL was plated on LB agar plates containing 50 µg/mL kanamycin to select for strains with kanamycin cassettes inserted in place of the gene that was removed. LB agar plates were then incubated at 37°C overnight statically, and next day single colony isolates were re-struck onto fresh LB agar plates supplemented with 50 µg/mL kanamycin and grown overnight at 37°C to confirm resistance to kanamycin. The kanamycin resistance cassette contained within these mutations was removed in some strains by FLP recombinase target (FRT)-mediated recombination to create markerless deletions so that plasmids with kanamycin resistance genes could be inserted into strains and selected for by the presence of growth on LB agar plates containing 50 µg/mL kanamycin (Hoang et al., 1998). FLP recombination was performed by transformation of the FLP2 plasmid into cells with kanamycin-resistant cassettes. The protocol for transformations is described in section 2.8.

2.5 Plasmid isolation

Isolation of plasmids referenced in this thesis (Table 2.1) was performed with a Sigma-Aldrich GenElute Plasmid Miniprep kit containing all reagents required. 5 mL of overnight cell culture, grown at 37ºC with aeration at 225 rotations per minute (rpm) in LB broth and antibiotics, and containing the plasmid of interest, was centrifuged at 4,000 rotations per minute (rpm) for ten minutes. Cells were resuspended in 200 µL of Lysis Solution and transferred to a 2 mL microcentrifuge tube 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 12,000 rpm for 1 minute. Then, a microcentrifuge column from the Miniprep kit was inserted into a 2 mL microcentrifuge tube and 500 µL Column Preparation Solution was added, and centrifuged at 12,000 rpm for 1 minute, and the flow through was discarded. The cell solution was then added to this column which was then centrifuged at 12,000 rpm for 1 minute. The flow-

through was then discarded and 750 µL Wash Solution was added to the column, which was centrifuged at 12,000 rpm for 1 minute. The flow-through was discarded and the column was inserted into a new 2 mL microcentrifuge tube and was centrifuged once more at 12,000 rpm for 1 minute. Finally, the column was added to a new 2 mL microcentrifuge tube and the DNA was eluted with 100 µL Elution buffer added to the column and centrifuged at 12,000 rpm for 1 minute. Plasmid DNA was then contained in the microcentrifuge tube and stored at -20˚C.

2.6 Polymerase Chain Reaction (PCR)

Deletion of genes by P1-*vir* transductions were confirmed by polymerase chain reaction (PCR). A Master-mix was used with the following formula per 50 µL reaction: 22 µL MilliQ H_2O, 10 µL betaine, 8 µL 5 mM dNTPs (deoxyribonucleotide triphosphates), 5 µL 10X PCR buffer, 2 µL 50 mM $MgSO_4$, 1 µL Forward primer, 1 µL Reverse primer, 1 µL Taq polymerase. Primers were constructed 200 base pairs upstream (forward primer) and 200 base pairs downstream (reverse primer) for each gene of interest to ensure a large band size could be visualized during gel electrophoresis. Cells were plated on LB agar plates supplemented with 50µg/mL kanamycin (for strains with the kanamycin cassette instead of the gene of interest), or just LB agar for wildtype controls to ensure the primers will bind upstream and downstream of the gene of interest when it is present. Plates were incubated overnight at 37°C in a stationary incubator. Next day, 4-5 single colonies were inoculated into 20 µL MilliQ H_2O in a 0.2 mL Eppendorf PCR tube and heat-treated (in the microwave for 30 seconds) to extract DNA. A master-mix was then created in a 0.2 mL Eppendorf PCR tube and 5 µL of each heated cell solution was added. A control of 5 µL MilliQ H_2O was also added to its own Master-mix 0.2 mL PCR tube to ensure that non-specific DNA contamination was not amplified. A brief centrifugation (10 seconds) was used to collect the solutions in the bottom of each tube. A thermal cycler was then set up with the following parameters: A 94°C denaturation step (1 minute), a 56°C annealing step (45 seconds), and a 72°C extension step (2 minutes), with 30 cycles in this order. After PCR, solutions in 0.2 mL PCR tubes were stored at 4°C until gel electrophoresis.

2.7 Gel electrophoresis

To identify if PCR products contained the amplified genes of interest, gel electrophoresis was performed. 1 gram of agarose was dissolved into 100 mL 1X TAE buffer (diluted from a 50X TAE stock solution: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 500 mM EDTA, pH 8.0, adjusted to 1 liter final volume with distilled $H_2 0$) in a microwave safe 250 mL Erlenmeyer flask. The flask was microwaved until boiling (roughly 1 minute) and set aside to cool (5-10 minutes). The agarose was then poured into the well tray of the gel electrophoresis machine with the well comb in place, avoiding production of bubbles in the gel while pouring. Once the agarose gel had solidified, the well comb was removed and 1X TAE buffer was added until the gel was completely submerged in buffer. 25 µL of DNA samples (as prepared in section 2.6, PCR protocol) were then each mixed with 5 µL 6x loading dye to visualize band migration (containing bromophenol blue and xylene cyanol FF) and loaded into the wells of the agarose gel. The first well was loaded with a 1 kb Plus DNA Ladder (purchased from ThermoFisher Scientific) to identify proper size of PCR-amplified bands. Gels were run at 100 volts for roughly 45 minutes or until bands reached near-end of the agarose gel. Gels were then soaked in ethidium bromide for 15 minutes and imaged. Band size was confirmed in the wildtype controls, and bands were not present in the P1-transduced strains harboring kanamycin insertions, and the DNA-free controls.

2.8 Calcium chloride-mediated transformations

All gene reporter plasmids and overexpression plasmids used in this study were constructed and described in previous studies (Table 2.1; Acosta et al., 2015; Raivio et al., 2013; Vogt et al., 2014). Insertion of overexpression and luminescent reporting plasmids into MC4100 and MG1655 used calcium chloride-mediated transformations. Single colonies of *E. coli* strains MC4100 and MG1655 were inoculated into 5 mL LB in a 15 mL glass tube and grown at 37°C with aeration overnight at 225 rotations per minute (rpm). Next day the overnight cultures of bacteria were subcultured 1:50 into 5 mL fresh LB in a 15 mL glass tube and grown for roughly 1-1.5 hours until cells reached an optical density (OD_{600}) of 0.2-0.3. Cultures were then centrifuged

at 4,000 rotations per minute (rpm) for 10 minutes, resuspended in 1 mL of Magic Formula (containing 0.1M $CaCl_2$, 0.1M MOPS (3-morpholinopropane-1-sulfonic acid) in dH_2O), transferred to a 1.5 mL microcentrifuge tube and were then incubated on ice for 30 minutes. Cultures were then centrifuged for 10 minutes at 4,000rpm, the supernatant was removed, and they were resuspended in 200 μL fresh Magic Formula. 1 μL of plasmid DNA was then added to the cultures (plasmids were isolated as per section 2.5), which were incubated on ice for another 10 minutes. Cultures were then heat-shocked in a water bath at 42.5°C for 45 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 1-1.5 hours grown at 37ºC in a stationary incubator. Following this recovery process, 100 μL of each mixture was plated on LB agar plates with antibiotics (depending on the antibiotic resistance genes in each plasmid, see Table 2.1) and incubated overnight at 37°C statically. The next day single colony isolates were struck onto fresh selective media and grown overnight at 37°C statically to confirm resistance. Overexpression plasmids were previously confirmed by Northern blotting that there was accumulation of the genes encoded on the plasmid when induced with IPTG (Vogt et al., 2014; Snyder et al., 1995).

For transformations of the FLP2 recombinant plasmid, additional steps were performed to ensure the kanamycin-resistance cassettes were removed from strains harboring insertional deletions after P1-*vir* transductions. After overnight growth of cells transformed with FLP2 plasmid, 50 single colonies were then streaked, per strain, on an LB agar plate containing 100 μg/mL ampicillin, and then on a plate containing 50 μg/mL kanamycin to identify cells that acquired the new FLP2 plasmid and those that have additionally lost the kanamycin-resistance cassette. These LB agar plates were then incubated overnight at 37ºC in a stationary incubator. Next day, cells that grew on LB agar plates with ampicillin but not the LB agar plates with kanamycin were inoculated into 5 mL LB in a 15 mL glass tube and incubated overnight at 37ºC with aeration at 225 rotations per minute (rpm). Overnight growth without antibiotics was sufficient for cells to reject the FLP2 plasmid. Next day, cells were struck onto a plain LB agar plate, then plates containing 100 μg/mL ampicillin, and then on a plate containing 50 μg/mL kanamycin and incubated overnight at 37ºC statically. Next day those that lost the FLP2 plasmid (ampicillin

sensitive) and the kanamycin-resistance cassette (kanamycin sensitive) were selected for further transformation of plasmids containing kanamycin-resistance genes into these strains.

2.9 Luciferase assays.

Luciferase reporter activity, quantified as counts per second (CPS) divided by optical density (OD_{600}), was measured as previously described (Price and Raivio, 2009). All strains were grown overnight in 2 mL LB broth in a 10 mL glass tube with appropriate antibiotics at 37°C with aeration at 225 rotations per minute (rpm). The next day, 1:50 subcultures were made in 2 mL fresh LB in a 10 mL tube and grown at 37°C with aeration at 225 rpm. Optical density and luminescence were measured every hour for 8 hours unless specified otherwise with a Perkin Elmer Victor 1420 plate reader. All luminescence readings were taken in a 96-well plate by transferring 200 µL of cell cultures into its wells and reading luminescent and optical density immediately. Normalized luminescence was calculated by dividing raw luminescence (in counts per second [cps]) by the optical density at 600nm (OD_{600}) of the same culture. When genes were over-expressed, IPTG was added to a final concentration of 0.05 mM after overnight growth or after 2 hours of subculture, where specified. In some experiments, Polymyxin B or EDTA was added to final concentrations of 0.1 mg/L or 4 mM, respectively, after 2 hours of subculture growth and re-incubated for an additional 4 hours (with EDTA), or 1 hour (with polymyxin B) before OD_{600} and luminescence were measured with the Wallac Victor2 Multilabel counter plate reader. Time points were chosen for polymyxin B and EDTA where the greatest induction of wildtype cells treated with either chemical was observed compared to the wildtype uninduced control. To measure the impact of pH on luminescent reporter gene expression, 2 mL overnight cultures in 10 mL glass tubes were centrifuged at 4,000 rpm for 10 minutes and the supernatent was removed. The bacteria pellet was resuspended in 2 mL fresh LB and antibiotics in a 10 mL glass tube and adjusted to alkaline (8.0), neutral (7.0) or acidic (5.5) pH by use of a pH probe and HCl or NaOH added dropwise. pH adjusted LB broth was filter-sterilized prior to use. Cells were incubated for 1 hour, then 200 µL of each culture was transferred to a 96-well polystyrene plate

(purchased from Sigma Aldrich) and optical density and luminescence readings were taken. Luminescence and optical density (OD_{600}) were measured with the Wallac Victor2 Multilabel counter plate reader at 1 hour where both gene reporters (*PcpxP-lux* and *PrprA-lux*) had greatest induction by pH changes, and to ensure pH of the growth medium did not become alkaline over time and influence the luminescence of each gene reporter. Luminescence assays were performed at least two times with three to five replicates per strain.

2.10 Measurement of gene expression in adhered bacteria.

Assays of gene expression in adhered bacteria were carried out in tissue culture treated 96-well polystyrene plates, purchased online from Sigma Aldrich. Frozen stocks of the indicated bacterial strains were struck for isolated colonies onto LB agar plates containing the appropriate antibiotics and incubated overnight at 37ºC statically. The next day, single colonies were inoculated into a 96-well plate (Sigma Aldrich) containing 200 µL LB broth and antibiotics. The plate was incubated at 37ºC on a rocker at 6 rotations per minute (rpm) for six hours. Planktonic cells were pipetted into new wells and 200 µL of LB was added to the wells containing adhered cells. The optical density (OD_{600}) of the planktonic cells, together with the luminescence of both planktonic and adhered bacteria were measured immediately with a Wallac Victor2 Multilabel counter plate reader. After the luminescence reading the wells containing adhered bacteria were scraped with a pipet tip to remove any cells on the sides and bottom of the well, and the bacteria were resuspended by pipetting and vortexing, and optical density readings were recorded and used to standardize the luminescence of the adhered cells. Normalized luminescence was calculated by dividing the raw luminescence (cps) by the OD_{600} of the same culture. Data for each strain represents the means and standard deviations of five replicate cultures, from two independent experiments.

2.11 pH survival assay.

All strains with either the *PcpxP-lux* or *PrprA-lux* reporters were grown overnight in 2 mL LB broth in 10 mL glass tubes with appropriate antibiotics at 37ºC with aeration at 225 rotations per minute (rpm). The next day, IPTG was added to a final concentration of 0.05mM in overnight tubes containing cultures of strains carrying overexpression plasmids and strains with the respective vector control plasmids, and all strains were grown for an additional 2 hours at 37ºC with aeration at 225 rpm. Overnight cultures were diluted 1:50 into 2 mL LB broth in 10 mL glass tubes containing appropriate antibiotics and adjusted with a pH probe to pH 8.5, 7.0 or 4.0 with HCl or NaOH added dropwise. pH adjusted LB broth was filter sterilized prior to use. pH was intensified greater than the luminescence assay by increasing alkaline pH from 8 to 8.5 and acid pH from 5.5 to 4.0 to visualize differences between Cpx and Rcs mutant strains compared to wildtype controls during acid and alkaline pH challenge. In addition, the preliminary screen with Cpx and Rcs mutants used acid pH 5.0 (Fig S3), however RprA was shown to be essential in previous studies at significantly lower pH (Bak et al., 2014), therefore we chose pH 4.0 to view how *rprA* overexpression affected cell survival under more extreme acid conditions. An additional 0.05 mM IPTG was added to cultures of strains carrying overexpression plasmids and strains harboring the respective vector control plasmids. Cells were grown for three hours at 37ºC with aeration at 225 rpm. The pH was readjusted every hour by centrifuging tubes at 4,000 rpm for ten minutes and resuspending the pelleted cells in new media by vortexing and pipetting. After the three hours cells were then serially diluted, plated on LB agar plates and incubated overnight at 37ºC statically. CFUs were imaged on each plate. Experiments were performed in duplicate with three replicates per strain.

2.12 Type six (VI) killing assay.

The susceptibility of wildtype and Cpx mutant strains to the *Vibrio cholerae* type six secretion system was evaluated with a killing assay (MacIntyre et al., 2010). Undergraduate student Tim Cho performed this killing experiment with prey strains constructed previously (Table

2.1). Type six active and inactive *V. cholerae* strains (provided by the Pukatzki lab), as well as prey MG1655 strains were streaked to cover the surface of their own LB agar plate containing appropriate antibiotics and were grown overnight at 37°C statically. *E. coli* strain MG1655 was used in this experiment rather than MC4100 as it is rifampicin resistant and can be selected for when quantifying survival of prey cells. Using a sterile loop, cells were recovered from each lawn of cells to fill the sterile loop and each individual strain was transferred to 2 mL of LB in a 2 mL microcentrifuge tube. These original 2 mL cultures were diluted to 1:50 into a new 2 mL microcentrifuge tube. Optical density of each tube was taken, and the original optical densities were calculated and converted to a CFU value (CFU enumeration was quantified based on the calculation in which an OD of 1= 1*10^9 CFU).

Volumes that resulted in 5x10^8 predator (V52 type VI active strain and V52 *ΔvasK*, type VI inactive strain) and 5x10^7 prey cells were calculated as previously described (MacIntyre et al., 2010). All *E. coli* strains with overexpression plasmids (pCpxP or pCA-*nlpE*)or their respective vector controls (pCMCP or pCA24N) were then incubated for 5 minutes with 0.1 mM IPTG to induce expression of *cpxP*- or *nlpE*- overexpression plasmids. The appropriate volume of predator and prey cells (10:1) were mixed in a microcentrifuge tube and centrifuged for 2 minutes at 15,000 rpms. Supernatants were aspirated, and cells were resuspended in 125 μL of LB broth in a 2 mL microcentrifuge tube. Each predator and prey combination were spotted on pre-warmed LB plates in 25 μL aliquots. Plates were incubated for 3 hours at 37°C, grown statically. Spots were then harvested with a sterile plastic loop and resuspended into 1 mL LB in a 2 mL microfuge tube. Cells were serially diluted and plated in duplicate on LB agar plates supplemented with 50 μg/mL rifampicin to select for viable prey cells. Cells were incubated at 37°C overnight, grown statically, and CFUs were counted and averaged the next day. Experiments were performed in triplicate, with three replicates per strain.

2.13 T6SS killing luminescence assay.

E. coli strain MG1655 harboring a *PcpxP*-luminescent reporter were used to quantify Cpx activity levels during type VI secretion attack by predator *V. cholerae.* Predator and prey mixtures with 10-fold higher predator than prey were prepared as written above, however cells were centrifuged and left in a pellet for co-incubation rather than pouring off the supernatant and resuspending cells in 125 µL LB broth and spotting 25µL cells for co-incubation (as described above, section 2.12). Co-incubation tubes (containing the predator and prey in a pellet) were set up at three time points to look for induction of the Cpx response during type VI secretion-mediated killing. At timepoints 0, 30 and 60 minutes, the *E. coli* pelleted with type VI active (V52) and type VI inactive (V52*ΔvasK*) predator *V. cholerae* were resuspended in 200 µL of fresh LB and transferred to wells of a 96-well plate. Optical density (OD_{600}) and luminescence readings were taken with a Wallac Victor2 Multilabel counter plate reader and optical density was used to standardize the luminescence readings from each well. Experiments were performed in triplicate, with three replicates per strain. Bars and standard deviations are averaged CFUs over all replicates and individual experiments.

2.14 Colicin killing assay.

A purified control lysate in HEPES buffer (chemical formula: $C_8H_{18}N_2O_4S$) and one containing colicins B and Ia in HEPES buffer was provided by PhD student Junshu Wang for these experiments, and prepared as described previously (Braun, 1974). A dilution of 1:100 from the original colicin B and Ia stock lysate was chosen to use in the killing assay, as it was the minimum inhibitory concentration, to test for survival of prey *E. coli*. *E. coli* strain MC4100 was chosen for this assay as it was the primary strain used in the laboratory and is susceptible to killing by type B colicins. Overnight cultures of wildtype and Cpx mutant strains were created by inoculating single colonies into 5 mL LB broth in 15 mL glass tubes and grown at 37ºC with aeration at 225 rotations per minute (rpm). Next day, overnight cultures were then subcultured

1:50 into 5mL fresh LB in a 15 mL glass tube. Cells were grown for two hours at 37ºC with aeration at 225 rotations per minute, then 10 µL colicin stock (1:100 dilution) or 10 µL of the control colicin-free lysate was added to each culture and incubated for two more hours with aeration at 37ºC to allow for colicin-mediated killing. Cells were then centrifuged and resuspended in 5 mL fresh LB to remove residual colicins and plated in serial dilutions. Plates were grown overnight at 37ºC statically and next day CFU enumeration was performed. Experiments were performed in triplicate, with three replicates per strain and the results were averaged.

2.15 Colicin killing luminescence assay.

E. coli strain MC4100 with a *PcpxP-lux* luminescent reporter was grown overnight in 2 mL LB with 50 µg/mL kanamycin in a 10 mL glass tube and incubated at 37ºC with aeration at 225 rotations per minute (rpm). Next day, 1:50 subcultures were made in 2 mL LB and antibiotics in a 10 mL glass tube and grown at 37ºC with aeration to mid-log phase (OD 0.5). Cultures were then co-incubated with either 10 µl colicin solution (1:200 dilution from stock solution, sub-MIC) or 10 µL control lysate (without any plasmid, diluted 1:200 from stock solution) in HEPES buffer (chemical formula: $C_8H_{18}N_2O_4S$) and luminescence was read over three minutes beginning at 30 seconds co-incubation with a Wallac Victor2 Multilabel counter plate reader. Luminescence readings were standardized to the optical density (OD_{600}) of the culture. Experiments were performed in triplicate, with three replicates per strain and the results were averaged.

2.16 Colicin killing beta-galactosidase assay.

E. coli strains, containing a chromosomal cpxP-lacZ reporter, were grown overnight in 5 mL LB in a 15 mL glass tube, incubated at 37ºC with aeration at 225 rotations per minute (rpm). Next day, 1:50 subcultures were made in 5 mL fresh LB in a 15 mL glass tube and grown at 37ºC with aeration at 225 rpm to mid-log phase (OD 0.5) and then co-incubated with either 10 µl colicin

lysate (1:200 dilution from stock solution sub-MIC) or 10 µL control lysate (1:200 dilution from colicin-free lysate) in HEPES buffer (chemical formula: $C_8H_{18}N_2O_4S$). Co-incubation mixtures were made for time points 15, 30 and 60 minutes to measure Cpx response activity during colicin entry and subsequent killing in triplicate (9 tubes total per replication). After each timepoint, cells were centrifuged at 4,000 rpm for ten minutes and resuspended in 5 mL 1X Z-buffer (chemical composition: 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), and 250 µL samples were taken from each replicate to measure optical density (OD_{600}) in a 96-well plate. Cells that were remaining in 1X Z-buffer 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 a well containing 195 µL 1X Z-buffer in a 96-well plate, then 50 µL 10 mg/mL ONPG (ortho-Nitrophenyl-β-galactoside) was added to each well and the A_{420} was measured with the plate reader. Beta-galatosidase activity was standardized to the optical density of each culture. Experiments were performed in triplicate, with three replicates per strain, per timepoint, and the results were averaged.

2.17 T4 phage killing assay.

Strains were grown overnight in 2 mL LB and 50 µg/mL kanamycin in a 10 mL glass tube, incubated at 37°C with aeration at 225 rotations per minute (rpm). The next day, 1:50 subcultures were made in 2 mL LB and 50 µg/mL kanamycin in a 10 mL glass tube and grown at 37°C with aeration at 225 rpm to mid-log phase (OD_{600} 0.5). Cultures were then co-incubated with 100 µL of T4 phage stock at a multiplicity of infection (MOI) of five or 100 µL phage buffer as a control. A one-hour timepoint was chosen to allow for killing of *E. coli* to observe differences in susceptibility between wild type and Cpx deletion strains without lysis of entire cultures. After one hour of T4 phage killing, cells were centrifuged at 4,000 rpm for ten minutes and washed with 2 mL fresh LB broth to remove residual phage. Cell cultures were then plated in serial dilution on LB agar plates. Agar plates were then incubated overnight at 37°C grown statically, and CFUs were enumerated

and compared to a phage free control for each strain. Experiments were performed three times with three replicates per strain and the results were averaged from all trials.

2.18 T4 phage killing luminescence assay.

E. coli strain MC4100 with a *PcpxP*-luminescent reporter was grown overnight in 2 mL LB and 50μg/mL kanamycin in a 10 mL glass tube incubated at 37°C with aeration at 225 rotations per minute (rpm). The next day, 1:50 subcultures were made in 2 mL LB and 50μg/mL kanamycin in a 10 mL glass tube and grown at 37°C with aeration at 225 rpm to mid-log phase (OD 0.5). Cells were then co-incubated with 100 μL T4 phage at a MOI of five or 100 μL phage buffer as a control. Cell cultures were then transferred to a 96-well plate. Luminescence readings were taken with a Wallac Victor2 Multilabel counter plate reader at timepoints: 1, 3, 5, 7, 9 and 30 minutes to account for any Cpx response activity that may sense attachment of phage, initial cell killing, and killing after 30 minutes from replicating bacteriophages. Luminescence readings were standardized to the optical density (OD_{600}) of the culture. Experiments were performed three times with three replicates per strain and the results were averaged from all trials.

2.19 Strains and plasmids

Table 2.1 Strains and plasmids for this study

Strain	**Description**	**Source of reference**
MG1655	Wildtype *E. coli,* F^-, lambda$^-$, rph-1, Rifampicin^R	(Price & Raivio, 2009)
SG1	MC4100 λRS88 *E. coli*	(Price & Raivio, 2009)
SG2	MC4100 λRS88 *cpxP-lux*	This study
SG3	MC4100 λRS88 *cpxA:kan*	(Danese et al., 1995)
SG4	MC4100 λRS88 *cpxR:spc*	(Danese et al., 1995)
TR50	MC4100 λRS88 *cpxP-lacZ*	(Raivio & Silhavy, 1997)
TR70	MC4100 λRS88 [*cpxP-lacZ*] *cpxR1:spc*	(Danese et al., 1995)
TR68	MC4100 λRS88 [*cpxP-lacZ*] *cpxA1:cam*	(Danese et al., 1995)
SG5	MC4100 λRS88 pCA24N	This study
SG6	MC4100 λRS88 pCA-*nlpE*	This study
SG7	MC4100 λRS88 pCMCP	This study
SG8	MC4100 λRS88 pCpxP	This study
C6706	*V. cholerae* V52 (O37 serogroup) El Tor C6706 strain, Strep^R	(MacIntyre et al., 2010)
C6706 Δ*vask*	*V. cholerae* V52 (O37 serogroup) El Tor C6706 strain, Δ*vask,* Strep^R	(Ma et al., 2009)
SG15	MG1655 *nlpE::kan*	This study
SG16	MG1655 *rcsF::kan*	This study
SG17	MG1655 *ompA::kan*	This study
SG18	MG1655 *tonB::kan*	This study
SG19	MG1655 *cpxA::kan*	This study
SG20	MG1655 *cpxR::kan*	This study
SG21	MG1655 *rcsC::kan*	This study
SG22	MG1655 *rcsB::kan*	This study
SG57	MG1655 *rprA::kan*	This study
SG23	MG1655 *cpxP-lux*	This study
SG24	MG1655 *cpxP-lux nlpE::kan*	This study
SG25	MG1655 *cpxP-lux rcsF::kan*	This study
SG26	MG1655 *cpxP-lux ompA::kan*	This study
SG27	MG1655 *cpxP-lux tonB::kan*	This study
SG28	MG1655 *cpxP-lux cpxA::kan*	This study
SG29	MG1655 *cpxP-lux cpxR::kan*	This study
SG30	MG1655 *cpxP-lux rcsC::kan*	This study
SG31	MG1655 *cpxP-lux rcsB::kan*	This study
SG32	MG1655 *rprA-lux*	This study
SG33	MG1655 *rprA-lux nlpE::kan*	This study
SG34	MG1655 *rprA-lux rcsF::kan*	This study
SG35	MG1655 *rprA-lux ompA::kan*	This study
SG36	MG1655 *rprA-lux tonB::kan*	This study

SG37	MG1655 *rprA-lux cpxA::kan*	This study
SG38	MG1655 *rprA-lux cpxR::kan*	This study
SG39	MG1655 *rprA-lux rcsC::kan*	This study
SG40	MG1655 *rprA-lux rcsB::kan*	This study
SG41	MG1655 *rprA-lux* pCA24N	This study
SG42	MG1655 *rprA-lux,* pCA-*nlpE*	This study
SG43	MG1655 *rprA-lux rcsF::kan* pCA24N	This study
SG44	MG1655 *rprA-lux rcsF::kan* pCA-*nlpE*	This study
SG45	MG1655 *rprA-lux rcsC::kan* pCA24N	This study
SG46	MG1655 *rprA-lux rcsC::kan* pCA-*nlpE*	This study
SG47	MG1655 *rprA-lux rcsF::kan rcsC::kan* pCA24N	This study
SG48	MG1655 *rprA-lux rcsF::kan rcsC::kan* pCA-*nlpE*	This study
SG49	MG1655 *rprA-lux ompA::kan* pCA24N	This study
SG50	MG1655 *rprA-lux ompA::kan* pCA-*nlpE*	This study
SG51	MG1655 *rprA-lux ompA::kan rcsF::kan* pCA24N	This study
SG52	MG1655 *rprA-lux ompA::kan rcsF::kan* pCA-*nlpE*	This study
SG53	MG1655 *rprA-lux cpxR::kan* pCA24N	This study
SG54	MG1655 *rprA-lux cpxR::kan* pCA-*nlpE*	This study
SG55	MG1655 *rprA-lux rcsB::kan* pCA24N	This study
SG56	MG1655 *rprA-lux rcsB::kan* pCA-*nlpE*	This study
SG58	MG1655 pBR322	This study
SG59	MG1655 pBR-*rprA*	This study
SG60	MG1655 Δ*rprA* pBR322	This study
SG61	MG1655 Δ*rprA* pBR-*rprA*	This study
SG62	MG1655 *cpxR::kan* pBR322	This study
SG63	MG1655 *cpxR::kan* pBR-*rprA*	This study
SG64	MG1655 *cpxA::kan* pBR322	This study
SG65	MG1655 *cpxA::kan* pBR-*rprA*	This study
Plasmids		
pFLP2	Broad host-range plasmid expressing the FLP recombinase from a temperature sensitive promoter	(Hoang et al., 1998)
pJW15*cpxP* (pJW25)	pJW15 luminescence reporter plasmid containing *cpxP* promoter: *cpxP-lux*; Kan^R	(MacRitchie et al., 2008)
pJW15*rprA*	pJW15 luminescence reporter	(Vogt et al., 2014)

	plasmid containing *rprA* promoter: *rprA-lux*; Kan^R	
pCA24N	Vector control for the ASKA library, containing $P_{T5\text{-}lac}$ IPTG-inducible promoter, Amk^R	(Kitagawa et al., 2006)
pCA-*nlpE*	$P_{T5\text{-}lac}$-*nlpE* overexpression strain, IPTG inducible, Amk^R	(Kitagawa et al., 2006)
pBR322	Cloning vector, Amp^R Tet^R	(Bolivar et al., 1977)
pBR-*rprA*	*rprA* cloned downstream of the IPTG inducible overexpression plasmid pBR322, Amp^R	(Vogt et al., 2014)
pCMCP	Overexpression vector for a cytoplasmic MBP-CpxP fusion protein, Amp^R	(Raivio et al., 1999)
pCpxP	*cpxP* cloned downstream of the IPTG inducible plasmid pCMCP, Amp^R	(Raivio et al., 1999)

Chapter 3: Results

Chapter 3: Results

3.1 Activation of the Rcs response by NlpE over-expression is dependent on the Rcs signal transduction cascade.

The Cpx and Rcs responses have several common inducing cues, each utilizes an outer membrane sensing lipoprotein, and both regulate the small RNA RprA (Vogt et al., 2014). We therefore hypothesized that these pathways could interact to respond to common cellular stresses and to control transcription of *rprA.* Previously, it was paradoxically shown that two methods of inducing the Cpx response (NlpE over-expression or mutational activation of CpxA) led to opposing effects on expression of the gene encoding the sRNA RprA (Vogt et al., 2014). Transcriptome studies showed that *rprA* transcription was up-regulated by over-expression of the outer membrane lipoprotein NlpE (Raivio et al., 2013), whereas a Cpx hyperactivating mutant (*cpxA24**) decreased this transcription (Vogt et al., 2014). To begin to elucidate these contradictory results, we created mutants lacking individual Rcs and Cpx signaling proteins and examined the ability of NlpE over-expression to alter Rcs- and Cpx-regulated gene transcription.

As previously observed, NlpE over-expression strongly increased *rprA:lux* expression in wild-type cells (Fig.3.1). We checked whether this activation required CpxR by analyzing the effect of NlpE over-expression on *PrprA:lux* activity in a *cpxR* deletion background. As in the wild-type strain, NlpE over-expression led to a significant increase in *PrprA:lux* activity, albeit not as strongly (Fig. 3.1). This suggests that CpxR is not strictly essential for activation of *rprA* transcription by NlpE over-expression. As previously observed, deletion of *cpxR* resulted in elevated basal levels of *rprA-lacZ* expression (Vogt et al., 2014). This observation is consistent with an inhibitory role for CpxR in *rprA* expression.

Since CpxA, like RcsC, functions as a phosphatase in the absence of envelope stress to maintain CpxR in an inactive state (Danese and Silhavy, 1998), these data also support a model in which the Cpx response represses *rprA* expression. Further, these results provide additional

evidence that NlpE over-expression does not induce *rprA* transcription through activation of the Cpx response by stimulating CpxA (Fig. 3.1). Interestingly, a deletion of *nlpE* represses *rprA* transcription slightly (Fig. S1). Altogether, these data indicate that CpxA and CpxR play a significant inhibitory role in regulating basal activity of the Rcs regulated gene *rprA but* are not involved in the induction of *rprA* expression when NlpE is over-expressed.

Since *rprA* is an Rcs regulon member, we wondered if NlpE might be activating its expression via this signaling cascade. To test this idea, we measured the ability of NlpE over-expression to stimulate luminescence of the *PrprA:lux* reporter in strains lacking Rcs signaling proteins localized to the outer membrane (RcsF, OmpA), the inner membrane (RcsC), or the cytoplasm (RcsB). Activation of the *rprA* reporter gene by NlpE over-expression was abolished in mutants harboring deletions of any of these genes (Fig. 3.1). The deletion of *rcsF* resulted in decreased luminescence produced from the *PrprA:lux* reporter relative to wild-type strain MG1655 and no significant increase in expression was observed upon NlpE over-expression (Fig. 3.1). Unlike the Cpx response that has only been shown to become activated by NlpE when cells are adhered to a surface, the Rcs response is quite sensitive to the presence of its outer membrane lipoprotein signaling member, RcsF, as most basal expression of the *PrprA*-luminescent reporter is abolished in an *rcsF* deletion mutant. This indicates that the basal activity of the Rcs response is attuned to the outer membrane, even in the absence of exogenous stresses (Fig. 3.1) (Castanié-Cornet et al., 2006; DiGiuseppe and Silhavy, 2003; Cho et al., 2014; Konovalova et al., 2016).

In the *rcsC* deletion mutant, expression of *rprA* was elevated in the vector control relative to the wild-type and no significant increase in luminescence occurred when NlpE was over-expressed (Fig. 3.1). In contrast, in a ∆*rcsB* mutant, expression of *PrprA-lux* was eliminated (Fig. 3.1). The stimulatory and inhibitory effects of deletions in *rcsC* and *rcsB,* respectively, on *PrprA:lux* expression are confirmative of previous studies (Fig. 3.1, Clarke et al., 2002; Majdalani and Gottesman, 2005; Takeda et al., 2001; Majdalani et al., 2002). RcsC, like CpxA, has phosphatase activity to maintain the response regulator, RcsB, in an off state under noninducing

conditions, and thus, in an *rcsC* mutant *rprA* transcription levels are increased (Majdalani et al., 2002). RcsB is the only known activator of *rprA* transcription. Consistent with this, we see virtually all *PrprA:lux* expression lost when *rcsB* is deleted (Fig. 3.1, Majdalani et al., 2002). We confirm that the Rcs response is activated by a deletion in *ompA,* previously shown to form a complex with RcsF (Fig. 3.1; Konovalova et al., 2014). This is consistent with published works showing that stresses that perturb lipoprotein trafficking prevent the Bam complex from tethering RcsF through OmpA, resulting in mislocalization of RcsF to the inner membrane where it induces RcsC strongly (Cho et al., 2014). Expression of *PrprA-lux* was not elevated further than the vector control strain by NlpE over-expression in a ∆*ompA* background, indicating that OmpA is necessary for induction of the Rcs response by overexpression of this lipoprotein NlpE (Fig. 3.1).

To gain insight into the mechanism by which NlpE over-expression, *ompA* or *rcsC* deletion activate the Rcs response, we created double mutants carrying deletion mutations in *rcsF* and either *rcsC* or *ompA.* In these double mutants, *PrprA:lux* expression was lowered in the vector control strain to levels similar to those observed in the single *rcsF* deletion mutant (Fig. 3.1). These data indicate that NlpE over-expression, *rcsC* or *ompA* deletion all lead to induction of *rprA* expression, and likely the whole Rcs regulon, in a fashion that requires RcsF, but not the response regulator CpxR (Fig. 3.1). It is interesting that the activation of *rprA* expression by removal of RcsC requires RcsF, since this implies the involvement of additional signaling proteins that affect RcsB phosphorylation status, and not simply nonspecific activation by molecules such as acetyl phosphate (Hu et al., 2013). Our data are consistent with a model in which NlpE over-expression could potentially be affecting the proper localization of RcsF to the outer membrane. Alternatively, NlpE might convey a signal to RcsF which activates the Rcs response.

3.2 The Cpx response is activated by surface adhesion independently of the Rcs response.

It has previously been reported that the Cpx response can sense adhesion to surfaces through the outer membrane lipoprotein NlpE (Otto and Silhavy, 2002). Our data indicate that

NlpE can influence Rcs signaling events through RcsF (Fig 3.1). Accordingly, we wondered if RcsF could be involved in sensing surface contact and conveying this signal to either the Rcs or Cpx inner membrane signal transduction proteins. To address this, we measured Cpx and Rcs pathway activity by means of a *PcpxP-* or *PrprA*-luminescent reporter in strains carrying deletions of genes encoding Cpx and Rcs signaling proteins in planktonic bacteria and those adhered to 96-well polystyrene plates (Fig. 3.2). As previously observed by Otto and Silhavy (2002), NlpE is required for sensing surface adhesion to activate the Cpx response, dependent on CpxA and CpxR (Fig. 3.2). Accordingly, expression of the Cpx-regulated *PcpxP-lux* reporter gene was significantly elevated (approximately two-fold) in wildtype bacteria adhered to polystyrene tissue culture treated plates, and this increase was abrogated when either NlpE, CpxA, or CpxR was absent (Fig. 3.2). As previously observed, the absence of CpxA phosphatase activity resulted in elevated *cpxP-lux* activity (Fig. 3.2). Interestingly, deletion of either the outer membrane protein OmpA or the iron transporter TonB result in insignificant differences between the adhered and planktonic bacteria as well (Fig. 3.2) (Noinaj et al., 2010; Wang, 2002). It was previously shown that deletions of *tonB* and *ompA* resulted in a decrease of *PcpxP-lux* activity (Wong, 2015, unpublished), but the reason for this reduction was unknown. The results presented indicate that these proteins are two newly identified signaling proteins necessary for signaling surface adhesion to activate the Cpx, in a currently unknown way. Their exact role in the signaling cascade, and whether these effects are additive to NlpE surface sensing requires further study. It is interesting to note, that at earlier timepoints (such as at five hours of adherence) or later timepoints (7 hours of adherence) this increase in Cpx activation in adhered cells is no longer observed. This indicates that Cpx signaling may be specific to cells undergoing specific cellular changes during the adhesion process and subsequent biofilm formation.

In an *rcsF* mutant, overall expression of the *PcpxP-lux* reporter was slightly elevated in adhered and planktonic cells compared to wildtype, but reporter expression was still increased during adhesion (Fig. 3.2). Accordingly, RcsF does not seem to be involved in the induction of the Cpx response upon adherence, as we still see greater induction in adhered cells versus planktonic cells in the *rcsF* mutant. These data also imply that the RcsF-dependent activation of

the Rcs response by NlpE over-expression does not involve a similar surface-sensing mechanism.

We wondered if the Rcs response, which also detects cell surface associated signals with an outer membrane lipoprotein, could also be activated under the same conditions as the Cpx response during adhesion. We thus measured luminescence expressed from the Rcs-activated *PrprA-lux* reporter in strains with deletions in genes encoding Rcs outer membrane (RcsF, OmpA), inner membrane (RcsC), and cytoplasmic (RcsB) signaling proteins, as well as in an *nlpE* deletion mutant to see if this pathway was activated under the same conditions as the Cpx response during surface adhesion, and whether it required NlpE. Our results indicate that the Rcs pathway is not induced in a wild-type strain under the conditions of adhesion used here that activate the Cpx response (Fig. S2). Further, no single mutant lacking any of the Rcs signaling proteins tested altered expression of the *PrprA-lux* reporter in planktonic versus adhered cells (Fig. S2). NlpE also had no effect on *PrprA-lux* expression in adhered vs. planktonic cells, indicating that NlpE cannot activate/repress this response during Cpx activating conditions of surface adhesion (Fig. S2).

3.3 The Cpx and Rcs responses are activated independently by signals generated in different cellular compartments during polymyxin B and EDTA exposure.

Our data indicate that the Cpx and Rcs responses are not similarly activated in adhered bacteria (Fig. 3.2, S2), however, both responses are commonly activated by other signals, including EDTA and polymyxin B (Audrain et al., 2013; Bury-Moné et al., 2009). Therefore, we sought to determine whether common signaling mechanisms could be involved in detecting these envelope stresses and transducing them to the cellular receptors of the Cpx and Rcs responses. Expression of luminescence from the Rcs-activated *rprA-lux* and Cpx-activated *PcpxP-lux* reporters was measured in the presence and absence of polymyxin B or EDTA and in strains lacking various components of the Rcs and Cpx signaling apparati.

In the presence of sub-MIC concentrations of either polymyxin B (0.1 mg/L) or EDTA (4 mM), *PcpxP* transcription is upregulated roughly two- to three-fold respectively (Figure 3.3A, 3.4A). This activation was dependent on CpxA and CpxR, as indicated by the lack of an increase in *PcpxP-lux* expression in the presence of polymyxin B or EDTA when either *cpxA* or *cpxR* was deleted (Fig. 3.3A, 3.4A). In contrast, in the absence of *nlpE, ompA or tonB,* exposure to either polymyxin B or EDTA still resulted in a two-fold induction of *PcpxP:lux* expression, comparable to that of the wild-type strain (Fig. 3.3A, 3.4A). This result suggests that the Cpx activating signal is not associated with the outer membrane, nor with TonB that spans to the outer and inner membrane in the periplasmic space. Surface adhesion may be a specific signal for these three proteins in generating an activating signal to CpxA. Further, induction of *PcpxP-lux* expression by these cues was independent of Rcs signaling proteins located in the outer membrane (OmpA and RcsF), inner membrane (RcsC), and cytoplasm (RcsB) (Fig 3.3A, 3.4A). These results demonstrate that polymyxin B and EDTA create an envelope stress that likely induces the Cpx response from the periplasm or inner membrane that can only be sensed first through CpxA.

Exposure to either polymyxin B or EDTA led to strong induction of *rprA* transcription (Figure 3.3B, 3.4B). This activation was dependent on all members of the Rcs signaling pathway tested: OmpA, RcsF, RcsC and RcsB (Fig. 3.3B, 3.4B). Deletion of any of the genes encoding these proteins abrogated activation of *PrprA-lux* expression by polymyxin B or EDTA, as compared to the non-induced control strains (Fig. 3.3B, 3.4B). These data indicate that these antimicrobial compounds generate an envelope stress that is sensed at the outer membrane, likely by an RcsF:OmpA complex. Induction of *PrprA-lux* expression was not dependent on NlpE, CpxA or CpxR as polymyxin B or EDTA exposure led to elevated levels of luminescence in strains harboring deletions in the genes encoding any of these Cpx signaling proteins (Fig. 3.3B, 3.4B). As previously seen, mutations in *cpxA* decreased overall activity of the *PrprA-lux* reporter and a *cpxR* deletion increased overall activity, with or without polymyxin B (Figs. S1, 3.3B, 3.4B). These data, again, are consistent with the conclusion that the Cpx response has an inhibitory effect on *rprA* expression levels. We conclude that polymyxin B and EDTA induce the Cpx and

Rcs responses independently and through signals that are generated at different locations of the cell envelope.

3.4 Acidic and alkaline pH are sensed independently by the Cpx and Rcs responses.

Both the Cpx and Rcs envelope stress responses have been implicated in responding to alterations in pH (Danese and Silhavy, 1998; Bak et al., 2014). The Cpx response is up-regulated by alkaline pH in a CpxA-dependent manner (Danese and Silhavy, 1998; Bak et al., 2014; Surmann, et al., 2016). Further, mutations that eliminate the Cpx response lead to sensitivity to alkaline pH. Additionally, activation of the Cpx response upon NlpE over-expression downregulates expression of the *gadA* gene encoding a glutamate decarboxylase that promotes acid resistance, indicating that the Cpx response plays a repressive role in the GAD acid stress response (Surmann et al., 2016). Conversely, acid stress up-regulates RprA expression, and RprA is capable of activating RpoS expression, which promotes the GAD acid stress response by stimulating expression of the gene encoding the transcriptional activator, GadX (Bak et al., 2014). Thus, despite their similar activation by EDTA and CAMPs, the Rcs and Cpx envelope stress responses seem to be oppositely regulated by, and involved in adaptation to, pH. We wondered if pH might be sensed, similarly to EDTA and CAMPs, through distinct mechanisms by the Rcs and Cpx responses.

To investigate this question, we again examined expression of the Cpx- and Rcs-activated *PrprA-lux* and *PcpxP-lux* reporter genes in various mutant backgrounds at acidic, neutral, and alkaline pHs. As previously observed, a *PcpxP-lux* reporter was strongly induced when *E. coli* K-12 was grown in alkaline pH, and this was dependent on both the sensor kinase CpxA and the response regulator CpxR (Fig. 3.5A; Danese and Silhavy, 1998). Our results demonstrate that the phosphatase activity of CpxA is especially critical for pH regulation of the Cpx response, since in a *cpxA* mutant, the Cpx response was highly induced at all pHs. Also, as previously observed, alkaline pH induction of the Cpx response did not require NlpE, OmpA nor TonB (Fig. 3.5A) (DiGiuseppe and Silhavy, 2003). This result suggests that the Cpx activating

cue that is generated under these conditions, like with EDTA and polymyxin B, does not originate at the outer membrane. Additionally, signals sensed by NlpE/OmpA may be a prerequisite to signal to TonB then CpxA based on their cellular location at the outer membrane (NlpE/OmpA), periplasm (TonB) and inner membrane (CpxA). Repetition of this experiment in *rcsF*, *rcsC* and *rcsB* mutants demonstrated that none of the Rcs signaling proteins play a role in regulating the Cpx response upon changes in pH (Fig. 3.5A).

Interestingly, examination of *PrprA-lux* expression under the same conditions revealed that the Rcs response is induced roughly 2-fold when exposed to either alkaline (8.0) or acidic pH (5.5) relative to neutral pH (7.0) (Fig. 3.5B) and requires all members of the Rcs signaling pathway tested (RcsF, OmpA, RcsC and RcsB). As with the other Rcs inducing signals we examined, pH regulation of *PrprA-lux* expression required Rcs signaling proteins found in the outer membrane (OmpA, RcsF), the inner membrane (RcsC), and the cytoplasm (RcsB). In strains carrying deletions in the genes encoding any of these four Rcs signaling proteins, no significant difference in *PrprA-lux* activity was observed at either acidic or alkaline pHs, compared to neutral pH (Fig. 3.5B). As previously observed, *PrprA-lux* was expressed at very low levels, if at all in both ∆*rcsF* and ∆*rcsB* mutants, demonstrating the critical role of these proteins in activating the Rcs signal transduction cascade (Fig. 3.5B). Also, as previously shown, removal of the RcsC hybrid sensor kinase resulted in constitutively elevated levels of *PrprA-lux* expression, as a result of the loss of RcsC phosphatase activity (Fig. 3.5B). Thus, like the Cpx response, the phosphatase activity of RcsC appears to be critical for pH regulation of the Rcs response. The ∆*ompA* mutant exhibited a similar phenotype, although the *PrprA-lux* reporter was not expressed at levels as high as those in the ∆*rcsC* mutant (Fig. 3.5B). These data indicate that OmpA, like RcsC, is necessary to maintain the Rcs pathway in an off state at neutral pH. As OmpA is found in a complex with RcsF at the outer membrane, this interaction must inhibit RcsF-mediated phosphorylation of RcsC (Gervais and Drapeau, 1992; Konovalova et al., 2016).

Removal of CpxR, CpxA, TonB or NlpE had no impact on the inducing effect of either acidic or alkaline pH on *rprA-lux* expression (Fig. 3.5B). RprA was, however, expressed at lower

levels at all pHs in the ∆*nlpE* ∆*tonB* and ∆*cpxA* mutants, and at higher levels at all pHs in the ∆*cpxR* strain background (Fig. 3.5B). As in previous experiments (Figs. 3.1, 3.2, and 3.3, S1), the elevated expression of *PrprA-lux* in the ∆*cpxR* mutant, and the decreased expression in the absence of CpxA (overactivating the Cpx response) are consistent with an inhibitory role for the Cpx response. Cumulatively, these results indicate that the Rcs-sensed signal for fluctuations in pH requires RcsF and OmpA, and is therefore likely generated at the outer membrane, while the Cpx response appears to be activated by a signal generated at the inner membrane and sensed by CpxA.

3.5 RprA expression levels are correlated with pH adaptation mediated by the Rcs and Cpx envelope stress responses.

The simultaneous induction of the Cpx and Rcs signaling pathways in the presence of many envelope stresses (CAMPs, EDTA, NlpE over-expression, alkaline pH) suggests that these responses are often beneficial under similar conditions (Figs 3.3-3.5). It is interesting that this does not appear to be the case for acidic pH, when the Rcs response is induced while the Cpx pathway is inhibited (Fig. 3.5). Given the demonstrated role that RprA plays in regulation of adaptation to acidic conditions (Gaida et al., 2013) we hypothesized that the ability of RprA to down-regulate the alkaline-responsive Cpx response might be crucial for acid tolerance, and further that CpxR-mediated repression of *rprA* transcription at elevated pH may be important for survival (Vogt et al., 2014; Bak et al., 2014). Additionally, under highly toxic conditions *rprA* repression through CpxR may not be essential (polymyxin B, EDTA). However, as acid stress is not critical for survival this regulation may be important to maintain the hydrogen ion concentration of intracellular pH solely.

To investigate this hypothesis, we compared the growth of serially diluted cultures of wild-type, ∆*rprA,* ∆*cpxR,* and ∆*cpxA* strains transformed with a vector control plasmid (pBR322) or an RprA overexpression plasmid that had been challenged by acidic or neutral pH (Fig. 3.6 3.7). At neutral pH all strains grew similarly (Fig. 3.6, panel B, 3.7, panel B). As previously shown (Bak et

al., 2014), we found that removal of RprA led to a slight survival defect at acid pH (Fig. 3.6, panel A). Over-expression of RprA complemented the growth defect and also resulted in enhanced survival of the wild-type strain at acidic pH (Fig. 3.6, panel A). At alkaline pH, the opposite result was observed (Fig. 3.6, panel C). Deletion of *rprA* resulted in a dramatic increase in survival, and this phenotype could be complemented upon expression of *rprA* from a plasmid (Fig. 3.6, panel C). Further, over-expression of RprA lead to decreased survival of the wild-type strain at alkaline pH compared to the vector control (Fig. 3.6, panel C).

Rcs-mediated activation of RprA expression has been shown to enhance survival at acidic pH (Bak et al., 2014). Consistent with this finding, we showed that deletion of *rcsB,* which is necessary for RprA expression, resulted in a survival defect at acidic pH. Conversely, mutation of *rcsC,* which results in overactivation of the Rcs pathway and therefore *rprA* transcription, improved growth slightly under this condition (Fig. S3). To determine if repression of *rprA* expression by the Cpx response (Figs. 3.1-3.4), and conversely inhibition of the Cpx response by RprA (Vogt et al., 2014), might also be important for coordinating the envelope stress response mediated adaptation to pH change, we measured survival of wild-type, ∆*cpxR,* and ∆*cpxA* strains, in the absence and presence of *rprA* over-expression at acidic (4.0), neutral (7.0), and alkaline pH (8.5). The acid pH was further decreased from the initial experiment with Cpx mutant strains (Fig. S3) to see distinguishable differences between cell survival, as RprA has been shown to benefit survival in extreme acid conditions (Bak et al., 2014). As previously seen, a ∆*cpxA* strain exhibited diminished survival at acidic pH while the ∆*cpxR* mutant survived better than the wild-type control (Fig. S3, panel A; Evans, 2015). Since the Cpx response is constitutively activated in the *cpxA* mutant background, we speculated that this phenotype could be due to Cpx-mediated inhibition of RprA expression, and therefore the Gad-dependent acid stress response which is activated by *rprA* overexpression (Surmann, et al., 2016; Bak et al., 2014). Indeed, over-expression of *rprA* in acidic pH restored growth of the ∆*cpxA* mutant to levels comparable to that of the wild-type strain and also facilitated growth of the wild-type control (Fig. 3.7, panel A). The ∆*cpxR* mutant, in which RprA is expressed at high levels, survived better at acidic pH, and over-expression of RprA had little impact on this phenotype (Fig. 3.7, panel A).

At alkaline pH, these phenotypes were essentially reversed. When the Cpx response was induced by mutation of *cpxA,* survival was improved compared to wild type cells (Fig. 3.7, panel C). Conversely, when the Cpx response was abrogated by deletion of *cpxR,* growth at alkaline pH was dramatically diminished (Fig. 3.7, panel C). Over-expression of RprA in all strains (wild-type, ∆*cpxA,* ∆*cpxR)* resulted in worsened survival at pH 8.5 (Fig. 3.7, panel C). Overall, these results support a model in which Rcs-mediated activation of RprA expression and its consequent inhibition of the Cpx response at low pH, together with the inhibition of RprA expression at elevated pH, are important components of pH adaptation in *E. coli*.

3.6 Investigation of Cpx response activity and cell survival during cell killing by the type VI secretion system of *Vibrio cholerae*.

The type VI machinery is one of six secretion systems used by bacteria to deliver proteins and DNA into target cells or the extracellular environment (Green and Mecsas, 2016). The needle-like structure injects effector proteins into prey cells and displays antibacterial activity against those without the associated immunity genes (MacIntyre et al., 2010). Within Gram-negative bacteria, roughly 25% contain a gene cluster for a type VI secretion system (Boyer et al., 2009) As type VI machinery appears conserved in a quarter of tested Gram-negative bacteria, we wanted to identify whether the Cpx stress response in *Escherichia coli* was inducible by attack and damage caused by a type VI secretion system as the Cpx response is attuned to damaging signals in the periplasm/inner membrane (Lazzaro et al., 2017). To test this, we measured luminescence produced by a Cpx regulated *PcpxP-lux* luminescent reporter plasmid in *E. coli* prey strain MG1655 upon exposure to *V. cholerae* predator strains containing either an active (V52) or inactive (V52 ∆*vasK*) type VI secretion system at a predator to prey ratio of 10:1 (MacIntyre et al., 2010). Both type VI active (V52) and inactive strains (V52*Δ vasK*) mixed with prey *E. coli* MG1655 showed a similar level of *PcpxP-lux* reporter activity over 60 minutes, however due to the high concentration of predator versus prey cells the results of the luminescence assay are inconclusive (Fig. 3.9). It is possible that the Cpx response could have

become activated by type VI machinery but as the optical density (OD_{600}) was used to standardize the luminescence values (counts per second, cps) recorded there may not have been a substantial amount of *E. coli* harboring the *PcpxP*-luminescent reporter to view any difference between cells attacked by *V. cholerae*'s type VI secretion system. To identify whether the Cpx response is induced by *V. cholerae*'s type VI secretion system additional experiments would need to be performed that can measure expression of a specific gene of interest (for our purposes a highly Cpx-regulated gene) without needing standardization, for example a quantitative polymerase chain reaction.

To see if *E. coli* strains with Cpx signaling protein deletions altered susceptibility to type VI secretion attack by *Vibrio cholerae*, we also performed a survival assay to quantify growth of the prey MG1655. This assay was not performed under the same conditions as the gene reporter assay (described above, and Fig 3.9) therefore cell killing cannot be confirmed in the previous experiment. After luminescence readings, cells would need to be serially diluted and plated on media selective for our prey *E. coli* strain (MG1655 would use LB agar plates supplemented with 50ug/mL rifampicin). In the present experiment, cells were co-incubated with type VI active (V52) and inactive (V52*ΔvasK*) strains of *V. cholerae* then selected for by serially diluting predator and prey mixtures, and plating on LB agar plates supplemented with 50ug/mL rifampicin. Rifampicin was used to select for rifampicin resistant prey *E. coli* strain MG1655 to visualize cell survival after overnight growth. Wildtype MG1655 prey cell survival was reduced roughly 100-fold when incubated with the type VI active strain versus the inactive strain, confirming that this *E. coli* strain was susceptible to type VI attack (Fig. 3.10). A ∆*cpxA* deletion mutant (hyperactivating the Cpx response) prey strain had similar growth to the wildtype strain with or without an active type VI *V. cholerae* predator, indicating overactivation of the Cpx response does not confer resistance against type VI secretion systems (Fig. 3.10). We then tested a prey strain harboring a pCMCP vector control or a pCpxP overexpression vector which results in inhibition of the Cpx response through an unknown interaction with CpxA (Fleischer et al., 2007; Raivio et al., 1999). We also tested a pCA24N vector control and PCA-*nlpE* overexpression strain that hyperactivates the Cpx

pathway, however the inducing signal has not been characterized as direct or indirect (Snyder et al., 1995). We tested the survival of type VI secretion attack of these strains against their empty vector controls to account for any activity of the vector that may influence survival. Both inhibition and overactivation of the Cpx response resulted in similar survival to the wildtype strain and the vector control strains, however the vector control strain (harboring the pCMCP plasmid) did slightly reduce survival with co-incubation of a type VI active strain versus the inactive control strain (Fig. 3.10). This could possibly be due to indirect effects of the pCMCP vector that are alleviated by insertion of a *cpxP* open reading frame (pCpxP) and allowing for overexpression of *cpxP*, however it would require further study. Overall, we can conclude that alterations in components of the Cpx response do not aid in survival upon type VI secretion system attack, but activity of the Cpx response during co-incubation with a type VI active or inactive strain of *Vibrio cholerae* will require further investigation.

3.7 T4 bacteriophage infection does not induce the Cpx response and activation/ repression of the Cpx response does not confer resistance.

Bacteriophages are another interesting and abundant organism capable of efficiently killing many types of bacteria and pose a unique model for therapeutic use against infectious bacteria (Lin et al., 2017). The T4 bacteriophage structure is similar to the type VI secretion machinery but rather than injecting proteins the viral DNA is injected into the host cell (Fokine and Rossmann, 2016; Pukatzki et al., 2007). If Gram-negatives can sense phage adsorption with stress response systems, they are potentially capable of evolving resistance mechanisms. We were interested in identifying whether the T4 lytic phage can induce the Cpx response upon attachment and entry into the cell. We used a T4 phage stock with a multiplicity of infection of five added to a culture of *E. coli* strain MC4100 with a *PcpxP-lux* reporter to monitor any Cpx activity during phage infection. We chose to measure gene expression over a 30-minute co-incubation with T4 phage to allow for attachment and subsequent lysis of *E. coli* to measure any Cpx activity

during each stage of the T4 infection cycle. Over 30 minutes we did not observe any *PcpxP-lux* activity upon phage addition, indicating that the Cpx response is unable to sense entry of T4 phage (Fig. 3.11).

To determine if there is a potential for greater survival against T4 phage by activating or repressing the Cpx response we created activating (∆*cpxA*) and repressing (∆*cpxR*) mutations in *E. coli* MC4100 to compare to wildtype cells. Wildtype cells had just under a 2-fold reduction in cell viability when treated with T4 phage as compared to a phage-free control experiment (Fig. 3.12). Both *cpxA* and *cpxR* deletion mutant strains had reduced viability, however the *cpxR* mutant strain survived slightly better than wildtype and the *cpxA* mutant, indicating repression of the Cpx response may benefit survival (Fig. 3.12). This is an interesting finding as overactivation of the Cpx response through a deletion of CpxA does not result in distinguishable differences in survival from wildtype cells. This may be a phenotype occurring independently of CpxA.

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3.8 Type B colicins (B and Ia) T4 bacteriophage infection does not induce the Cpx response and activation/ repression of the Cpx response does not confer resistance.

Colicins are efficient molecules created by bacteria for killing many closely related species that do not contain the respective immunity genes (Cascales et al., 2017). Additionally, they pose as an inexpensive alternative for future nonantibiotic antibacterial treatments on food products (Schulz et al., 2015). If colicins can enter through the cell envelope without triggering a stress response it is less likely that cells will develop resistance to them. We chose to test type B colicins for their potential to activate the Cpx response because of their pore forming abilities at the cell envelope where the Cpx response responds to perturbations to this protective barrier (Cascales et al., 2017). Within the type B colicins we chose colicins Ia and B which were available from previous graduate student work in our lab. The lysate containing both colicins was

extracted in HEPES buffer and a lysate free control in HEPES buffer, made by PhD student Junshu Wang as previously described (Braun et al.,1974). These lysates were used to test Cpx activation with a *PcpxP-lux* reporter in *E. coli* strain MC4100. We chose to report the results for the incubation over one hour to allow for colicin uptake and subsequent bactericidal activity (pore formation in the cell envelope) against the *E. coli* cells. Over the one hour co-incubation Cpx activity seemed to drop rather than increase in cell cultures containing the colicin lysate versus the colicin-free control lysate (Fig. 3.13), indicating the Cpx response is downregulated during colicin attack. As the Cpx response is classically activated during envelope stress we hypothesized that the loss in energy potential created by these pore-forming bacteriocins may be affecting the luminescent reporter which is a highly sensitive measure of cellular activity (Riss et al., 2004). To determine whether the Cpx response is downregulated or the reporter we are using is too sensitive we performed a beta galactosidase assay as it is placed on the chromosome and may offer less ambiguous luminescent reporter activity. We used a chromosomal *cpxP-lacZ* fusion to monitor Cpx activation when co-incubated with a lysate of colicins B and Ia. The results of the beta galactosidase assay provided additional support that the Cpx response is downregulated when in the presence of type B colicins (Fig 3.14). The Cpx response is most commonly known for its regulation of protein folding and degrading factors to alleviate stress in the cell envelope, therefore this detrimental and opposing effect to cell envelope damage caused by pore forming colicins indicates Cpx activity may harm cells when combatting these toxic molecules (Danese and Silhavy, 1997; Danese et al.,1995; Dartigalongue and Raina, 1998).

To clarify whether activation of the Cpx response was harmful during colicin treatment we tested whether Cpx activating (∆*cpxA*) or repressing (∆*cpxR*) mutations had any effect on survival during colicin-mediated attack. Wildtype cell survival was reduced two-fold by type B colicin treatment (Fig 3.15). The *cpxA* and *cpxR* mutant strains also exhibited reduced CFU counts consistent with wildtype (Fig. 3.15). Activation or inhibition of the Cpx response does not appear to directly contribute to greater cell death or survival during type B colicin infection.

3.9 Figures

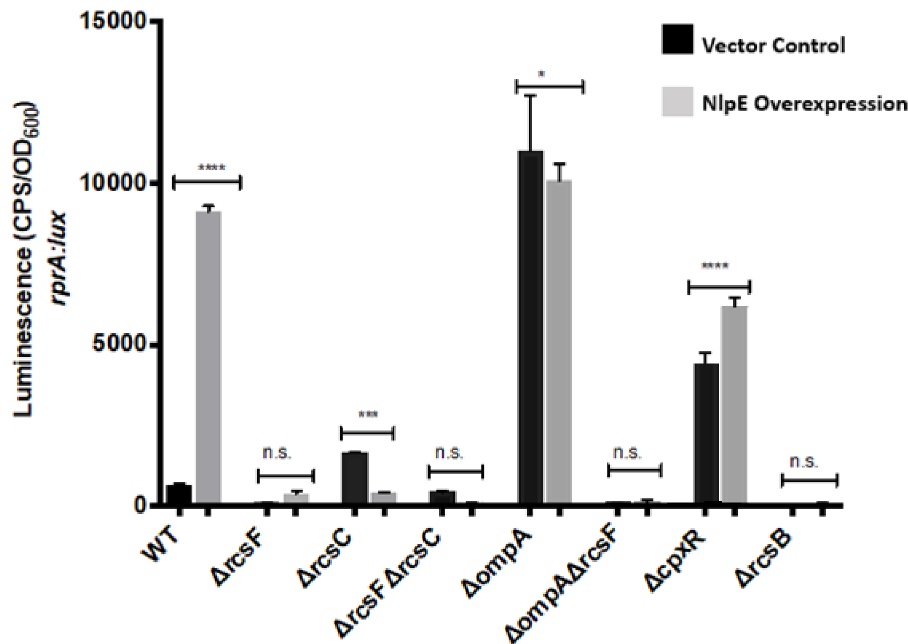


Figure 3.1. The Rcs response is induced by NlpE overexpression, dependent on OmpA/RcsF/RcsC/RcsB. Strains bearing *PrprA*-luminescent reporter plasmids with single and double gene deletions encoding Rcs (*rcsF, rcsC, ompA, rcsB*) and Cpx (*cpxR*) response signaling proteins and either pCA-*nlpE* overexpression vector or pCA24 vector control were subcultured 1:50 from 2 mL overnight cultures into 2 mL fresh LB broth and antibiotics and grown at 37ºC with aeration at 225rpm for two hours. *nlpE* overexpression was induced at 2 hours growth by addition of 0.05 mM IPTG to cell cultures. After an additional two hours of growth *PrprA-lux* activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Data for each strain represents the means and standard deviations of five replicate cultures, from two independent experiments where results were averaged. Asterisks denote a statistically significant difference from the relevant vector control, pCA24N (****, $P \leq 0.0001$; ***, $P \leq 0.001$, *, $P \leq 0.05$; One-way ANOVA with multiple comparison test).

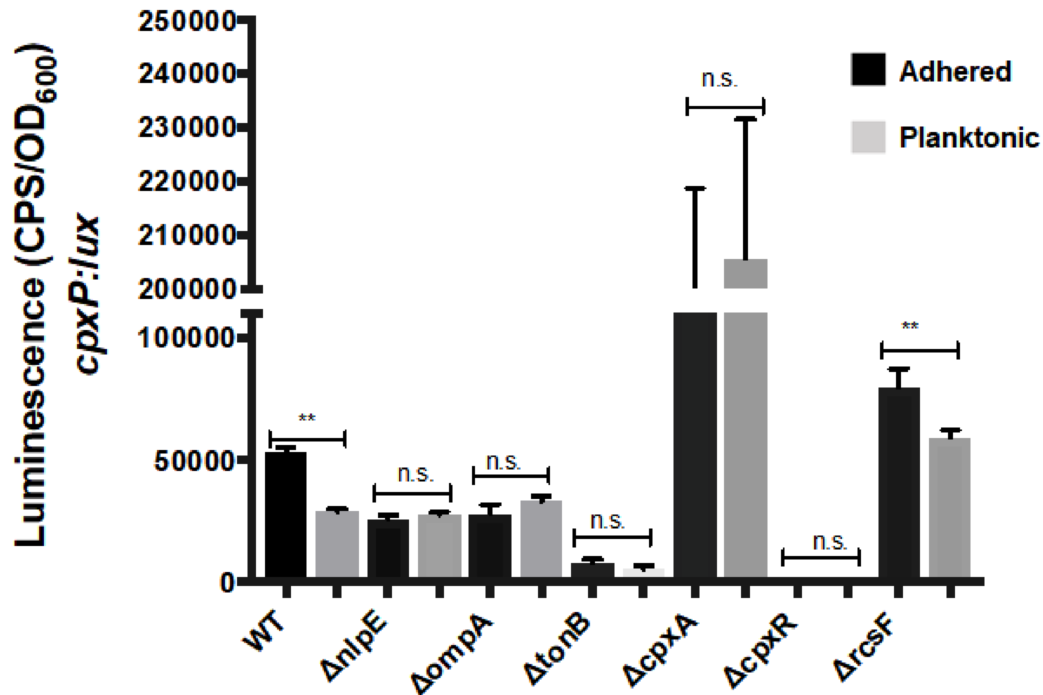
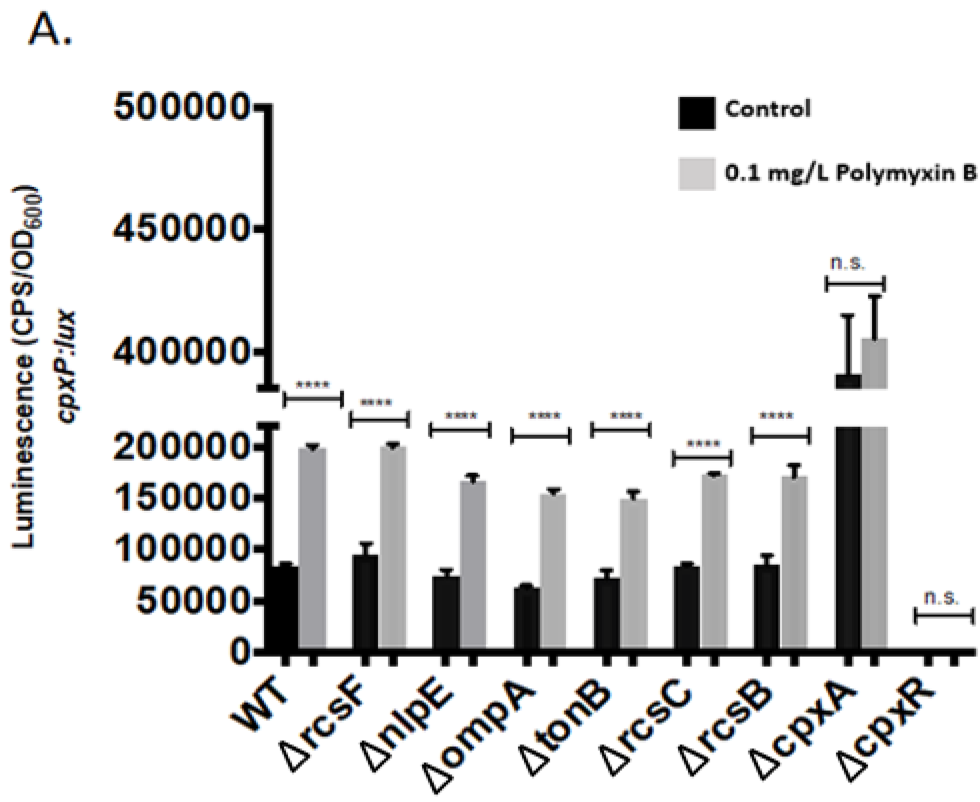


Figure 3.2. The Cpx response is induced during surface adhesion requiring NlpE, OmpA, TonB, CpxA and CpxR. Strains bearing *PcpxP*-luminescent reporter plasmids and single gene deletions encoding Cpx (*nlpE, cpxA, cpxR*) and Rcs (*rcsF*) response signaling proteins, and two suggested Cpx response signaling proteins (*ompA* and *tonB*) were grown overnight at 37ºC on LB agar plates with antibiotics. Single colonies were inoculated into 200 µL LB broth with antibiotics in a tissue culture-treated 96-well plate. The 96-well plate was then grown at 37ºC with rocking at 6 rotations per minute (6rpm) for six hours to allow for adherence to wells of the plate. At the six-hour timepoint planktonic cells were pipetted into new wells of a 96-well plate and the adhered cells had 200 µL LB added to each well. *PcpxP-lux* activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Cells were scraped from each well with a pipette tip and resuspended with pipetting and vortexing and optical density readings were taken and used to standardize adhered cell luminescence readings. Data for each strain represents the means and standard deviations of five replicate cultures, from two independent experiments where results were averaged. Asterisks denote a statistically significant difference from the adhered versus planktonic cells (**, $P \leq 0.01$; One-way ANOVA with multiple comparison test).

A.



B.

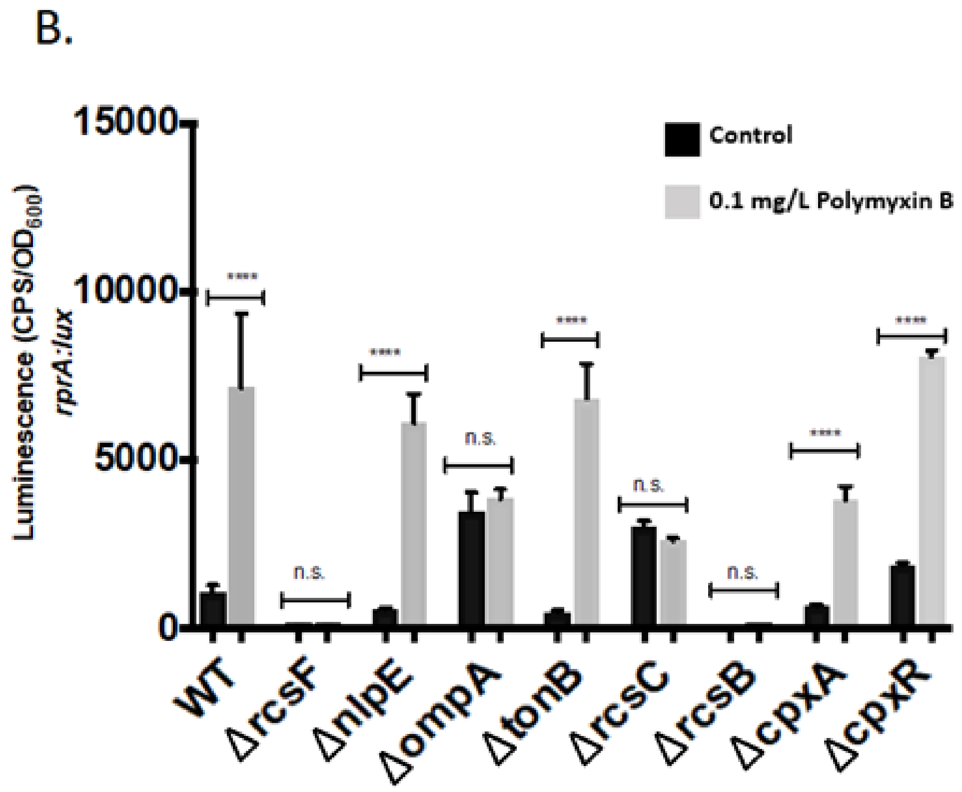


Figure 3.3. Polymyxin B induces the Cpx and Rcs responses independently of one another, at different cellular compartments. Strains bearing A) *PcpxP* or B) *PrprA*-luminescent reporter plasmids in single gene deletion strains encoding Cpx (*nlpE, ompA, tonB, cpxA, cpxR*) and Rcs (*rcsF, ompA, rcsC, rcsB*) response signaling proteins were subcultured 1:50 from 2 mL overnight cultures into 2 mL fresh LB broth and antibiotics and grown at 37ºC with aeration at 225rpm for two hours. At two hours growth, polymyxin B was added to each experimental cell culture to a final volume of 0.1mg/L and cells were re-incubated at 37ºC with aeration at 225rpm for an additional hour (greatest induction determined at this timepoint). After one hour *PcpxP-lux* and *PrprA-lux* activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Data for each strain in each experiment (A and B) represents the means and standard deviations of five replicate cultures, with the entire experiment repeated three times, and the results were averaged. Asterisks denote a statistically significant difference from the relevant control (****, $P \leq 0.0001$; One-way ANOVA with multiple comparison test).

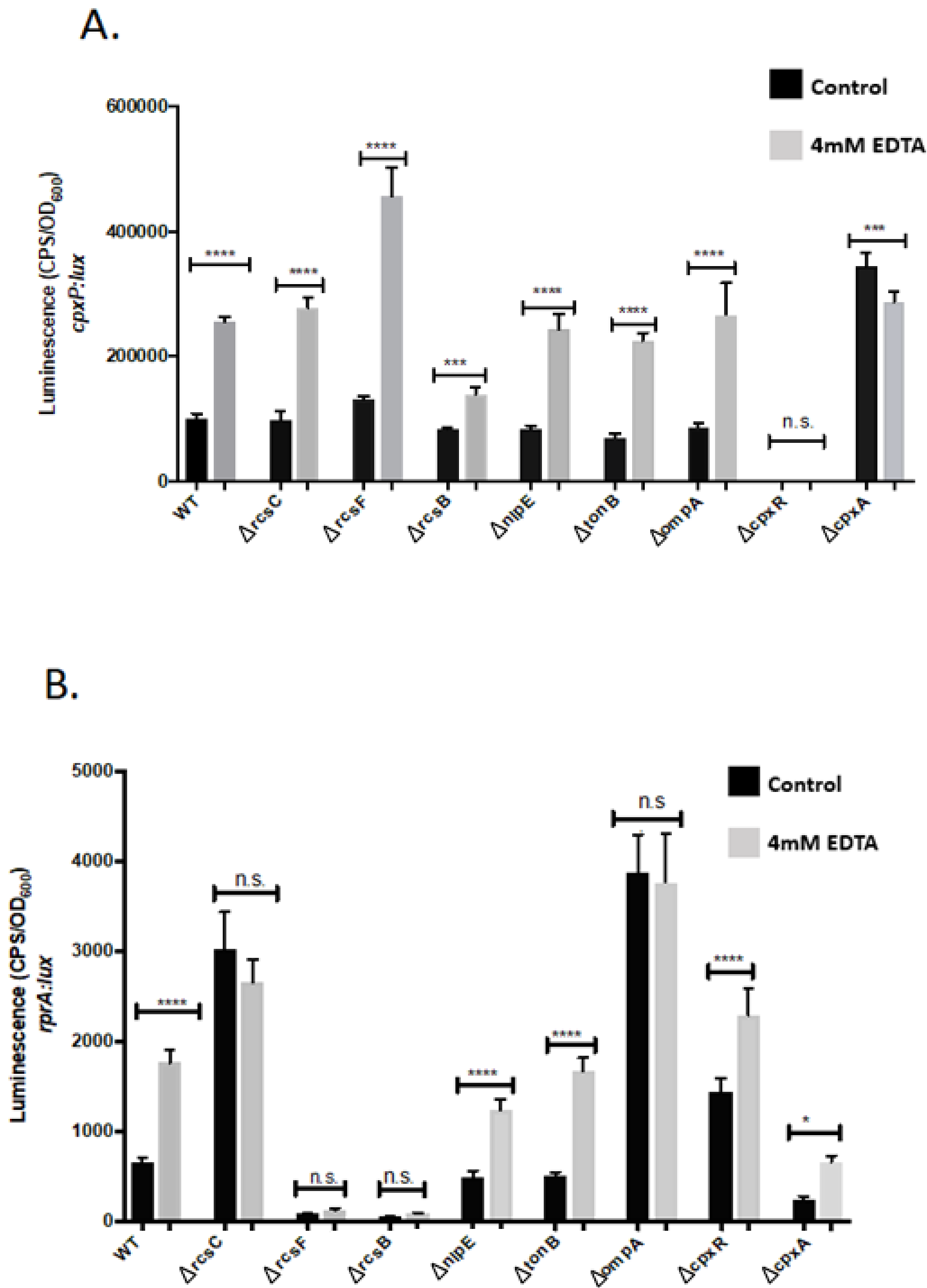
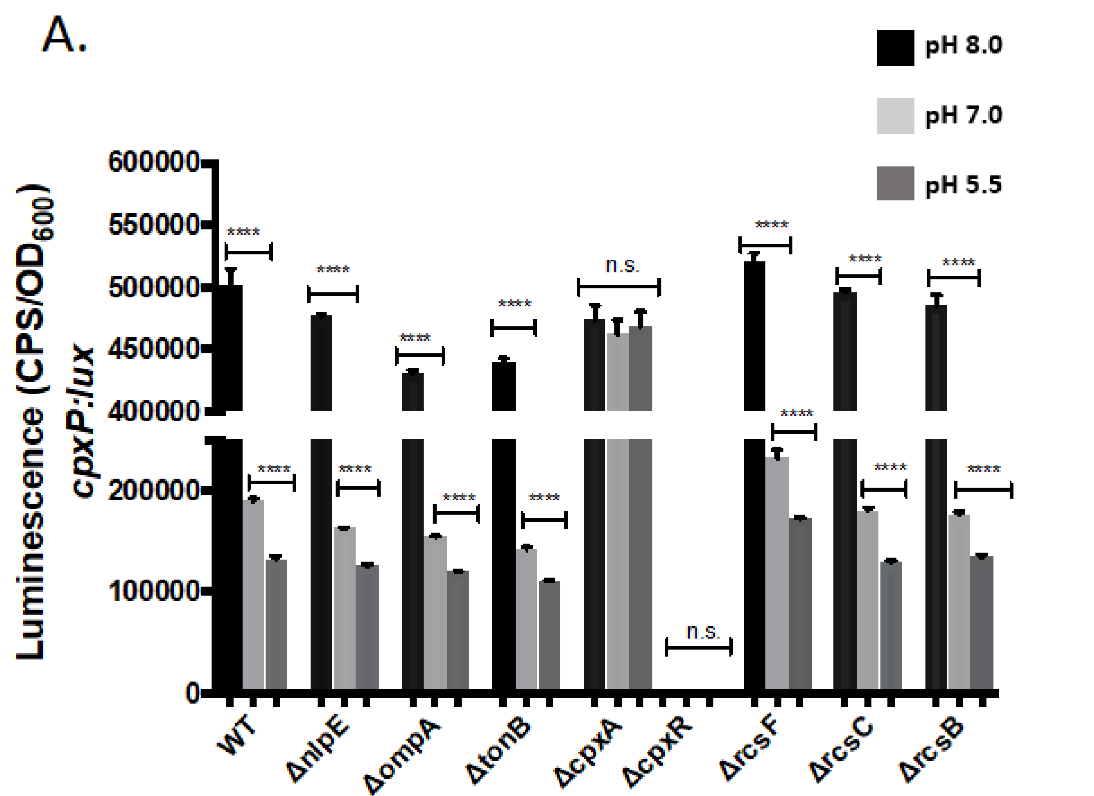


Figure 3.4. EDTA induces the Cpx and Rcs responses independently of one another, in different cellular compartments. Strains bearing A)*PcpxP* or B) *PrprA*-luminescent reporter plasmids in single gene deletions encoding Cpx (*nlpE, ompA, tonB, cpxA, cpxR*) and Rcs (*rcsF,*

ompA, rcsC, rcsB) signaling proteins were subcultured 1:50 from 2mL overnight cultures grown at 37ºC with aeration (225 rpm) into 2mL fresh LB broth and antibiotics and grown at 37ºC with aeration at 225rpm for two hours. At two hours growth, EDTA was added to each experimental cell culture to a final volume of 4mM and cells were re-incubated at 37ºC with aeration at 225rpm for an additional four hours (greatest induction was found at four hours). After four hours *PcpxP-lux* and *PrprA-lux* activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Data for each strain in each experiment (A and B) represents the means and standard deviations of five replicate cultures, with the entire experiment repeated three times, and the results were averaged. Asterisks denote a statistically significant difference from the relevant control (****, $P \leq 0.001$; *, $P \leq 0.05$; One-way ANOVA with multiple comparison test).


A.
pH 8.0
pH 7.0
pH 5.5
Luminescence (CPS/OD600)
cpxP:lux
600000
550000
500000
450000
400000
200000
100000
0
****
n.s.
WT
ΔnlpE
ΔompA
ΔtonB
ΔcpxA
ΔcpxR
ΔrcsF
ΔrcsC
ΔrcsB

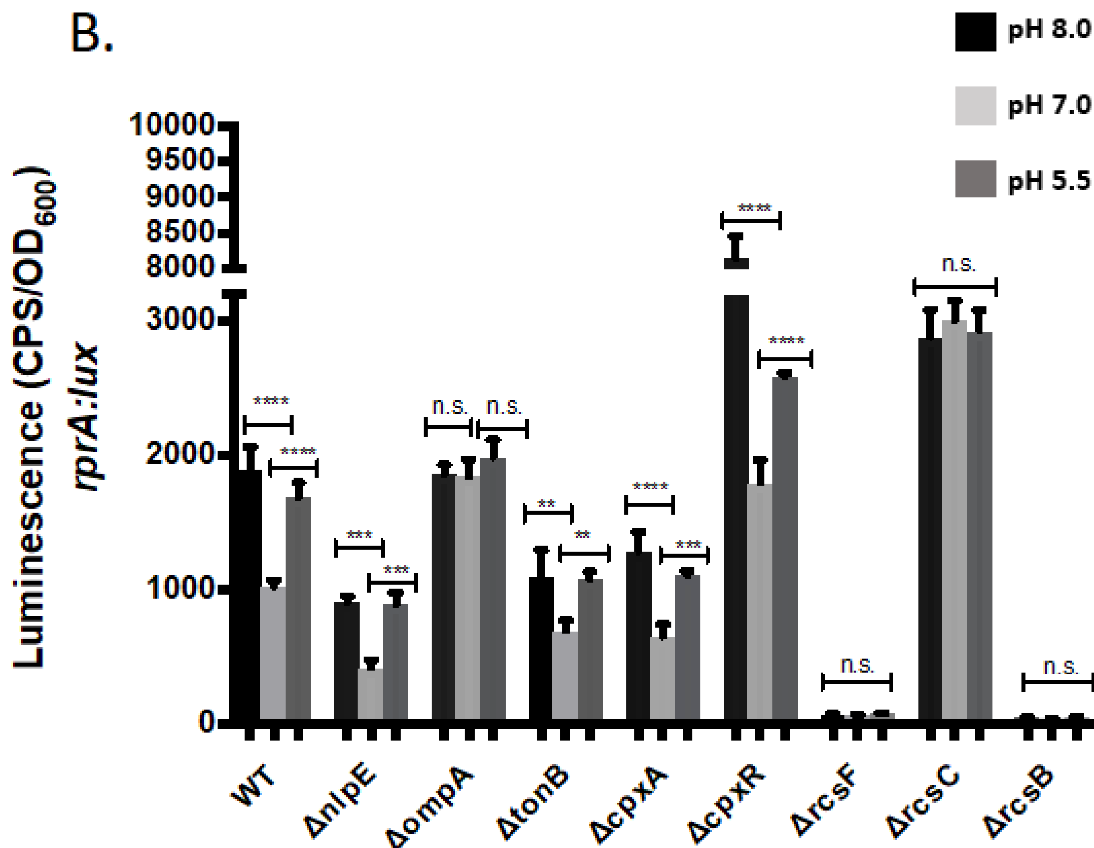

B.
pH 8.0
pH 7.0
pH 5.5
Luminescence (CPS/OD600)
rprA:lux
10000
9500
9000
8500
8000
3000
2000
1000
0
****
***
**
n.s.
WT
ΔnlpE
ΔompA
ΔtonB
ΔcpxA
ΔcpxR
ΔrcsF
ΔrcsC
ΔrcsB

Figure 3.5. The Cpx and Rcs responses are both induced in alkaline pH, but only the Rcs is activated in acidic pH, downregulating the Cpx response. Strains bearing A)*PcpxP* or B) *PrprA*-luminescent reporter plasmids in single gene deletion strains encoding Cpx (*nlpE, ompA, tonB, cpxA, cpxR*) and Rcs (*rcsF, ompA, rcsC, rcsB* signaling proteins were grown in 2 mL LB and antibiotics overnight at 37ºC with aeration at 225 rpm. Next day, cultures were centrifuged at 4,000rpm for 10 minutes and the supernatant was removed. Cells were resuspended in 2 mL fresh LB broth and antibiotics, adjusted to alkaline (8.0), neutral (7.0) or acidic (5.5) pH by addition of HCl or NaCl added dropwise to the new LB broth, and grown at 37ºC with aeration at 225rpm for one hour. After one hour *PcpxP-lux* (A) and *PrprA-lux* (B) activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Data for each strain in each experiment (A and B) represents the means and standard deviations of five replicate cultures, with the entire experiment repeated three times, and results representing the average of all trials and standard deviations. Asterisks denote a statistically significant difference from the relevant neutral (pH 7.0) control (****, $P \leq 0.0001$; ***, $P \leq 0.001$, **, $P \leq 0.01$; One-way ANOVA with multiple comparison test).

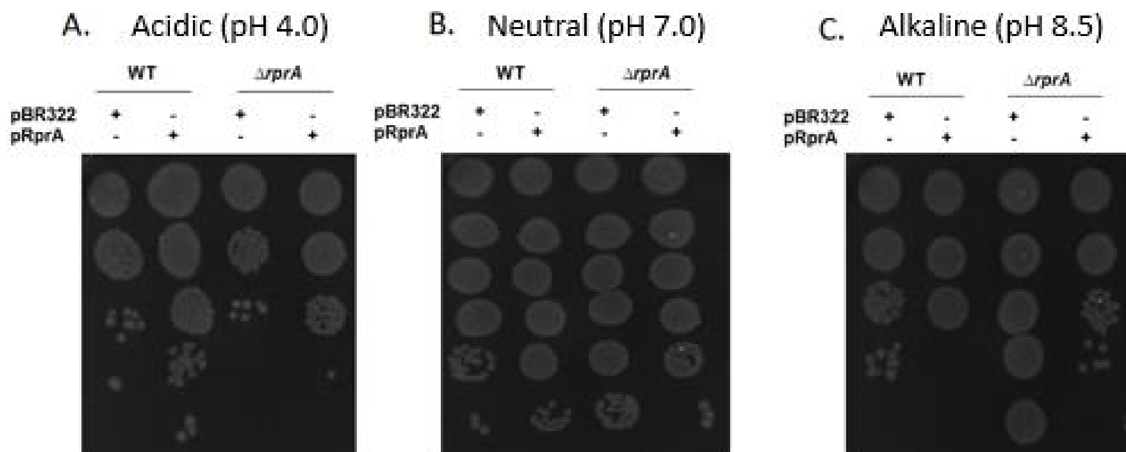


Figure 3.6. Overexpression of *rprA* confers resistance in acidic pH and sensitivity in alkaline pH. Strains bearing pBR-*rprA* overexpression vector or a pBR322 vector control reporter plasmid in wildtype and *rprA* mutants were grown in 2 mL LB and antibiotics overnight with aeration (225 rpm) at 37ºC. Next day, IPTG at a final concentration of 0.05mM was added to each culture and cells were grown for an additional two hours with aeration (225 rpm) at 37ºC. Cells were then subcultured 1:50 into 2 mL fresh LB broth and antibiotics, adjusted to A) acidic (4.0), B) neutral (7.0) or C) alkaline (8.5) pH. An additional 0.05mM IPTG was added to all strains carrying overexpression plasmids and strains harboring the respective vector control plasmids. pH of the LB broth was adjusted by addition of HCl or NaCl added dropwise to the fresh LB broth and grown at 37ºC with aeration at 225 rpm for three hours, readjusting the pH every hour by centrifuging tubes at 4,000 rpm for ten minutes and resuspending pelleted cells in new, pH adjusted media by vortexing and pipetting. After the three hours cells were serially diluted and plated on LB agar plates and incubated overnight at 37ºC, grown statically. Next day, CFUs were imaged on each plate. Experiments were performed in duplicate with three replicates per strain. The above image is representative of one trial and is consistent with replicates.

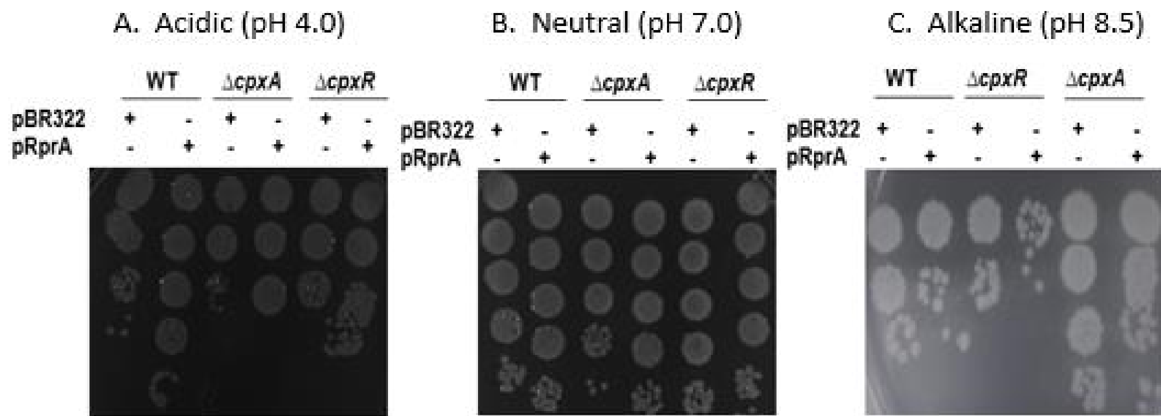


Figure 3.7. Overexpression of *rprA* confers resistance in acidic pH and sensitivity in alkaline pH in Cpx mutants. Strains bearing pBR-*rprA* overexpression vector or a pBR322 vector control reporter plasmid with single gene deletions encoding Cpx response signaling proteins were grown in 2mL LB and antibiotics overnight with aeration (225 rpm) at 37ºC. Next day, IPTG at a final concentration of 0.05mM was added to each culture and cells were grown for an additional two hours with aeration (225 rpm) at 37ºC. Cells were then subcultured into 2 mL fresh LB broth and antibiotics, adjusted to A)acid (4.0), B)neutral (7.0) or C)alkaline (8.5) pH by addition of HCl or NaCl added dropwise to the fresh LB broth, and grown at 37ºC with aeration at 225rpm for three hours, readjusting the pH every hour by centrifuging tubes at 4,000 rpm for ten minutes and resuspending pelleted cells in new media by vortexing and pipetting. After the three hours cells were serially diluted and plated on LB agar plates and incubated overnight at 37ºC statically. CFUs were imaged on each plate. Experiments were performed in duplicate with three replicates per strain. The above image is representative of one trial and is consistent with replicates.

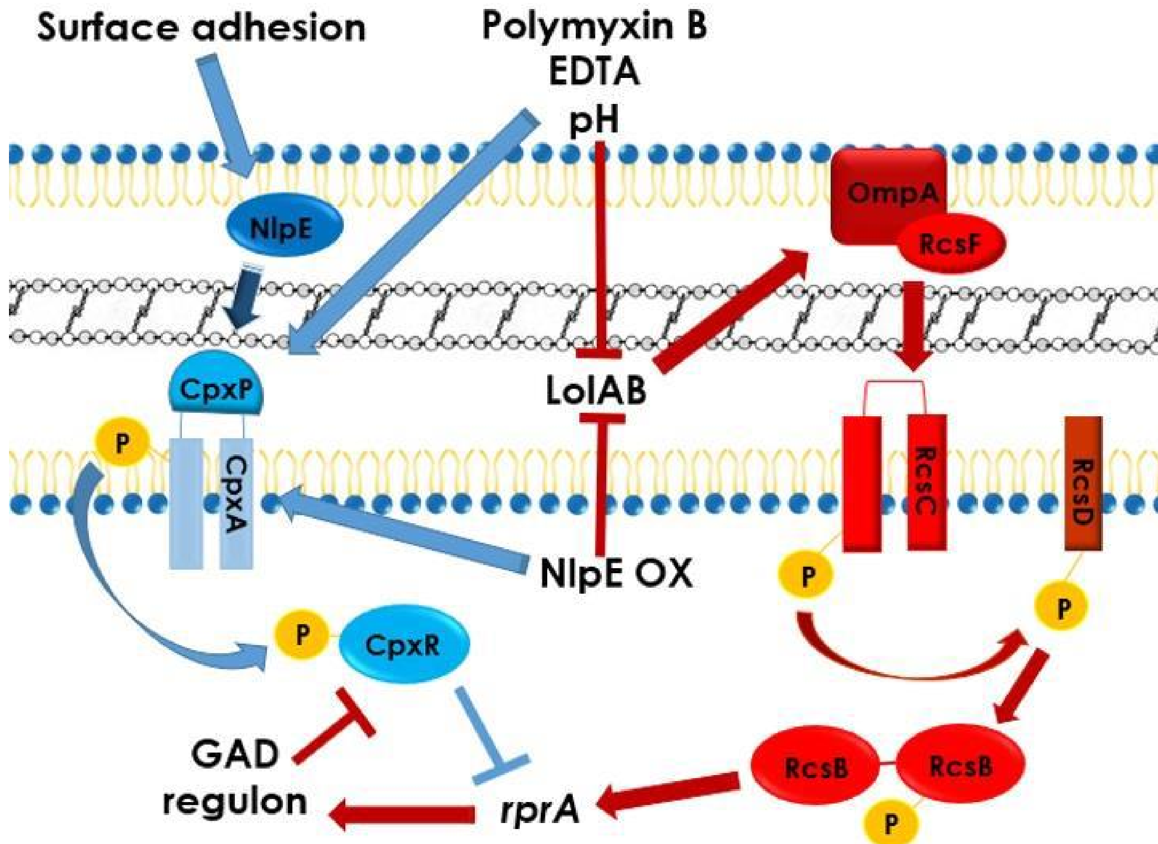


Figure 3.8 A model for signaling by, and coordination of, the Rcs and Cpx envelope stress responses. The Rcs and Cpx envelope stress responses are both activated by polymyxin B, EDTA, alkaline pH and NlpE over-expression, but through different mechanisms. These signals induce the Rcs response through RcsF and OmpA, either by altering the RcsF:OmpA complex at the cell surface or by disrupting its formation by the Lol and Bam assembly machineries. These same cues induce the Cpx response in an NlpE-independent manner, through the sensor histidine kinase CpxA at the inner membrane. Activation of the Cpx response leads to inhibition of *rprA* transcription as well as down-regulation of the GDAR response (Vogt et al., 2014), blocking export of protons from the cytoplasm during alkaline pH conditions. Under acidic conditions, the Rcs response promotes expression of RprA, which up-regulates the GDAR response through binding to RpoS and elevates transcription of the Gad regulon member transcription factors, GadX and GadE (Bak et al., 2014). Arrows indicate positive regulation, whereas lines with a perpendicular line indicate repression of the gene/ gene cluster.

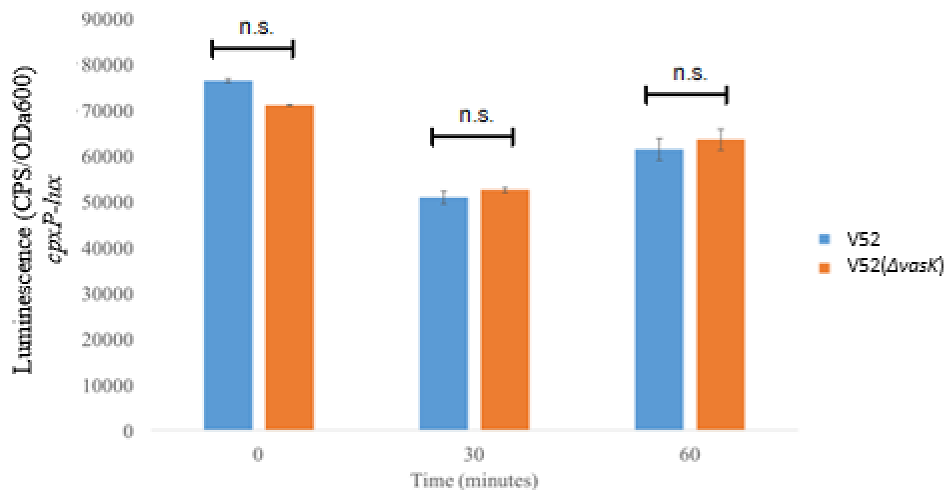


Figure 3.9 It is indeterminate whether the Cpx response is activated by the Type VI secretion system of *Vibrio cholerae*. Wildtype *E. coli* strain MG1655 harboring a *PcpxP*-luminescent reporter and predator *V. cholerae* strains with a type VI active (V52) and type VI inactive (V52*ΔvasK*) were streaked on their own LB agar plates with appropriate antibiotics to create a lawn of growth with overnight incubation at 37ºC, grown statically. Next day, using a sterile plastic loop, cells were recovered from each lawn of cells to fill a sterile loop each, and were transferred to 2 mL LB broth in a 2 mL microcentrifuge tube and resuspended by pipetting. The original 2 mL culture tubes were subcultured 1:50 into 2 mL fresh LB. Optical density readings of each dilution were taken (OD_{600}), and the original optical densities were calculated and converted to a colony forming unit value (CFU, enumeration was quantified based on the calculation in which an OD of 1=1*10^9 CFU). Volumes chosen that resulted in 5*10^8 predator and 5*10^7 prey cells were calculated. The above mentioned 10:1 predator and prey mixtures were mixed in triplicate for each of three time points we were interested in measuring (9 tubes in total) in a microcentrifuge tube and centrifuged for two minutes at 15,000 rpms. Cell mixtures were left in a pellet for 0, 30 and 60 minutes and then the supernatant was removed and cells were resuspended in 200 µL fresh LB broth and transferred to the wells of a 96-well plate. Optical density (OD_{600}) and luminescence readings were taken; optical density was used to standardize the luminescence readings (counts per second, cps) from each well. Experiments were performed in triplicate, with three replicates per strain, and the results were averaged. N.S.

denotes a non-significant statistical difference from the control (n.s., $P > 0.05$; One-way ANOVA with multiple comparison test).

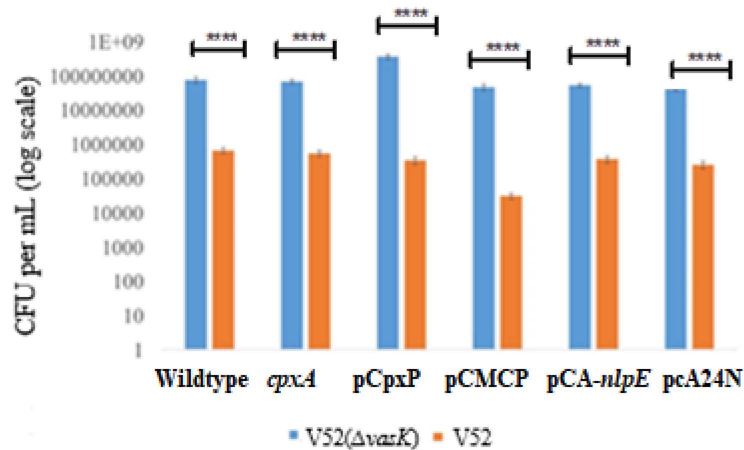


Figure 3.10 Mutations in the Cpx response do not alter susceptibility to the Type VI secretion system of *Vibrio cholerae*. Wildtype *E. coli* strain MG1655, a *cpxA* deletion strain, *cpxP* and *nlpE* overexpression vectors (pCpxP and pCA-*nlpE*), and their vector controls (pCMCP and pCA24N, respectively) were used as prey in this experiment. Prey strains and predator *V. cholerae* strains that were type VI active (V52) or type VI inactive (V52*ΔvasK*) were streaked on their own LB agar plates with appropriate antibiotics to create a lawn of growth with overnight incubation at 37ºC, grown statically. Next day, using a sterile plastic loop, cells were recovered from each lawn of cells to fill the sterile, circular loop each, and were transferred to 2mL LB broth in a 2mL microcentrifuge tube and resuspended by pipetting. The original 2mL culture tubes were subcultured 1:50 into 2mL fresh LB. Optical density readings of each dilution were taken (OD_{600}), and the original optical densities were calculated and converted to a colony forming unit value (CFU, enumeration was quantified based on the calculation in which an OD of 1=1*10^9 CFU). Volumes chosen that resulted in 5*10^8 predator and 5*10^7 prey cells were calculated. Prey *E. coli* strains harboring a vector control plasmid (pCMCP or pCA24N) or an overexpression vector plasmid (pCpxP or pCA-*nlpE*) were incubated with 0.1mM IPTG for 5 minutes to induce expression of *cpxP* or *nlpE*. The calculated 10:1 predator and prey mixtures were mixed in a microcentrifuge tube and centrifuged for two minutes at 15,000 rpms. Supernatants were removed and cells were resuspended in 125 µL LB broth. Each predator and prey combination were spotted on pre-warmed LB agar plates in 25 µL aliquots. Plates were incubated for three

hours at 37ºC statically, then spots were harvested with a sterile loop and resuspended in 1 mL LB broth. Cells were then serially diluted and plated on LB agar plates in duplicate to ensure accuracy of spot harvesting. LB agar plates used in serial dilution contained 50ug/mL rifampicin to select for rifampicin resistant prey *E.coli.* Cells were incubated overnight at 37ºC statically, and next day CFUs were counted and averaged. Experiments were performed in triplicate, with three replicates per strain, and results were averaged. Asterisks denote a statistically significant difference from the relevant control (****, $P \leq 0.0001$; One-way ANOVA with multiple comparison test).

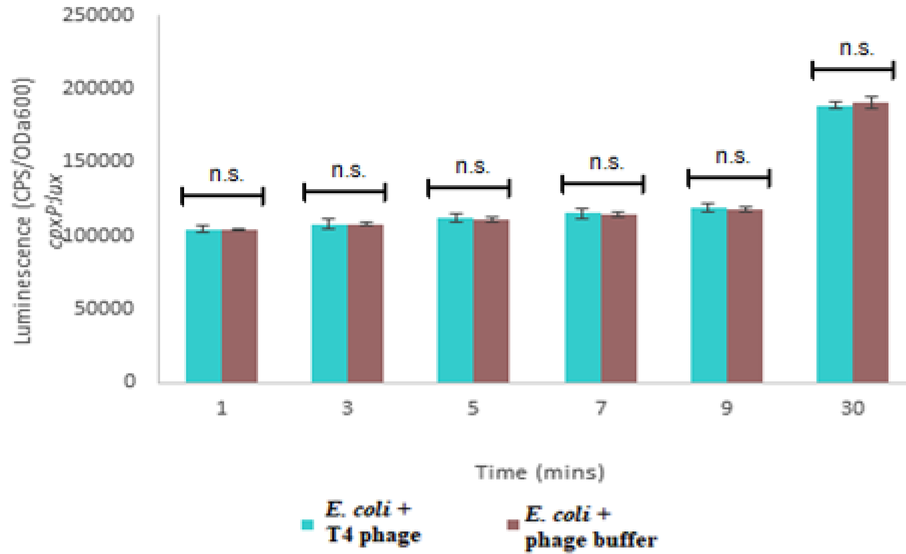


Figure 3.11 T4 phage infection does not induce the Cpx response. *E. coli* strain MC4100 with a *PcpxP*-luminescent reporter was grown overnight in 2 mL LB and 50 µg/mL kanamycin at 37ºC with aeration at 225 rpm. Next day, 1:50 subcultures were made in 2mL LB and antibiotics, grown at 37ºC with aeration (225 rpm) to mid-log phase (OD 0.5) and then co-incubated with either 100µL T4 phage at a MOI (multiplicity of infection) of five or 100 µL phage buffer as a control. Cell mixtures were transferred to a 96-well plate. Luminescence and optical density (OD_{600}) were taken over three minutes beginning at 1 minute from co-incubation. Luminescence readings (counts per second, cps) were standardized to the optical density (OD_{600}) of the culture, and experiments were performed three times with three replicates per strain and the results were averaged from all trials. N.S. denotes a non-significant statistical difference from the control (n.s., $P > 0.05$; One-way ANOVA with multiple comparison test).

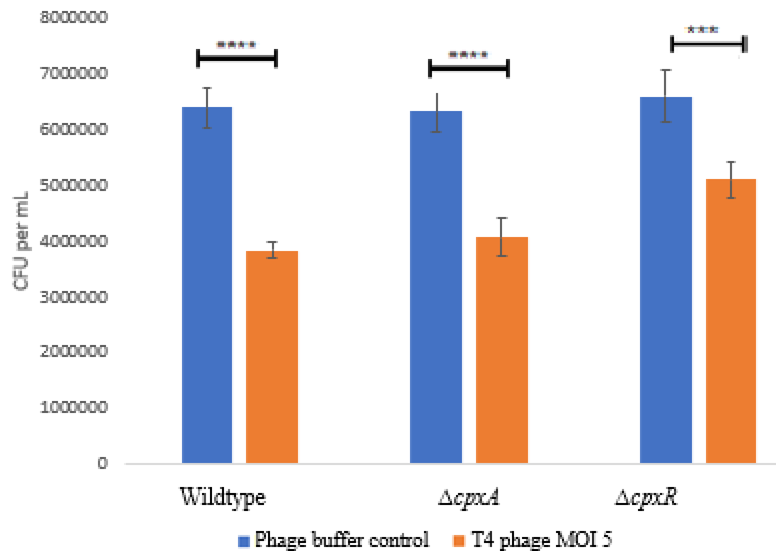


Figure 3.12 Activation/ repression of the Cpx response does not confer resistance to T4 bacteriophage. *E. coli* strain MC4100 and Cpx deletion mutants (*ΔcpxA*, activating mutation and *ΔcpxR,* inactivating mutation) were grown overnight in 2 mL LB at 37ºC with aeration (225 rpm). Next day, 1:50 subcultures were made in 2 mL LB, grown at 37ºC with aeration (225 rpm) to mid-log phase (OD 0.5) and then co-incubated with either 100 µL of a T4 phage stock at a multiplicity of infection (MOI) of five, or 100 µL phage buffer as a control. Cell mixtures were then incubated at 37ºC with aeration (225 rpm) for another hour. Cells were then centrifuged at 4,000 rpm for ten minutes and the resuspended in 2 mL fresh LB to remove residual phage and plated on LB agar plates in serial dilutions, in duplicate for each strain. Cells were grown overnight at 37ºC, grown statically and next day CFU enumeration was performed. Experiments were performed three times with three replicates per strain and the results were averaged from all trials. Asterisks denote a statistically significant difference from the relevant control (****, $P \leq 0.0001$; ***, $P \leq 0.001$; One-way ANOVA with multiple comparison test).

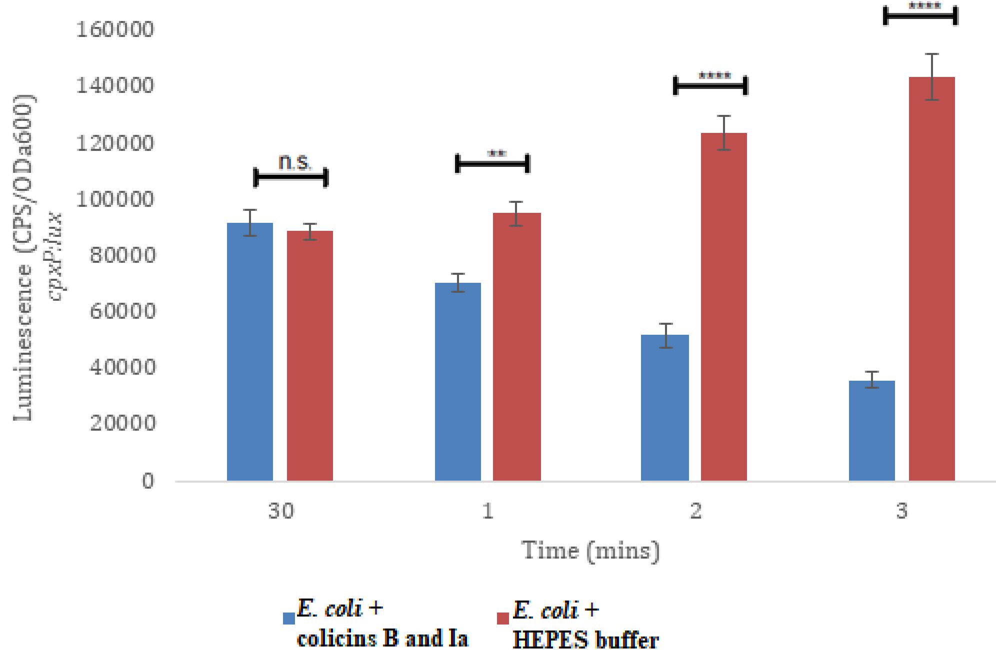


Figure 3.13 Type B colicins (B and Ia) do not induce the Cpx response. *E. coli* strain MC4100 with a *PcpxP*-luminescent reporter was grown overnight in 2 mL LB and 50 µg/mL kanamycin at 37ºC with aeration (225 rpm). Next day, 1:50 subcultures were made in 2 mL LB and antibiotics, grown at 37ºC with aeration (225 rpm) to mid-log phase (OD 0.5) and then co-incubated with either 10µl colicin lysate (1:200 dilution from stock solution) or 10µL control lysate (1:200 dilution from colicin-free stock lysate) in HEPES buffer (chemical formula: $C_8H_{18}N_2O_4S$) and transferred to a 96-well plate. Luminescence and optical density (OD_{600}) were read over three minutes beginning at 30 seconds co-incubation. Luminescence readings (counts per second, cps) were standardized to the optical density (OD_{600}) of the culture. Experiments were performed in triplicate, with three replicates per strain and the results were averaged. Asterisks denote a statistically significant difference from the relevant control (****, $P \leq 0.0001$; **, $P \leq 0.01$; One-way ANOVA with multiple comparison test).

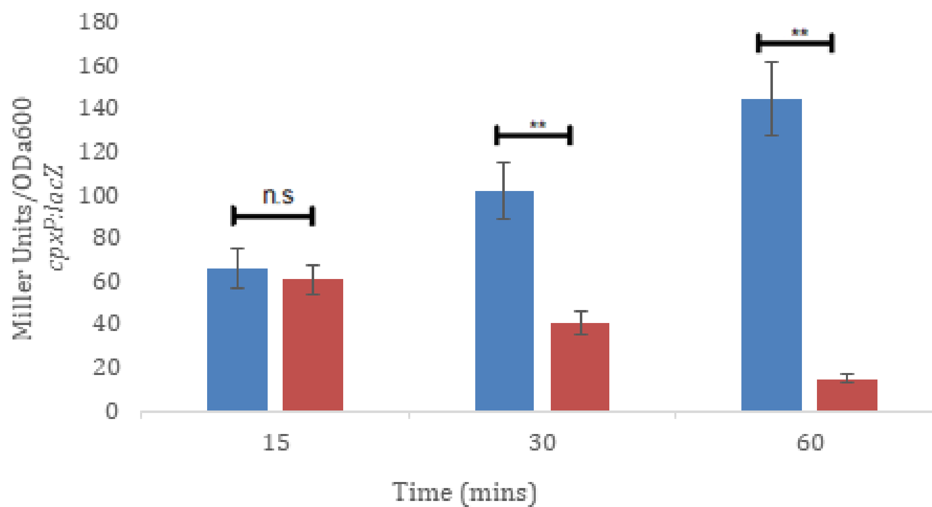


Figure 3.14 Type B colicins (B and Ia) do not induce transcription of the *cpxP::lacZ* chromosomal reporter. *E. coli* strain MC4100 with a *cpxP:lacZ* chromosomal reporter (named TR50) was grown overnight in 5 mL LB at 37ºC with aeration (225rpm). Next day, 1:50 subcultures were made in 5mL LB and grown at 37ºC with aeration (225 rpm) to mid-log phase (OD 0.5) and then co-incubated with either 10µl colicin lysate (1:200 dilution from stock solution) or 10µL control lysate (1:200 dilution from colicin-free lysate) in HEPES buffer (chemical formula: $C_8H_{18}N_2O_4S$). Co-incubation mixtures were made for time points 15, 30 and 60 minutes to measure Cpx response activity during colicin entry and subsequent killing in triplicate (9 tubes total, per replication of the experiment). After each timepoint, cells were centrifuged at 4,000rpm for ten minutes and resuspended in 5mL 1X Z-buffer (chemical composition: 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), and 250uL samples were taken from each replicate to measure optical density (OD_{600}) in a 96-well plate. Cells that were remaining in 1X Z-buffer 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 a well containing 195 µL 1X Z-buffer in a 96-well plate, then 50 µL 10 mg/mL ONPG (ortho-Nitrophenyl-β-galactoside) was added to each well and the A_{420} was measured with the plate reader. Beta-galatosidase activity was standardized to the optical density of each culture. Experiments were performed in triplicate, with three replicates per strain and the

results were averaged. Asterisks denote a statistically significant difference from the relevant control (**, $P \leq 0.01$; One-way ANOVA with multiple comparison test).

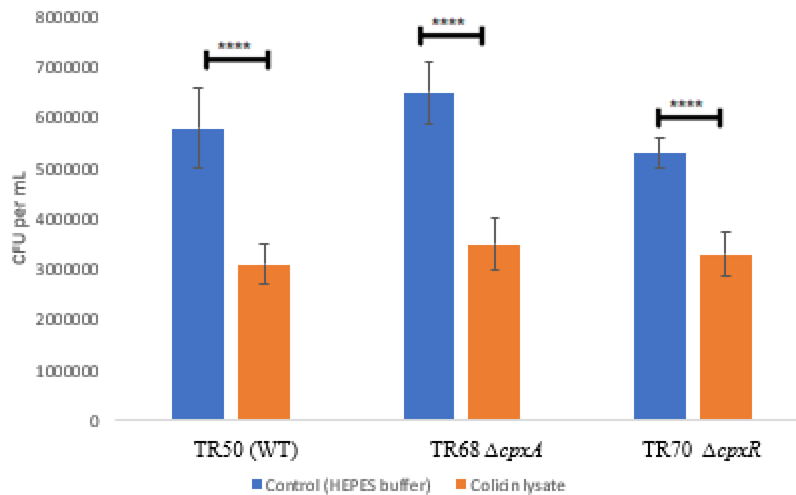


Figure 3.15 Neither an activating mutation (∆*cpxA*) nor repressing (∆*cpxR*) of the Cpx confers resistance to colicin-mediated killing. *E. coli* strain MC4100 and Cpx deletion mutants (*ΔcpxA*, activating mutation and *ΔcpxR,* inactivating mutation) harboring a *cpxP:lacZ* reporter (named TR50, TR68 and TR70 respectively) were grown overnight in 5 mL LB at 37ºC with aeration at 225 rpm. Next day, 1:50 subcultures were made in 5 mL fresh LB, grown at 37ºC with aeration (225 rpm) to mid-log phase (OD 0.5) and then co-incubated with either 10µl colicin stock solution (1:100 dilution, minimum inhibitory concentration) or 10µL control lysate (1:100 dilution from colicin-free lysate) in HEPES buffer (chemical formula: $C_8H_{18}N_2O_4S$) and incubated at 37ºC with aeration for another 2 hours. Cells were then centrifuged and the resuspended in 5 mL fresh LB to remove residual colicins and plated on LB agar plates in serial dilutions in duplicate. Cells were grown overnight at 37ºC statically and next day CFU enumeration was performed. Experiments were performed in triplicate, with three replicates per strain and the results were averaged. Asterisks denote a statistically significant difference from the relevant control (****, P ≤ 0.0001; One-way ANOVA with multiple comparison test).

Chapter 4: Discussion

Chapter 4: Discussion

How different envelope stress responses interact, and the cellular signal that induces them are important areas of investigation for understanding how microbes coordinate discrete adaptations to maximize survival. The results presented here indicate that the Cpx and Rcs envelope stress responses of *E. coli* sense envelope stress signals that are often commonly generated, using independent mechanisms. Our data and others also demonstrate that envelope stress responses are likely coordinated through impacts on lipoprotein trafficking and by proteins and sRNAs that modulate the activity of signaling pathways or downstream targets. We have further shown that two newly identified proteins, TonB and OmpA, play a role in signalling surface adhesion to CpxA. The second portion of the results section of this thesis examined the role of the Cpx in sensing detrimental molecules and appendages. This was a preliminary screen for potential antibacterials that could either bypass the Cpx signalling pathway or still efficiently kill *E. coli* regardless of this stress response defense mechanism.

4.1 Independent induction of the Cpx and Rcs responses by toxic molecules

We show that the Cpx and Rcs envelope stress responses, although activated by many similar cues, sense these signals independently of one another and potentially in different cellular compartments. In all cases we examined here, common inducing cues of both pathways activated the Rcs response through the outer membrane localized lipoprotein RcsF and the Cpx response through the inner membrane sensor kinase CpxA. None of the common Rcs and Cpx envelope stresses we analyzed were sensed by the Cpx response in an NlpE-dependent manner (Figs. 3.2-3.5), in line with the previously published findings of DiGiuseppe and Silhavy (2003). These results strongly suggest that the activating signals generated by common envelope stresses disrupt discrete cellular processes that lead to induction of each response independently, as outer membrane anchored complexes (RcsF:OmpA) are required for all signals to activate the Rcs response, whereas the inner membrane histidine kinase, CpxA, is required to sense all tested signals except surface adhesion.

Signaling through the Rcs pathway is very sensitive to RcsF (Majdalani and Gottesman, 2005) and has been proposed to involve two separate mechanisms. RcsF is tethered to the outer membrane and contains a surface exposed region where it can sense perturbations to LPS, including those generated by polymyxin B treatment (Konovalova et al., 2014; Konovalova et al., 2016). Signaling is proposed to result from a change in the OMP:RcsF complex such that RcsF can extend across the periplasm to interact with the inner membrane localized signaling machinery (Asmar et al., 2017). Alternatively, RcsF can also strongly activate Rcs signaling when it becomes mislocalized to the periplasm by virtue of problems with OMP or lipoprotein trafficking (Cho et al., 2014). EDTA is known to perturb LPS by chelating stabilizing cations and so we consider it likely that this signal is sensed, like polymyxin B, via an RcsF:OMP complex (Fig. 3.8). Whether pH similarly disrupts LPS, or, alternatively, results in defects in OMP assembly or lipoprotein trafficking remains to be determined (Fig. 3.8).

We demonstrate here that NlpE overexpression also induces the Rcs response in an RcsF-dependent manner (Fig. 3.1). Since this induction is still seen in a *cpxR* mutant, albeit to a lesser degree, it seems probable that NlpE overexpression creates an Rcs activating signal, as previously proposed (Cho et al., 2014), by titrating the Lol lipoprotein trafficking machinery and disrupting RcsF localization. Alternatively, or in addition, it is possible that NlpE overexpression generates an additional signal via alteration of the envelope through its effect on the Cpx response. The Cpx response was recently found to be involved in regulating an alternative pathway for lipoprotein trafficking (Grabowicz and Silhavy, 2017), and it will be interesting to determine what this pathway is, and if it could impact the coordinated regulation of the Rcs, Cpx, and possibly other responses. Interestingly, the Cpx and Rcs responses also appear to be connected in some fashion through the cell wall. The Cpx response mediates changes in cell wall cross-linking (Bernal-Cabas et al., 2015; Delhaye and Collet, 2016) and the Rcs response is activated by a variety of cell wall active antibiotics (Laubacher and Ades, 2008). Further, the Rcs response was recently shown to be activated when multiple PBPs are deleted, in a Cpx-dependent manner (Evans et al., 2013). Thus, another potential explanation for our observations could be that activation of the Cpx response by NlpE over-expression leads to cell wall changes

that induce the Rcs pathway in a RcsF-dependent manner. Whatever the mechanism, the results presented here indicate that outer membrane localized lipoproteins with signaling roles can potentially impact the activities of other adaptive responses in multiple ways. Indeed, it may be that events of both types ultimately lead to the high level of Rcs pathway induction observed upon NlpE over-expression.

Cpx activating signals such as a buildup of misfolded pilus proteins PapE and PapG, perturbations to phospholipid composition and changes in proteolysis at the inner membrane are all sensed through the histidine kinase, CpxA (DiGiuseppe and Silhavy, 2003; Kim et al., 2018; Mileykovskaya and Dowhan, 1997; Price and Raivio, 2009). The Cpx response also requires this inner membrane protein to sense the envelope stresses studied here, indicating the signal(s) for activation generated by pH changes, polymyxin B and EDTA is likely generated in the periplasm and/or at the inner membrane (Fig. 3.8). CpxA has a periplasmic sensing domain, however the exact nature of the signal it receives during conditions of stress remains to be determined (Keller et al., 2011; Weber and Silverman, 1988). The periplasmic sensing domain is required to activate the Cpx response through CpxA upon signal generation. Recently it was shown that CpxA is sensitive to the presence of large respiratory protein complexes in the inner membrane (Guest et al., 2017). Given that CAMPs have been shown to alter respiration and displace peripheral membrane proteins (Choi et al., 2017; Wenzel et al., 2014) one intriguing possibility is that CpxA senses damage to membrane proteins and/or defects in their function.

4.2 Unique surface signals are sensed by different OM lipoproteins

The ability of NlpE to induce the Cpx response in bacteria adhered to a polystyrene surface does not extend to the outer membrane lipoprotein RcsF or other members of the Rcs phosphorelay (Fig. S2). Similarly, other stressors (polymyxin B, EDTA, pH) that require RcsF and OmpA for transduction to the Rcs signaling machinery are not sensed by NlpE to activate the Cpx pathway (Figs. 3.3-3.5, Fig. 3.8). Accordingly, it seems that independent signals and sensing mechanisms exist for the detection of unique signals at the outer membrane. While RcsF has

been demonstrated to sense extracellular signals via a surface exposed domain in an RcsF:Omp complex (Konovalova et al., 2014; Konovalova et al., 2016), whether NlpE has a similar domain capable of sensing surfaces is not known. As newly identified proteins for activating the Cpx during adherence to a surface, it is possible that NlpE acts through OmpA or TonB in the periplasm to phosphorylate CpxA (Fig. 3.2). If NlpE can form a complex with OmpA it is an interaction that does not result in the overactivation of the Cpx in the absence of OmpA (whereas the Rcs becomes overactivated by excess RcsF in the periplasm) (Fig. 3.2, S1). It also seems that OmpA and TonB, like NlpE, are not induced by any of the other tested inducing cues in this study. The necessity of these proteins during surface adhesion must therefore generate a specific signal for activation of the Cpx response. Notably, our assay for surface-induced Cpx signaling involves incubating bacteria for several hours in a microtitre plate, and so theoretically it is possible that the Cpx response is receptive to a signal that is generated in adhered bacteria, but *after* adherence, and potentially during subsequent biofilm formation. Furthermore, NlpE-dependent signalling to the Cpx response during adhesion is not observed at 5 hours and 7 hours adherence using this protocol, indicating a specific cellular event during the adhesion process requires activation of the Cpx response. Overall, this leaves open the possibility that the relevant signal occurs within the periplasm, rather than at the cell surface. Future work addressing the topology of NlpE, and these important identified, and potential interacting proteins will shed light on the unique nature of the signals that are sensed by these outer membrane localized lipoproteins.

4.3 The Cpx response represses expression of the RprA sRNA

We provide clear evidence here for an inhibitory role of the Cpx response in expression of the sRNA RprA. Previous work examining Cpx regulation of *rprA* involved the use of the *E. coli* non-pathogenic K-12 strain MC4100 and the enteropathogenic type strain E2348/69 (Vogt et al., 2014). In this study, activation of the Cpx response by NlpE overexpression resulted in activation

of *rprA* expression, while the presence of the *cpxA24* mutation conferring constitutive activation of CpxA resulted in repression of *rprA*. Our results help explain this contradictory finding and indicate that overexpression of NlpE induces *rprA* transcription in a RcsF, OmpA, RcsC, and RcsB-dependent manner (Figs. 3.1, 3.8). Previous examples of genes that are differentially regulated by mutational activation of the Cpx response by the *cpxA24* allele as compared to NlpE overexpression, such as we found in the present study, have been noted (Price and Raivio, 2009). It therefore seems clear that these two commonly used methods for inducing the Cpx response generate different envelope stress signals that are interpreted uniquely and lead to distinct outcomes based on the signaling pathways involved.

Much of our current and previous data indicate that the Cpx response negatively regulates expression of *rprA.* Activation of the Cpx response by removal of CpxA phosphatase activity (Figs. 3.3-3.5) or mutational activation (Vogt et al., 2014) led to inhibition of *rprA* expression, while removal of CpxR resulted in elevated transcription (Figs. 3.1-3.4). None the less, Vogt et al (2014) found that the *cpxA24* allele led to induction of an *rprA-lacZ* reporter gene in *E. coli* K-12 strain MC4100. At present we cannot explain this discrepancy but note that *rprA* is not detectable in this strain unless an *rcsC* mutation is introduced (Raivio et al., 2013; Vogt et al., 2014). Further, the Cpx response was recently proposed to regulate an alternative lipoprotein trafficking pathway in MC4100 (Grabowicz and Silhavy, 2017). Thus, one possibility is that strain differences in MC4100 versus MG1655 (used in this study of Rcs and Cpx response interactions) with regard to lipoprotein trafficking, its Cpx regulation, or both are responsible for the observed differences in regulation of *rprA* transcription.

The mechanism by which CpxR regulates *rprA* expression is not currently known, however both RcsB and CpxR bind upstream of *rprA* (Majdalani et al., 2002; Vogt et al., 2014). It therefore seems likely that CpxR may impede RcsB-activated transcription from the *rprA* promoter. Consistent with this conclusion, it was observed that the addition of CpxR resulted in multiple shifted nucleic acid species in an EMSA (Vogt et al., 2014).

4.4 Coordination of envelope stress responses

The Cpx and Rcs two-component systems sense a number of similar stresses in the cell envelope, however their pathways act independently of one another to transduce an activating signal to the cytoplasm where the response regulators control a number of downstream gene targets (Fig. 3.8). Our data suggest that the coordination of the Rcs and Cpx envelope stress responses through the sRNA RprA plays an important physiological role in harmonizing these responses for optimal adaptation to pH changes. High level RprA expression confers acid resistance (Fig. 3.6, 3.7), which is likely due to its up-regulation of the GadX (Bak et al., 2014) and GadE (Evans, 2015) transcriptional activators of the GDAR response mediated by chaperone activity of Hfq (Henderson et al., 2013). Conversely, the absence of RprA increases survival at alkaline pH (Fig. 3.6, 3.7), perhaps by lowering any basal expression of the GDAR response and preventing export of protons from the cytoplasm. Alternatively, or in addition, deletion of *rprA* could facilitate survival under alkaline conditions because it no longer inhibits the Cpx response. The Rcs response promotes RprA expression at acid pH, and mutants in which the response is activated (ie. *ΔrcsC*) or inhibited (*ΔrcsB*, *ΔrcsF*) exhibit elevated or diminished survival at acid pH respectively (Fig. S3, panel A). Conversely, the Cpx response inhibits RprA expression, and mutants in which this response is activated (*ΔcpxA*) or inhibited (*ΔcpxR*) are noticeably more or less resistant to alkaline pH (Fig. 3.7, panel C; S3, panel C). Cumulatively, these data support a role for Rcs activation and Cpx inhibition of RprA expression in adaptation to both acid and alkaline pH (Fig. 3.8).

In contradiction to this model, RprA expression is activated compared to neutral pH at alkaline pHs (Fig. 3.5B). We propose that RprA expression serves as an additional feedback inhibitory control on the Cpx response. Our data suggest that RprA inhibits CpxR activity in part through the GDAR pathway, since removal of GadA or GadE reduces RprA mediated decreases in Cpx pathway activity (Evans, 2015). It is also known that the GDAR pathway is repressed by the Cpx response (Surmann et al., 2016). Although we do not currently know the mechanism by which the GDAR pathway regulates CpxR, perhaps increased removal of protons from the

cytoplasm by the GDAR pathway (and potentially others) as Cpx-sensed envelope stress is relieved and repression of the GDAR system is alleviated creates a signal that results in diminished CpxR activity, notifying the cell that the alkaline stress has been dealt with. A similar feedback inhibitory model has been proposed for the Cpx-regulated periplasmic chaperone CpxP, which is degraded as long as misfolded envelope proteins are present (Isaac et al., 2005).

The Rcs response facilitates the GDAR response and the removal of protons from the cytoplasm (Evans, 2015; Bak et al., 2014), while the Cpx response down-regulates the production of aerobic electron transport complexes (Guest et al., 2017), which could conceivably result in the accumulation of protons. We hypothesize based on results presented here that the coordination of the Rcs and Cpx envelope stress responses through the sRNA RprA plays an important physiological role in coordinating these opposed responses for optimal adaptation to pH changes (Fig. 3.8). The sigma^{E} and Cpx responses both up-regulate the expression of sRNAs that down-regulate either entire stress responses (Coornaert et al., 2010) or individual members of other stress regulons (Chao and Vogel, 2016; Grabowicz et al., 2016). Our work takes the role of sRNAs in envelope stress response a step further, suggesting a physiological logic for opposing control of the sRNA RprA and delineating a feedback inhibitory role that balances the Cpx and Rcs pathways under conditions where their joint induction is not beneficial.

4.5 The Cpx response is not induced by T4 bacteriophage and type B colicins, and does not confer resistance to type VI secretion systems, T4 bacteriophage nor type B colicins.

The second set of experiments of this study focused on Cpx stress response inducing cues by toxins. Studies of inducing cues by toxins not only help elucidate the cellular signal for activation of the response but identifies potential nonantibiotic antibacterials that could pose as novel therapeutics to battle Gram-negative pathogen infections. With the rise of multi-drug resistant bacteria, exploration of new interventions continue to be explored (Opal, 2016). We show that cell killing mediated by either the T4 bacteriophage, and type B colicins all efficiently kill *E. coli* cells without inducing the Cpx two-component system (Figs 3.11, 3.12, 3.13, 3.14, 3.15).

Activity of the Cpx response during induction by type VI secretion systems is still indeterminate due to the nature of the experimental design (Fig. 3.9). Specifically, a 10:1 predator to prey mixture was used and standardized to the optical density of the mixture (*V. cholerae* and prey *E. coli*) therefore activity of the reporter could be indistinguishable from the control mixture with type VI inactive *V. cholerae,* simply due to the large number of *V. cholerae* compared to *E. coli*. Further experiments to look at this interaction would benefit from a method of examining Cpx gene reporting where optical density would not need to be standardized such as quantitative PCR or looking at a ratio of prey to predator that is significantly higher in the quantity of prey used. To note, overall activity of the Cpx response increases with growth, and later timepoints with higher basal *cpxP-lux* activity can be attributed to this.

Looking at cell survival with wildtype and Cpx deletion strains, neither hyperactivation (∆*cpxA*), nor inactivation (∆*cpxR*) of this stress response was capable of restoring growth significantly greater than wildtype cells in killing assays with *V. cholerae* type VI active strains, nor type B colicins (Figs. 3.10, 3.15). In the T4 phage killing assay a deletion of *cpxR* resulted in slightly greater survival compared to wildtype cells (Fig. 3.12), however the mechanism behind this phenotype is yet to be determined. Potentially, basal activity of the Cpx response resulted in the use of cellular energy that increased either phage uptake or replication of the viral phage genome. Another interesting observation, this time in the type B colicin killing assay, was the reduced survival of the *cpxP* overexpression vector control strain (pCMCP) that had reduced growth compared to all other strains tested (Fig. 3.15). The reduced survival was abolished when *cpxP* was overexpressed on the vector, indicating that the control vector itself may have had an indirect detrimental effect on growth (Fig. 3.15).

Overall, the molecules and complexes tested are efficient at mediating cell killing when the Cpx stress response is functioning normally (as in wildtype cells) or hyperactivated (with *nlpE*-overexpression or a deletion of *cpxA*) indicating activity of the Cpx response is not sufficient to rescue cells from killing (Stratton, 2003). Ultimately, further studies that look at how these toxins affect virulence-associated two-component systems in Gram-negative bacteria will be important

in determining whether they can be modified for clinical use or if they will spur resistance in already multi-drug resistant pathogens.

Appendices

Appendix Figures

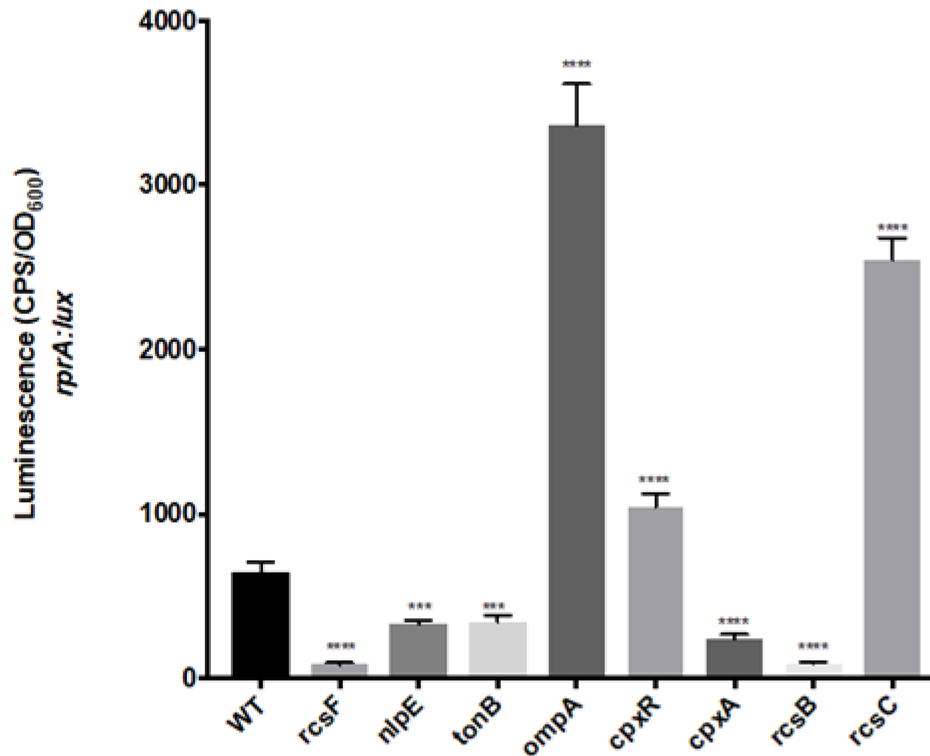


Figure S1. Cpx and Rcs response members can affect downstream transcription of their regulons. Strains bearing *PrprA*-luminescent reporter plasmid and deletions of genes encoding Cpx (*cpxA, nlpE, tonB, ompA, cpxR*) and Rcs signaling proteins (*rcsF, ompA, rcsC, rcsB*) were subcultured 1:50 from 2 mL overnight cultures grown at 37ºC with aeration (225 rpm) into 2 mL fresh LB broth and antibiotics and grown at 37ºC with aeration (225 rpm) for six hours to identify any altered expression of the *PrprA-lux* reporter by single gene mutations. After six hours *PrprA-lux* activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Data for each strain represents the means and standard deviations of five replicate cultures, with the entire experiment repeated three times, and results were averaged. Asterisks denote a statistically significant difference from the wildtype control (****, $P \leq 0.0001$; ***, $P \leq 0.001$; One-way ANOVA with multiple comparison test).

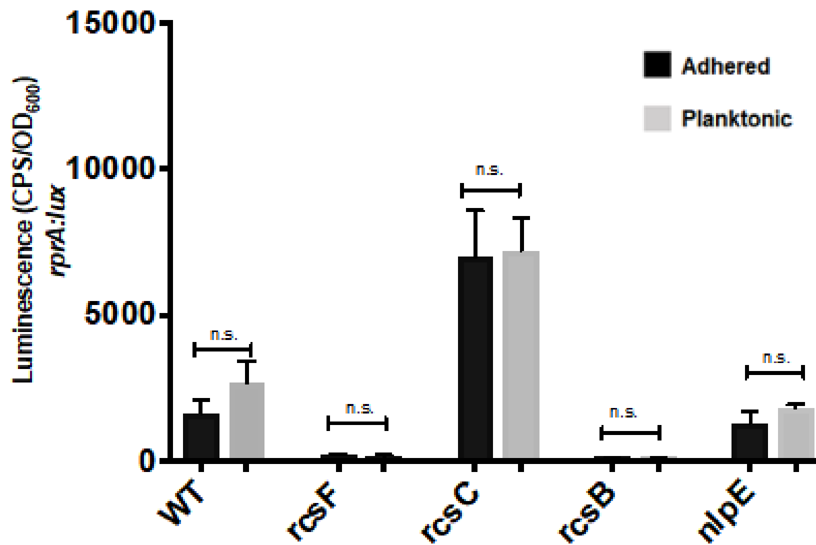


Figure S2. The Rcs is not induced during surface adhesion. Strains bearing *PrprA*-luminescent reporter plasmids and deletions of genes encoding Rcs (*rcsF, rcsC, rcsB*) and Cpx (*nlpE*) signaling proteins were grown overnight statically at 37ºC on LB agar plates (streaked for isolated colonies) with antibiotics. Single colonies were inoculated into 200 µL LB broth with antibiotics in a tissue culture-treated 96-well plate. The 96-well plate was then incubated at 37ºC with rocking at 6 rotations per minute (rpm) for six hours to allow for adherence to wells of the plate. At the six-hour timepoint planktonic cells were pipetted into new wells of a 96-well plate and the adhered cells had 200 µL LB added to their wells. *PrprA-lux* activity was measured for each strain by recording CPS (counts per second) absorbance values and adjusting for cell density measured as optical density (OD_{600}). Importantly, to avoid missing any cells adhered to the sides of the wells, adhered cells were scraped from each well with a pipette tip and resuspended with pipetting and vortexing and optical density readings were taken and used to standardize adhered cell luminescence readings. Data for each strain represents the means and standard deviations of five replicate cultures, and results were averaged. N.S. denote a non-significant difference from the adhered versus planktonic cells (n.s., $P > 0.05$; One-way ANOVA with multiple comparison test).

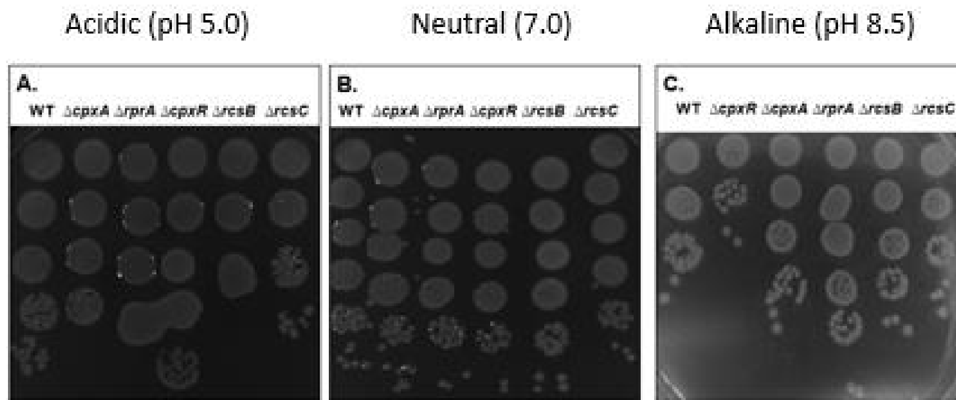


Figure S3. Deletion of *rprA* and *rcsB* increases fitness in alkaline pH; Under acidic conditions a deletion in *rprA*, *cpxA*, *rcsB* or *rcsC* decrease survival. Wildtype and Cpx mutants (*ΔcpxA*, activating mutation and *ΔcpxR* inactivating mutation) were grown in 2 mL LB overnight with aeration (225 rpm) at 37ºC. Overnight cultures were then subcultured into 2 mL fresh LB broth adjusted to A) acidic (5.0), B) neutral (7.0) or C) alkaline (8.5) pH. pH of the LB broth was adjusted by addition of HCl or NaCl added dropwise to the fresh LB broth. Cells were then grown at 37ºC with aeration at 225 rpm for three hours, readjusting the pH every hour by centrifuging tubes at 4,000 rpm for ten minutes and resuspending pelleted cells in new media by vortexing and pipetting. After the three hours cells were serially diluted and plated on LB agar plates and incubated overnight at 37ºC statically. CFUs were imaged on each plate. Experiments were performed in duplicate with three replicates per strain.

References

Ackermann, H.-W., & Prangishvili, D. (2012). Prokaryote viruses studied by electron microscopy. *Archives of Virology*, *157*(10), 1843–1849.

Acosta, N., Pukatzki, S., & Raivio, T. (2015). The *Vibrio cholerae* Cpx envelope stress response senses and mediates adaptation to low iron. *Journal of Bacteriology*, *197*(2), 262–276.

Allen, W., Phan, G., & Waksman, G. (2009). Structural Biology of Periplasmic Chaperones. *Advances in Protein Chemistry and Structural Biology*, *78*, 51–97.

Alteri, C., & Mobley, H. (2012). *Escherichia coli* physiology and metabolism dictates adaptation to diverse host microenvironments. *Current Opinion in Microbiology*, *15*(1), 3–9.

Asmar, A., Ferreira, J., Cohen, E., Cho, S.-H., Beeby, M., Hughes, K., & Collet, J.-F. (2017). Communication across the bacterial cell envelope depends on the size of the periplasm. *PLOS Biology*, *15*(12), 303-318.

Audrain, B., Ferrières, L., Zairi, A., Soubigou, G., Dobson, C., Coppée, J.-Y., … Ghigo, J.-M. (2013). Induction of the Cpx envelope stress pathway contributes to *Escherichia col*i tolerance to antimicrobial peptides. *Applied and Environmental Microbiology*, *79*(24), 7770–7779.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., … Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*, *2*(1), 8-21.

Bak, G., Han, K., Kim, D., & Lee, Y. (2014). Roles of *rpoS*- activating small RNAs in pathways leading to acid resistance of *Escherichia coli*. *Microbiology Open*, *3*(1), 15–28.

Bakelar, J., Buchanan, S., & Noinaj, N. (2016). The structure of the β-barrel assembly machinery complex. *Science (New York, N.Y.)*, *351*(6269), 180–186.

Banin, E., Brady, K., & Greenberg, E. (2006). Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Applied and Environmental Microbiology*, *72*(3), 2064–2069.

Barr, D. (2017). Research Guides: Lab Rats and More: Model Organisms in Research Today: *E.*

coli. [Web] Harvard Library, Harvard University.

Basler, M. (2015). Type VI secretion system: secretion by a contractile nanomachine. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *370*(1679), 834-852.

Bernal-Cabas, M., Ayala, J., & Raivio, T. (2015). The Cpx envelope stress response modifies peptidoglycan cross-linking via the L,D-transpeptidase LdtD and the novel protein YgaU. *Journal of Bacteriology*, *197*(3), 603–614.

Black, L., & Rao, V. (2012). Structure, Assembly, and DNA Packaging of the Bacteriophage T4 Head. *Advances in Virus Research*, *82*, 119–153.

Blount, Z. (2015). The unexhausted potential of *E. coli*. *ELife*, *4*, e05826.

Bolivar, F., Rodriguez, R., Greene, P. , Betlach, M., Heyneker, H., Boyer, H., … Falkow, S. (1977). Construction and characterization of new cloning vehicle. II. A multipurpose cloning system. *Gene*, *2*(2), 95–113.

Bontemps-Gallo, S., Madec, E., & Lacroix, J.-M. (2015). The two-component system CpxAR is essential for virulence in the phytopathogen bacteria *D ickeya dadantii* EC3937. *Environmental Microbiology*, *17*(11), 4415–4428.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC Genomics*, *10*(1), 104-116.

Braun, V., Schaller, K., & Wabl, M. (1974). Isolation, Characterization, and Action of Colicin M. *Antimicrobial agents and Chemotherapy, 5*(5), 520-533.

Bryan, D., El-Shibiny, A., Hobbs, Z., Porter, J., & Kutter, E. (2016). Bacteriophage T4 Infection of Stationary Phase *E. coli*: Life after Log from a Phage Perspective. *Frontiers in Microbiology*, *8*(7), 1391.

Bryan, L., & Van Den Elzen, H. (1977). Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. *Antimicrobial Agents and Chemotherapy*, *12*(2), 163–177.

Bury-Moné, S., Nomane, Y., Reymond, N., Barbet, R., Jacquet, E., Imbeaud, S., … Bouloc, P. (2009). Global Analysis of Extracytoplasmic Stress Signaling in *Escherichia coli*. *PLoS Genetics*, *5*(9), 651-670.

Cao, J., Woodhall, M., Alvarez, J., Cartron, M., & Andrews, S. (2007). EfeUOB (YcdNOB) is a tripartite, acid-induced and CpxAR-regulated, low-pH Fe^{2+} transporter that is cryptic in *Escherichia coli* K-12 but functional in *E. coli* O157:H7. *Molecular Microbiology*, *65*(4), 857–875.

Cascales, E., Buchanan, S., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., … Cavard, D. (2007). Colicin Biology. *Microbiology and Molecular Biology Reviews*, *71*(1), 158–229.

Castanié-Cornet, M.-P., Cam, K., Bastiat, B., Cros, A., Bordes, P., & Gutierrez, C. (2010). Acid stress response in *Escherichia coli*: mechanism of regulation of *gadA* transcription by RcsB and GadE. *Nucleic Acids Research*, *38*(11), 3546–3554.

Castanié-Cornet, M.-P., Cam, K., & Jacq, A. (2006). RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia coli*. *Journal of Bacteriology*, *188*(12), 4264–4270.

Chao, Y., & Vogel, J. (2016). A 3′ UTR-Derived Small RNA Provides the Regulatory Noncoding Arm of the Inner Membrane Stress Response. *Molecular Cell*, *61*(3), 352–363.

Cho, S.-H., Szewczyk, J., Pesavento, C., Zietek, M., Banzhaf, M., Roszczenko, P., … Collet, J.-F. (2014). Detecting Envelope Stress by Monitoring β-Barrel Assembly. *Cell*, *159*(7), 1652–1664.

Choi, H., Yang, Z., & Weisshaar, J. (2017). Oxidative stress induced in *E. coli* by the human antimicrobial peptide LL-37. *PLOS Pathogens*, *13*(6), 81-97.

Clarke, D., Joyce, S., Toutain, C., Jacq, A., & Holland, I. (2002). Genetic analysis of the RcsC sensor kinase from *Escherichia coli* K-12. *Journal of Bacteriology*, *184*(4), 1204–1208.

Clokie, M., Millard, A., Letarov, A., & Heaphy, S. (2011). Phages in nature. *Bacteriophage*, *1*(1), 31–45.

Coornaert, A., Lu, A., Mandin, P., Springer, M., Gottesman, S., & Guillier, M. (2010). MicA sRNA links the PhoP regulon to cell envelope stress. *Molecular Microbiology*, *76*(2), 467–479.

Cosma, C., Danese, P., Carlson, J., Silhavy, T., & Snyder, W. (1995). Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope-associated stresses. *Molecular Microbiology*, *18*(3), 491–505.

Costa, T., Felisberto-Rodrigues, C., Meir, A., Prevost, M., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature Reviews Microbiology*, *13*(6), 343–359.

Danese, P., & Silhavy, T. (1997). The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. *Genes & Development*, *11*(9), 1183–1193.

Danese, P., & Silhavy, T. (1998). CpxP, a stress-combative member of the Cpx regulon. *Journal of Bacteriology*, *180*(4), 831–839.

Danese, P., Snyder, W., Cosma, C., Davis, L., & Silhavy, T. (1995). The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes & Development*, *9*(4), 387–398.

Dartigalongue, C., & Raina, S. (1998). A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. *The EMBO Journal*, *17*(14), 3968–3980.

Das, S., & Chaudhuri, K. (2003). Identification of a unique IAHP (IcmF associated homologous proteins) cluster in *Vibrio cholerae* and other proteobacteria through in silico analysis. *In Silico Biology*, *3*(3), 287–300.

Davies, B., Kohanski, M., Simmons, L., Winkler, J., Collins, J., & Walker, G. (2009). Hydroxyurea Induces Hydroxyl Radical-Mediated Cell Death in *Escherichia coli*. *Molecular Cell*, *36*(5), 845–860.

Davies, J., & Reeves, P. (1975). Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group A. *Journal of Bacteriology*, *123*(1), 102–117.

De Wulf, P., McGuire, A., Liu, X., & Lin, E. (2002). Genome-wide Profiling of Promoter Recognition by the Two-component Response Regulator CpxR-P in *Escherichia coli*.

Journal of Biological Chemistry, *277*(29), 26652–26661.

Debnath, I., Norton, J., Barber, A., Ott, E., Dhakal, B., Kulesus, R., & Mulvey, M. (2013). The Cpx stress response system potentiates the fitness and virulence of uropathogenic *Escherichia coli*. *Infection and Immunity*, *81*(5), 1450–1459.

Delhaye, A., & Collet, J. (2016). Fine-Tuning of the Cpx Envelope Stress Response Is Required for Cell Wall Homeostasis in *Escherichia coli, 7*(1), 1–10.

DiGiuseppe, P., & Silhavy, T. (2003). Signal detection and target gene induction by the CpxRA two-component system. *Journal of Bacteriology*, *185*(8), 2432–2440.

Domínguez-Bernal, G., Pucciarelli, M., Ramos-Morales, F., García-Quintanilla, M., Cano, D., Casadesús, J., & García-del Portillo, F. (2004). Repression of the RcsC-YojN-RcsB phosphorelay by the IgaA protein is a requisite for *Salmonella* virulence. *Molecular Microbiology*, *53*(5), 1437–1449.

Dong, T., Ho, B., Yoder-Himes, D., & Mekalanos, J. (2013). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(7), 2623–2628.

Dorel, C., Vidal, O., Prigent-Combaret, C., Vallet, I., & Lejeune, P. (1999). Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation. *FEMS Microbiology Letters*, *178*(1), 169–175.

Ebel, W., & Trempy, J. (1999). *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions to activate its own expression. *Journal of Bacteriology*, *181*(2), 577–584.

Elizabeth Hall-asheshov, B., & Asheshov, I. (1956). The Induction of the Lytic Cycle in Lysogenic Bacteria by Phagolessin A *58*. *Journal of General Microbiology*, *14*(1), 174-187.

Evans, K., Kannan, S., Li, G., de Pedro, M., & Young, K. (2013). Eliminating a set of four penicillin binding proteins triggers the Rcs phosphorelay and Cpx stress responses in *Escherichia coli*. *Journal of Bacteriology*, *195*(19), 4415–4424.

Farris, C., Sanowar, S., Bader, M., Pfuetzner, R., & Miller, S. (2010). Antimicrobial peptides activate the Rcs regulon through the outer membrane lipoprotein RcsF. *Journal of*

Bacteriology, *192*(19), 4894–4903.

Finnegan, S., & Percival, S. (2015). EDTA: An Antimicrobial and Antibiofilm Agent for Use in Wound Care. *Advances in Wound Care*, *4*(7), 415–421.

Fleischer, R., Heermann, R., Jung, K., & Hunke, S. (2007). Purification, Reconstitution, and Characterization of the CpxRAP Envelope Stress System of *Escherichia coli*. *Journal of Biological Chemistry*, *282*(12), 8583–8593.

Flores-Kim, J., & Darwin, A. (2012). Phage Shock Protein C (PspC) of *Yersinia enterocolitica* Is a Polytopic Membrane Protein with Implications for Regulation of the Psp Stress Response. *Journal of Bacteriology*, *194*(23), 6548–6559.

Fokine, A., & Rossmann, M. (2016). Common Evolutionary Origin of Procapsid Proteases, Phage Tail Tubes, and Tubes of Bacterial Type VI Secretion Systems. *Structure*, *24*(11), 1928–1935.

Franzin, F., & Sircili, M. (2015). Locus of enterocyte effacement: a pathogenicity island involved in the virulence of enteropathogenic and enterohemorragic *Escherichia coli* subjected to a complex network of gene regulation. *BioMed Research International*, *2015*, 1-10.

Gaida, S., Al-Hinai, M., Indurthi, D., Nicolaou, S., & Papoutsakis, E. (2013). Synthetic tolerance: three noncoding small RNAs, DsrA, ArcZ and RprA, acting supra-additively against acid stress. *Nucleic Acids Research*, *41*(18), 8726–8737.

Gerken, H., Charlson, E., Cicirelli, E., Kenney, L., & Misra, R. (2009). MzrA: a novel modulator of the EnvZ/OmpR two-component regulon. *Molecular Microbiology*, *72*(6), 1408–1422.

Gerlach, R., & Hensel, M. (2007). Protein secretion systems and adhesins: The molecular armory of Gram-negative pathogens. *International Journal of Medical Microbiology*, *297*(6), 401–415.

Gervais, F., & Drapeau, G. (1992). Identification, cloning, and characterization of rcsF, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation ftsZ84 in *Escherichia coli* K-12. *Journal of Bacteriology*, *174*(24), 8016–8022.

Glauert, A., & Thornley, M. (1969). The Topography of the Bacterial Cell Wall. *Annual Review of Microbiology*, *23*(1), 159–198.

Goemans, C., Denoncin, K., & Collet, J.-F. (2014). Folding mechanisms of periplasmic proteins. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1843*(8), 1517–1528.

Gottesman, S., Trisler, P., & Torres-Cabassa, A. (1985). Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. *Journal of Bacteriology*, *162*(3), 1111–1119.

Grabowicz, M., Koren, D., & Silhavy, T. (2016). The CpxQ sRNA Negatively Regulates Skp To Prevent Mistargeting of β-Barrel Outer Membrane Proteins into the Cytoplasmic Membrane. *MBio*, *7*(2), 312-316.

Grabowicz, M., & Silhavy, T. (2017). Redefining the essential trafficking pathway for outer membrane lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(18), 4769–4774.

Green, E., & Mecsas, J. (2016). Bacterial Secretion Systems: An Overview. *Microbiology Spectrum*, *4*(1), 1-2.

Guckes, K., Breland, E., Zhang, E., Hanks, S., Gill, N., Algood, H., … Hadjifrangiskou, M. (2017). Signaling by two-component system noncognate partners promotes intrinsic tolerance to polymyxin B in uropathogenic *Escherichia coli*. *Science Signaling*, *10*(461), 23-29.

Guest, R., Wang, J., Wong, J., & Raivio, T. (2017). A Bacterial Stress Response Regulates Respiratory Protein Complexes To Control Envelope Stress Adaptation. *Journal of Bacteriology*, *199*(20), 153-217.

Guo, X.-P., & Sun, Y.-C. (2017). New Insights into the Non-orthodox Two Component Rcs Phosphorelay System. *Frontiers in Microbiology*, *8,* 2014.

Hagan, C., Silhavy, T., & Kahne, D. (2011). β-Barrel Membrane Protein Assembly by the Bam Complex. *Annual Review of Biochemistry*, *80*(1), 189–210.

Hayashi, S., & Wu, H. (1990). Lipoproteins in bacteria. *Journal of Bioenergetics and Biomembranes*, *22*(3), 451–471.

Henderson, C.., Vincent, H., Casamento, A., Stone, C., Phillips, J. , Cary, P., … Callaghan, A. (2013). Hfq binding changes the structure of *Escherichia coli* small noncoding RNAs OxyS and RprA, which are involved in the riboregulation of rpoS. *RNA*, *19*(8), 1089–1104.

Hershey, A. (1952). Reproduction of Bacteriophage. *International Review of Cytology*, *1*, 119–134.

Hirakawa, H., Nishino, K., Yamada, J., Hirata, T., & Yamaguchi, A. (2003). Beta-Lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli. Journal of Antimicrobial Chemotherapy*, *52*(4), 576–582.

Hoang, T. , Karkhoff-Schweizer, R., Kutchma, A., & Schweizer, H. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene*, *212*(1), 77–86.

Hodgkinson, J., Horsley, A., Stabat, D., Simon, M., Johnson, S., da Fonseca, P., … Blocker, A. (2009). Three-dimensional reconstruction of the *Shigella* T3SS transmembrane regions reveals 12-fold symmetry and novel features throughout. *Nature Structural & Molecular Biology*, *16*(5), 477–485.

Howell, A., Dubrac, S., Noone, D., Varughese, K., & Devine, K. (2006). Interactions between the YycFG and PhoPR two-component systems in *Bacillus subtilis*: the PhoR kinase phosphorylates the non-cognate YycF response regulator upon phosphate limitation. *Molecular Microbiology*, *59*(4), 1199–1215.

Hu, L., Chi, B., Kuhn, M., Filippova, E., Walker-Peddakotla, A., Bäsell, K., … Wolfe, A. (2013). Acetylation of the response regulator RcsB controls transcription from a small RNA promoter. *Journal of Bacteriology*, *195*(18), 4174–4186.

Isaac, D., Pinkner, J., Hultgren, S., & Silhavy, T. (2005). The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. *Proceedings of the National Academy of Sciences*, *102*(49), 17775–17779.

Jakes, K., & Finkelstein, A. (2010). The colicin Ia receptor, Cir, is also the translocator for colicin Ia. *Molecular Microbiology*, *75*(3), 567–578.

Joshi, A., Kostiuk, B., Rogers, A., Teschler, J., Pukatzki, S., & Yildiz, F. (2017). Rules of Engagement: The Type VI Secretion System in *Vibrio cholerae*. *Trends in Microbiology*,

25(4), 267–279.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J.-M., Lazzaroni, J.-C., Lejeune, P., & Dorel, C. (2005). CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in Escherichia coli. *Journal of Bacteriology*, *187*(6), 2038–2049.

Keller, R., Havemann, J., & Hunke, S. (2011). Different regulatory regions are located on the sensor domain of CpxA to fine-tune signal transduction. *Research in Microbiology*, *162*(4), 405–409.

Todar, K. (2012). Pathogenic *E. coli. Textbook of Bacteriology*, [Web], p.1-4.

Kim, S., Song, I., Eom, G., & Kim, S. (2018). A Small Periplasmic Protein with a Hydrophobic C-Terminal Residue Enhances DegP Proteolysis as a Suicide Activator. *Journal of Bacteriology*, *200*(3), 519-527.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., & Mori, H. (2006). Complete set of ORF clones of *Escherichia coli* ASKA library (A Complete Set of E. coli K-12 ORF Archive): Unique Resources for Biological Research. *DNA Research*, *12*(5), 291–299.

Klein, A., Shulla, A., Reimann, S., Keating, D., & Wolfe, A. (2007). The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators. *Journal of Bacteriology*, *189*(15), 5574–5581.

Kohanski, M., Dwyer, D., & Collins, J. (2010). How antibiotics kill bacteria: from targets to networks. *Nature Reviews. Microbiology*, *8*(6), 423–435.

Kohanski, M., Dwyer, D., Wierzbowski, J., Cottarel, G., & Collins, J. (2008). Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell*, *135*(4), 679–690.

Konovalova, A., Mitchell, A., & Silhavy, T. (2016). A lipoprotein/β-barrel complex monitors lipopolysaccharide integrity transducing information across the outer membrane. *ELife*, *5*, 1-12.

Konovalova, A., Perlman, D., Cowles, C., & Silhavy, T. (2014). Transmembrane domain of surface-exposed outer membrane lipoprotein RcsF is threaded through the lumen of -barrel

proteins. *Proceedings of the National Academy of Sciences*, *111*(41), E4350–E4358.

Laloux, G., & Collet, J.-F. (2017). Major Tom to Ground Control: How Lipoproteins Communicate Extracytoplasmic Stress to the Decision Center of the Cell. *Journal of Bacteriology*, *199*(21), 216-222.

Landman, D., Georgescu, C., Martin, D., & Quale, J. (2008). Polymyxins revisited. *Clinical Microbiology Reviews*, *21*(3), 449–465.

Laubacher, M., & Ades, S. (2008). The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. *Journal of Bacteriology*, *190*(6), 2065–2074.

Lazdunski, C. J., Bouveret, E., Rigal, A., Journet, L., Lloubès, R., & Bénédetti, H. (1998). Colicin import into *Escherichia coli* cells. *Journal of Bacteriology*, *180*(19), 4993–5002.

Lazzaro, M., Feldman, M., & García Véscovi, E. (2017). A Transcriptional Regulatory Mechanism Finely Tunes the Firing of Type VI Secretion System in Response to Bacterial Enemies. *MBio*, *8*(4), 559-567.

Leive, L. (1965). Release of lipopolysaccharide by EDTA treatment of *E. coli*. *Biochemical and Biophysical Research Communications*, *21*(4), 290–296.

Levengood-Freyermuth, S., Click, E., & Webster, R. (1993). Role of the carboxyl-terminal domain of TolA in protein import and integrity of the outer membrane. *Journal of Bacteriology*, *175*(1), 222–228.

Lin, D., Koskella, B., & Lin, H. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *8*(3), 162–173.

Ma, A., McAuley, S., Pukatzki, S., & Mekalanos, J. (2009). Translocation of a *Vibrio cholerae* Type VI Secretion Effector Requires Bacterial Endocytosis by Host Cells. *Cell Host & Microbe*, *5*(3), 234–243.

Ma, Z., Gong, S., Richard, H., Tucker, D., Conway, T., & Foster, J. (2003). GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in *Escherichia coli* K-12. *Molecular Microbiology*, *49*(5), 1309–1320.

MacIntyre, D., Miyata, S., Kitaoka, M., & Pukatzki, S. (2010). The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences*, *107*(45), 19520–19524.

MacRitchie, D., Buelow, D., Price, N., & Raivio, T. (2008a). Two-Component Signaling and Gram-Negative Envelope Stress Response Systems. *Bacterial Signal Transduction: Networks and Drug Targets*, *631*, 80–110.

MacRitchie, D., Ward, J., Nevesinjac, A., & Raivio, T. (2008b). Activation of the Cpx envelope stress response down-regulates expression of several locus of enterocyte effacement-encoded genes in enteropathogenic *Escherichia coli*. *Infection and Immunity*, *76*(4), 1465–1475.

Mahoney, T., & Silhavy, T. (2013). The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. *Journal of Bacteriology*, *195*(9), 1869–1874.

Majdalani, N., & Gottesman, S. (2005). The Rcs phosphorelay: A Complex Signal Transduction System. *Annual Review of Microbiology*, *59*(1), 379–405.

Majdalani, N., Hernandez, D., & Gottesman, S. (2002). Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Molecular Microbiology*, *46*(3), 813–826.

Mileykovskaya, E., & Dowhan, W. (1997). The Cpx two-component signal transduction pathway is activated in *Escherichia coli* mutant strains lacking phosphatidylethanolamine. *Journal of Bacteriology*, *179*(4), 1029–34.

Miot, M., & Betton, J.-M. (2004). Protein quality control in the bacterial periplasm. *Microbial Cell Factories*, *3*(1), 4-11.

Miyata, S., Kitaoka, M., Brooks, T., McAuley, S., & Pukatzki, S. (2011). *Vibrio cholerae* requires the type VI secretion system virulence factor VasX to kill *Dictyostelium discoideum*. *Infection and Immunity*, *79*(7), 2941–2949.

Nagahama, H., Sakamoto, Y., Matsumoto, K., & Hara, H. (2006). RcsA-dependent and -independent growth defects caused by the activated Rcs phosphorelay system in the *Escherichia coli pgsA* null mutant. *The Journal of General and Applied Microbiology*, *52*(2),

91–98.

Narita, S., & Tokuda, H. (2010). Sorting of Bacterial Lipoproteins to the Outer Membrane by the Lol System. *Methods in molecular biology, 619,* 117–129.

Nevesinjac, A., & Raivio, T. (2005). The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic *Escherichia coli. Journal of Bacteriology*, *187*(2), 672–686.

Noinaj, N., Guillier, M., Barnard, T., & Buchanan, S. (2010). TonB-Dependent Transporters: Regulation, Structure, and Function. *Annual Review of Microbiology*, *64*(1), 43–60.

Ogasawara, H., Teramoto, J., Hirao, K., Yamamoto, K., Ishihama, A., & Utsumi, R. (2004). Negative regulation of DNA repair gene (ung) expression by the CpxR/CpxA two-component system in *Escherichia coli* K-12 and induction of mutations by increased expression of CpxR. *Journal of Bacteriology*, *186*(24), 8317–8325.

Okuda, S., & Tokuda, H. (2009). Model of mouth-to-mouth transfer of bacterial lipoproteins through inner membrane LolC, periplasmic LolA, and outer membrane LolB. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(14), 5877–5882.

Opal, S. (2016). Non-antibiotic treatments for bacterial diseases in an era of progressive antibiotic resistance. *Critical Care*, *20*(1), 397.

Otto, K., & Silhavy, T. (2002). Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(4), 2287–2292.

Pannen, D., Fabisch, M., Gausling, L., & Schnetz, K. (2016). Interaction of the RcsB Response Regulator with Auxiliary Transcription Regulators in *Escherichia coli*. *The Journal of Biological Chemistry*, *291*(5), 2357–2370.

Plate, C., Seely, S., & Laffler, T. (1986). Evidence for a protonmotive force related regulatory system in *Escherichia coli* and its effects on lactose transport. *Biochemistry*, *25*(20), 6127–6132.

Price, N., & Raivio, T. (2009). Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *Journal of Bacteriology*, *191*(6), 1798–1815.

Pukatzki, S., Ma, A., Revel, A., Sturtevant, D., & Mekalanos, J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proceedings of the National Academy of Sciences*, *104*(39), 15508–15513.

Pukatzki, S., Ma, A., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W., … Mekalanos, J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(5), 1528–1533.

Raivio, T. (2014). Everything old is new again: An update on current research on the Cpx envelope stress response. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1843*(8), 1529–1541.

Raivio, T., Leblanc, S., & Price, N. (2013). The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *Journal of Bacteriology*, *195*(12), 2755–2767.

Raivio, T., Popkin, D., & Silhavy, T. (1999). The Cpx envelope stress response is controlled by amplification and feedback inhibition. *Journal of Bacteriology*, *181*(17), 5263–5272.

Raivio, T., & Silhavy, T. (1997). Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *Journal of Bacteriology*, *179*(24), 7724–7733.

Ricci, D., & Silhavy, T. (2012). The Bam machine: A molecular cooper. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1818*(4), 1067–1084.

Riss, T., Moravec, R., Niles, A., Duellman, S., Benink, H., Worzella, T., & Minor, L. (2004). Cell Viability Assays. *Assay Guidance Manual*. [Web]. Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Russell, A., Peterson, S., & Mougous, J. (2014). Type VI secretion system effectors: poisons with a purpose. *Nature Reviews Microbiology*, *12*(2), 137–148.

Schaechter, M. (2009). Outer membrane, Gram-negative bacteria. *Encyclopedia of microbiology*, *2*, 198-209.

Schmöe, K., Rogov, V., Rogova, N., Löhr, F., Güntert, P., Bernhard, F., & Dötsch, V. (2011). Structural Insights into Rcs Phosphotransfer: The Newly Identified RcsD-ABL Domain

Enhances Interaction with the Response Regulator RcsB. *Structure*, *19*(4), 577–587.

Shands, J., & Chun, P. (1980). The dispersion of Gram-negative lipopolysaccharide by deoxycholate. *Journal of Biological Chemistry*, *255*(3), 1221-1226.

Sheahan, K.-L., Cordero, C., & Fullner Satchell, K. (2004). Identification of a domain within the multifunctional *Vibrio cholerae* RTX toxin that covalently cross-links actin. *Proceedings of the National Academy of Sciences*, *101*(26), 9798–9803.

Shiba, Y., Miyagawa, H., Nagahama, H., Matsumoto, K., Kondo, D., Matsuoka, S., … Hara, H. (2012). Exploring the relationship between lipoprotein mislocalization and activation of the Rcs signal transduction system in *Escherichia coli. Microbiology*, *158*(5), 1238–1248.

Shimohata, N., Chiba, S., Saikawa, N., Ito, K., & Akiyama, Y. (2002). The Cpx stress response system of *Escherichia coli* senses plasma membrane proteins and controls HtpX, a membrane protease with a cytosolic active site. *Genes to Cells : Devoted to Molecular & Cellular Mechanisms*, *7*(7), 653–62.

Sikdar, R., Simmons, A., & Doerrler, W. (2013). Multiple Envelope Stress Response Pathways Are Activated in an *Escherichia coli* Strain with Mutations in Two Members of the DedA Membrane Protein Family. *Journal of Bacteriology*, *195*(1), 12–24.

Silhavy, T., Berman, M., Enquist, L., Lynn W. (1984). Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Silhavy, T., Kahne, D., & Walker, S. (2010). The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives in Biology*, *2*(5), 414.

Snyder, D., & McIntosh, T. (2000). The lipopolysaccharide barrier: correlation of antibiotic susceptibility with antibiotic permeability and fluorescent probe binding kinetics. *Biochemistry*, *39*(38), 11777–11787.

Snyder, W., Davis, L., Danese, P., Cosma, C., & Silhavy, T. (1995). Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. *Journal of Bacteriology*, *177*(15), 4216–4223.

South, T., Larsen, R., Jill, A., Scott, Z.-R., & Rogers, O. (2009). Cross-talk between the TonB and TolA energy transduction systems in *Escherichia coli*. *Ohio Link,* 1-70.

Srinivasan, V., & Rajamohan, G. (2013). KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, *57*(9), 4449–4462.

Srinivasan, V., Vaidyanathan, V., Mondal, A., & Rajamohan, G. (2012). Role of the Two Component Signal Transduction System CpxAR in Conferring Cefepime and Chloramphenicol Resistance in *Klebsiella pneumoniae* NTUH-K2044. *PLoS ONE*, *7*(4), 33777.

Stratton, C. (2003). Dead bugs don't mutate: susceptibility issues in the emergence of bacterial resistance. *Emerging Infectious Diseases*, *9*(1), 10–6.

Surmann, K., Ćudić, E., Hammer, E., & Hunke, S. (2016). Molecular and proteome analyses highlight the importance of the Cpx envelope stress system for acid stress and cell wall stability in *Escherichia coli*. *Microbiology Open*, *5*(4), 582–596.

Takeda, S., Fujisawa, Y., Matsubara, M., Aiba, H., & Mizuno, T. (2001). A novel feature of the multistep phosphorelay in *Escherichia col*i: a revised model of the RcsC YojN RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. *Molecular Microbiology*, *40*(2), 440–450.

Taniguchi, N., Matsuyama, S.-I., & Tokuda, H. (2005). Mechanisms underlying energy-independent transfer of lipoproteins from LolA to LolB, which have similar unclosed {beta}-barrel structures. *The Journal of Biological Chemistry*, *280*(41), 34481–34488.

Thede, G., Arthur, D., Edwards, R., Buelow, D., Wong, J., Raivio, T., & Glover, J. (2011). Structure of the periplasmic stress response protein CpxP. *Journal of Bacteriology*, *193*(9), 2149–2157.

Thorbjarnardottir, S., Magnosdottir, R., Eggertsson, G., Kagan, S., & Androsson, O. (1978). Mutations determining generalized resistance to aminoglycoside antibiotics in *Escherichia coli*. *MGG Molecular & General Genetics*, *161*(1), 89–98.

Unden, G., Becker, S., Bongaerts, J., Schirawski, J., & Six, S. (1994). Oxygen regulated gene expression in facultatively anaerobic bacteria. *Antonie van Leeuwenhoek*, *66*(1–3), 3–22.

Vianney, A. (2005). *Escherichia coli tol* and *rcs* genes participate in the complex network affecting curli synthesis. *Microbiology*, *151*(7), 2487–2497.

Vogt, S., Evans, A., Guest, R., & Raivio, T. (2014). The Cpx Envelope Stress Response Regulates and Is Regulated by Small Noncoding RNAs. *Journal of Bacteriology*, *196*(24), 4229–4238.

Vogt, S., & Raivio, T. (2012). Just scratching the surface: an expanding view of the Cpx envelope stress response. *FEMS Microbiology Letters*, *326*(1), 2–11.

Wang, Y. (2002). The Function of OmpA in *Escherichia coli*. *Biochemical and Biophysical Research Communications*, *292*(2), 396–401.

Waterman, S., & Small, P. (2003). Transcriptional Expression of *Escherichia coli* Glutamate-Dependent Acid Resistance Genes *gadA* and *gadBC* in an *hns rpoS* Mutant. *Journal of Bacteriology*, *185*(15), 4644–4647.

Weber, R., & Silverman, P. (1988). The Cpx proteins of *Escherichia coli* K12: Structure of the CpxA polypeptide as an inner membrane component. *Journal of Molecular Biology*, *203*(2), 467–478.

Wenzel, M., Chiriac, A., Otto, A., Zweytick, D., May, C., Schumacher, C., … Bandow, J. (2014). Small cationic antimicrobial peptides delocalize peripheral membrane proteins. *Proceedings of the National Academy of Sciences*, *111*(14), 1409–1418.

Wilson, M., & Bernstein, H. (2016). Surface-Exposed Lipoproteins: An Emerging Secretion Phenomenon in Gram-Negative Bacteria. *Trends in Microbiology*, *24*(3), 198–208.

Wittebole, X., De Roock, S., & Opal, S. (2014). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence*, *5*(1), 226–235.

Yamamoto, K., & Ishihama, A. (2006). Characterization of Copper-Inducible Promoters Regulated by CpxA/CpxR in *Escherichia coli*. *Bioscience, Biotechnology, and Biochemistry*, *70*(7), 1688–1695.

Yu, Z., Qin, W., Lin, J., Fang, S., & Qiu, J. (2015). Antibacterial Mechanisms of Polymyxin and Bacterial Resistance. *BioMed Research International*, *2015*, 1–11.

Zhou, X., Keller, R., Volkmer, R., Krauss, N., Scheerer, P., & Hunke, S. (2011). Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. *The Journal of Biological Chemistry*, *286*(11), 9805–9814.