Regulation of Respiration by the
Cpx Response in Enteropathogenic *Escherichia coli*

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ABSTRACT

Gram-negative bacteria are separated from the environment by a multilayered structure known as the envelope, which is comprised of the outer membrane, the inner membrane and the aqueous periplasmic space that lies between the two membranes. Biogenesis of the envelope is a complex process that requires the coordinated activity of several different processes. To maintain a contiguous barrier, *Escherichia coli* utilize several signal transduction pathways that sense and mediate adaptation to defects in envelope biogenesis or envelope integrity. One such pathway is the Cpx envelope stress response, which mediates adaptation to stresses that affect protein folding within the envelope. To alleviate protein-folding stress, the Cpx response activates the transcription of several envelope-localized chaperones and proteases while also repressing the expression of macromolecular envelope protein complexes. While it is clear that regulation of chaperones and proteases would generate an effective adaptive response to misfolded proteins, the role of multisubunit protein complexes in this process is not yet known. Recent analysis of the Cpx regulon in enteropathogenic *E. coli* suggests that the Cpx response inhibits the expression of several respiratory complexes of the aerobic electron transport chain. The purpose of this thesis was to better understand the role of respiration in the Cpx envelope stress response, with a focus on the primary dehydrogenase NADH dehydrogenase I and terminal oxidase cytochrome bo_3. First, we provide evidence to suggest that the Cpx response regulates expression of these complexes at the transcriptional and post-transcriptional level. Transcription of the genes encoding both complexes is directly repressed by the Cpx response. Furthermore, we found that activation of the Cpx response increases proteolysis of both NADH dehydrogenase I and cytochrome bo_3 in a manner that may involve the zinc metalloprotease FtsH. We also show that regulation of these complexes facilitates adaptation to several stresses that activate the Cpx response. Next, we investigated the

relationship of respiration to the stress that is sensed by the Cpx response. We found that both NADH dehydrogenase I and cytochrome bo_3 contribute to basal levels of Cpx pathway activity, but are not required for activation of the Cpx response by overproduction of the outer-membrane lipoprotein NlpE or through mutation of the inner membrane protease HtpX. However, we found that respiration may play a role in activation of the Cpx response in *E. coli* lacking *tolC*, which encodes the outer membrane channel of multidrug efflux systems. We found that deletion of *tolC* activates the Cpx response through accumulation of the siderophores and that this decreases activity of the NADH-dependent aerobic electron transport chain. We provide evidence to suggest that enterobactin accumulation disrupts heme biosynthesis and that this may disrupt biogenesis of respiratory complexes. Finally, we have found that the Cpx response inhibits expression of genes involved in enterobactin biosynthesis. The results presented in this thesis make a significant contribution to the study of envelope biogenesis in gram-negative bacteria. We believe that activation of the Cpx response reduces expression of envelope-localized macromolecular protein complexes to prevent damage to the inner membrane. These data provide support for the hypothesis that the Cpx response functions to monitor and maintain the integrity of the inner membrane.

PREFACE

Some of the content presented in chapter one has been published as RL Guest and TL Raivio “Role of the gram-negative envelope stress response in the presence of antimicrobial agents” 2016, *Trends in Microbiology*, volume 24, issue 5, pages 377-390, doi: 10.1016/j.tim.2016.03.001. I was responsible for reviewing published literature and manuscript composition. TL Raivio was also responsible for reviewing published literature and manuscript composition.

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Content presented in chapter two has been accepted for publication as RL Guest, J Wang, JL Wong, and TL Raivio “A bacterial stress response regulates expression of respiratory protein complexes to control envelope stress adaptation”, 2017, Journal of Bacteriology volume 199, issue 20, e00152-17, doi: 10.1128/JB.00153-17. I was responsible for collection and analysis of data presented in figures 1, 2, 3, 1-4A, 1-4B, and 1-5. I was also responsible for manuscript composition. J Wang and JL Wong assisted with the collection and analysis of the data presented in figure 1-4C. J Wang also assisted with manuscript composition. TL Raivio was the supervisory author and was involved with concept formation, manuscript composition, and edits.

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Research Complete!

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LIST OF SYMBOLS, NOMENCLATURE, AND ABBREVIATIONS

Symbols

Δ: Deletion of the specified gene locus

:: Interruption of the genetic locus by insertion

Abbreviations

Amk: amikacin

Amp: ampicillin

ATP: adenosine triphosphate

Cam: chloramphenicol

DNA: deoxyribonucleic acid

IM: inner membrane

IPTG: isopropyl-β-D-thiogalactopyranoside

Kan: kanamycin

LPS: lipopolysaccharide

NADH: Nicotinamide adenine dinucleotide

NDH: NADH dehydrogenase

OM: outer membrane

PG: peptidoglycan

PMF: proton motive force

RNA: ribonucleic acid

sRNA: small RNA

Str: streptomycin

VC: vector control

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

General Introduction

1.1 *Escherichia coli*

The gram-negative bacterium *Escherichia coli* is arguably one of the most well characterized model organisms. Outside of the laboratory, *E. coli* live as commensals in the gastrointestinal tract of warm-blooded animals and reptiles (1). Commensal *E. coli* colonize the large intestine and reside within the mucus layer (2). As facultative anaerobes, *E. coli* are able to grow both aerobically using oxygen, anaerobically using an alternative terminal electron acceptor, or by fermentation (2).

Some *E. coli* have evolved the capacity to cause disease. Collectively, these pathogenic *E. coli* are thought to kill more than two million humans per year (3). Pathogenic *E. coli* can be classified into two groups based on the site of infection (4). Intestinal *E. coli* pathogens colonize the small or large intestine and induce diarrhea, while extraintestinal *E. coli* pathogens colonize outside of the gastrointestinal tract and have been associated with urinary tract infections, sepsis, and meningitis. There are at least six intestinal *E. coli* pathotypes (4), including enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC).

EPEC colonize the small intestine and are a major cause of infantile diarrhea in developing countries (5). EPEC colonization progresses through a series of three steps: adherence, signal transduction, and intimate attachment (5). EPEC initially attach to host epithelial cells via the bundle-forming pilus. Bundle-forming pili also attach EPEC cells to each other to create a microcolony in a phenomenon called localized adherence. Next, the type three secretion system secretes the translocated intimin receptor into the host cells. Translocated intimin receptor expressed on the surface of the host cell interacts with intimin on the surface of the EPEC cell to stabilize the interaction between EPEC and the host. Effectors are secreted into the host cell to induce cytoskeletal rearrangements that form the characteristic attaching and effacing lesion associated with EPEC infection. Some of the genetic and biochemical tools used to study commensal *E. coli* can be exploited for the study of EPEC making this a good model organism for the study of diarrheal pathogens.

1.2 The Gram-Negative Bacterial Envelope

Gram-negative bacteria, such as *E. coli*, are defined by the structure of their envelope, which consists of the outer membrane, the inner membrane, and the

intervening periplasmic space that contains the peptidoglycan layer. A brief overview of each layer of the envelope is described in this section (Figure 1-1).

1.2.1 The outer membrane

The outer membrane is found only in gram-negative bacteria and is an asymmetric lipid bilayer consisting of lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet (6). Each LPS molecule is composed of three regions: the lipid A glycolipid, the conserved core polysaccharide, and the variable O-antigen, which is made up of repeating units of oligosaccharide. The unique LPS layer substantially slows the entry of hydrophobic compounds into the cell, and this is thought to occur for two reasons (6). First, lipid A contains only saturated fatty acids under standard laboratory conditions, which increases the rigidity of the LPS layer. Second, neighboring LPS molecules engage in lateral, non-covalent interactions that stabilize the LPS leaflet.

The outer membrane contains several porin proteins that facilitate the entry of molecules into the cell. Porin proteins can be non-specific or substrate specific, and may or may not require energy for transport (7). Energy-dependent transport systems interact with the inner membrane TonB-ExbB-ExbD complex to actively transport substrates into the cell at the expense of the proton motive force (PMF) (7). The outer membrane also contains several protein channels that are involved in the export of substances out of the cell. One such export channel, TolC, is described in greater detail in section 1.5. The outer membrane contains approximately 90% of the lipoproteins found in *E. coli* (8). Such lipoproteins function in a variety of processes including biogenesis of the LPS leaflet, insertion of outer membrane porins, and signal sensing (9-11). Lipoproteins also play a structural role by attaching the outer membrane to the underlying peptidoglycan layer (12).

1.2.2 The periplasm

The periplasm is the aqueous compartment that lies between the inner and outer membranes. Proteins that reside within the periplasm participate in solute and protein transport, detoxification, nutrient degradation, and protein folding (13). It is noteworthy that the periplasm is devoid of an obvious energy source, and the mechanism by which proteins function within this environment is the subject of intense study (8). The periplasm also contains the peptidoglycan layer. This layer is comprised of a series of

interconnected glycopeptides that protect the cell from lysis from the internal turgor pressure, which has been estimated to be approximately 29 kPa (8, 14). The peptidoglycan layer is relatively thin in gram-negative bacteria compared to gram-positive bacteria.

1.2.3 The inner membrane

The inner membrane, which is also referred to as the cytoplasmic membrane, is a phospholipid bilayer that surrounds the cytoplasm. The major phospholipids in the *E. coli* inner membrane are the zwitterionic phospholipid phosphatidylethanolamine and the negatively charged phospholipids phosphatidylglycerol and cardiolipin (8). Phosphatidylethanolamine is the most abundant phospholipid comprising 70-80% of the total lipids, followed by phosphatidylglycerol (15-20%) and cardiolipin (≤5%) (8).

The inner membrane contains the greatest protein diversity in the envelope, and it has been estimated that 20-30% of the *E. coli* proteome is associated with the inner membrane (15, 16). Proteins that reside within the inner membrane perform a variety of functions, including solute transport, electron transport and oxidative phosphorylation, biogenesis of envelope components, signal transduction, toxic waste disposal, and secretion of proteins and metabolites (13). A more detailed description of the *E. coli* electron transport chain can be found in section 1.4. The Sec translocon and the YidC insertase/assembly factor are responsible for inserting proteins into the *E. coli* inner membrane (17, 18). SecY, SecE, and SecG of the Sec translocon comprise the protein-conducting channel that is embedded within the inner membrane, while the SecA ATPase provides the energy required to translocate proteins across the membrane using both ATP and the PMF (17). The accessory proteins SecD, SecF, and YajC optimize the transfer process (17). Proteins destined for the inner membrane are secreted through the Sec translocon as they emerge from the ribosome (17). The transmembrane segments move out of the Sec translocon into the membrane through a lateral gate in SecY (18). The YidC insertase interacts with the Sec translocon complex, and is thought to facilitate insertion of membrane proteins (19). YidC is also able to insert proteins into the inner membrane in a Sec-independent manner (18).

Inner membrane proteins that are not folded or assembled properly are degraded by the inner membrane zinc metalloprotease FtsH (20). The membrane proteins HflC, HflK, and YccA directly interact with FtsH and are thought to modulate its activity (21, 22). FtsH directly interacts with YidC, suggesting that FtsH may degrade inner

membrane proteins that do not insert into the membrane properly (23). FtsH is thought to be redundant with a second zinc metalloprotease, HtpX (24). Several cytoplasmic proteins are also substrates of FtsH (20).

1.2.4 Envelope stress responses

The envelope is the foundation that separates the cell from its environment and *E. coli* survival is dependent on the envelope forming a contiguous barrier. As such, *E. coli* possess several stress responses that monitor and maintain envelope integrity (25). First, the σ^E stress response generates a response to stresses that affect the biogenesis of outer membrane proteins (26). Second, the Bae (bacterial adaptive response) stress response regulates expression of multidrug efflux pumps and promotes resistance to several toxic compounds (27). Third, the Rcs (regulator of capsule biosynthesis) stress response senses several stresses that weaken the envelope and regulates the expression of genes involved in capsule production and biofilm formation (28, 29). Fourth, the Psp (phage shock protein) response facilitates adaptation to stresses that disrupt membrane integrity and dissipate the proton motive force (30). Finally, the Cpx (conjugative plasmid expression) envelope stress response is believed to mediate adaptation to stresses that affect envelope protein folding, and is discussed in greater detail in section 1.3 (Figure 1-2).

1.3 The Cpx Envelope Stress Response

The Cpx response is regulated by a typical two-component signal transduction system consisting of the inner membrane bound sensor CpxA (31-33) and the cytoplasmic response regulator CpxR (34). The first phenotypes associated with mutations at the *cpx* locus were diverse and seemingly unrelated. Mutations mapping to *cpxA* were found to decrease elaboration of the conjugative pilus, for which *cpxA* was named (conjugative plasmid expression) (35). Mutations in *cpxA* were also associated with isoleucine and valine auxotrophy (36, 37), the ability to use L-serine as a source of carbon and nitrogen (38), and altered expression of envelope proteins (39). Other work suggested that CpxA might be involved in a process related to respiration. Like mutations in genes known to affect energy generation, mutations in *cpxA* promoted resistance to low levels of aminoglycoside antibiotics and prevented the use of non-fermentable carbon sources for growth (37, 38, 40, 41). Furthermore, mutations in *cpxA*

were associated with a decrease in the secondary active transport of proline and lactose, but not the primary active transport of glutamine (37, 41-43), suggesting that CpxA may affect the generation or maintenance of the proton motive force. In support of this hypothesis, *cpxA* mutants were resistant to colicin K, which is a pore-forming cytotoxin that dissipates the proton motive force (41, 44). However, mutations in *cpxA* do not alter the proton motive force or affect ATP hydrolase activity (41, 45, 46). Instead, research supported a model in which CpxA functions to balance the activity of secondary active transporters in accordance with the proton motive force (47).

Data gathered in the decades since, however, support a role for CpxA and CpxR in envelope stress resistance. Mutations in *cpxA* that activate the Cpx response suppress the toxicity conferred by a LamB-LacZ-PhoA fusion protein, which forms toxic disulfide-bonded protein aggregates within the periplasm, and a variant of LamB that inappropriately localizes to the inner membrane (48-50). Activation of the Cpx response was found to increase expression of the periplasmic protease/chaperone DegP, which is required for CpxA-mediated resistance to the LamB-LacZ-PhoA fusion protein, but not the LamB variant (50). Additional envelope-localized protein folding and degrading factors are regulated by the Cpx response, including the disulfide bond oxidoreductase DsbA, the peptidyl-prolyl isomerase PpiA, and the chaperones Spy and CpxP (51-54). Together, these results suggest that activation of the Cpx response reduces envelope stress by regulating factors that rid the envelope of toxic proteins. Furthermore, several conditions known or predicted to generate misfolded or mislocalized envelope proteins activate the Cpx response, including overproduction of pilin subunits in the absence of their cognate chaperone (55, 56), alkaline pH (54), expression of a misfolded variant of the periplasmic maltose binding protein MalE (57), and overexpression of the outer-membrane lipoprotein NlpE (58, 59). This work has established the view of the Cpx system as an envelope stress response tasked with monitoring and maintaining protein folding within the envelope.

1.3.1 Signal Transduction

CpxA is comprised of two transmembrane segments separated by a periplasmic sensing domain and a cytoplasmic signal transmitter domain (33). Support for the role of the periplasmic domain in signal sensing came from analysis of a series of *cpxA** mutants that activate the Cpx response (60). Mutations that cluster within the periplasmic domain prevented further activation of the Cpx response by several inducing

signals, while activation was still observed in *E. coli* harboring a mutation within the cytoplasmic domain of CpxA. Thus, it was concluded that the periplasmic domain is essential for signal detection. Furthermore, the periplasmic domain of CpxA contains a PAS (per-arnt-sim) domain (61) (RM Malpica, G. Thede, JNM Glover, TL Raivio, unpublished observation) that is involved in ligand binding in other sensory proteins (62). The molecular nature of the ligand that interacts with CpxA has not yet been identified. Nonetheless, upon sensing an inducing signal, conformational changes in the cytoplasmic transmitter domain stimulate autophosphorylation at a conserved histidine residue within CpxA and subsequent transfer of the phosphate to a conserved aspartate residue within CpxR (60). Once phosphorylated, CpxR functions as a transcription factor that binds to a 5'-GTAAA(N_5)GTAAA-3' DNA sequence to activate or repress transcription (51, 63). In *E. coli*, the CpxR regulon consists of over 100 genes, most of which are associated with inner membrane processes (64). Genes that are most strongly activated by CpxR contain a CpxR-binding site approximately 100 base pairs upstream of the translation start site (65). In the absence of an inducing signal, CpxA functions as a phosphatase to dephosphorylate CpxR, thus ensuring the response is not inappropriately activated (60).

CpxR may also be phosphorylated in a CpxA-independent manner by the high-energy phosphodonor acetyl-phosphate. Acetyl-phosphate is synthesized from acetyl-CoA via the Pta-AckA pathway during the metabolism of acetate (66). Acetyl-phosphate is thought to accumulate and activate the Cpx response in the presence of excess glucose or pyruvate, and during stationary phase (54, 66-69). Notably, accumulation of acetyl-CoA upon inactivation of the Pta-AckA pathway inhibits the expression of Cpx-regulated genes (69, 70). While the reason for this is not fully understood, current evidence suggests that acetylation of the alpha-subunit of RNA polymerase by acetyl-CoA is responsible (69, 70).

1.3.1.1 Accessory signaling proteins

CpxRA signal transduction can be influenced by two auxiliary regulators: the outer membrane lipoprotein NlpE and the periplasmic chaperone CpxP. A role for NlpE in the Cpx response was first observed when overexpression of NlpE suppressed the toxicity of the LamB-LacZ-PhoA fusion protein in a CpxR-dependent manner (58). Likewise, overexpression of NlpE increases the transcription of several genes positively

regulated by CpxR (51), suggesting that NlpE promotes activation of the Cpx response. NlpE is required for sensing surface adhesion (71) and defects in lipoprotein trafficking (72), and communicating these signals to CpxA. The mechanism by which NlpE senses surface adhesion is not well understood, but may involve conformational changes in NlpE within the outer membrane that bring NlpE in proximity to CpxA (73). Conversely, accumulation of NlpE within the inner membrane is believed to activate the Cpx response in lipoprotein trafficking mutants (72). In this regard, overexpression of NlpE variants that remain in the outer-leaflet of the inner membrane activate the Cpx response to a greater extent than overexpression of wildtype NlpE (59, 74). NlpE does not appear to participate in sensing all conditions that activate the Cpx pathway, as misfolded pilin subunits and alkaline pH activate the Cpx response in *E. coli* lacking *nlpE* (67). Nonetheless, it is clear that NlpE conveys signals associated with surface adhesion and lipoprotein trafficking to CpxA.

In contrast to NlpE, overproduction of the periplasmic chaperone CpxP inhibits the Cpx response (75). This function appears to be specific to CpxP, as overexpression of other periplasmic chaperones does not alter Cpx pathway activity (76). While the mechanism by which CpxP inhibits the Cpx response is not clear, it is believed to involve direct interaction between CpxP and the periplasmic sensing domain of CpxA (53, 75, 77, 78). Clues as to how CpxP and CpxA interact have come from structural studies on CpxP. CpxP is a dimer that forms a bowl-shaped structure, with each monomer forming a long, bent, and hooked hairpin (79, 80). The positively charged interior concave surface is predicted to interact with the negatively charged sensory domain of CpxA (80). However, studies in *Vibrio paraheamolyticus* and *E. coli* have failed to identify an interaction between CpxP and the sensing domain of CpxA *in vitro* (61) (RM Malpica, G. Thede, JNM Glover, TL Raivio, unpublished observation). Furthermore, microscale thermophoresis data suggest that CpxP has low affinity for CpxA (81). As such, it is unclear whether CpxP directly interacts with CpxA *in vivo*.

The inhibitory function of CpxP is thought to be relieved upon binding of misfolded proteins to the outer convex surface of CpxP (80). In support of this idea, overproduction of CpxP does not inhibit the Cpx response in the presence of misfolded pilin subunits or alkaline pH (67). Under these conditions, CpxP may function as a chaperone to facilitate degradation of misfolded proteins by DegP (82). Furthermore, CpxP is able to reduce protein aggregation *in vitro* and stabilize misfolded periplasmic fusion proteins *in vivo,* providing further support for its function as a chaperone (80, 83).

It is possible that misfolded proteins titrate CpxP away from CpxA, thus allowing for activation of the Cpx response (67). However, only a slight increase in Cpx pathway activity is observed in *E. coli* lacking *cpxP* (75), and Cpx pathway activity can be further increased in this mutant by alkaline pH, misfolded pilin subunits, and NlpE overexpression (67). Thus, it appears that CpxA does not require CpxP to sense the protein-folding status of the envelope. Instead, CpxP is thought to fine-tune activation of the Cpx response in the presence of misfolded proteins, or alternatively, to promote rapid inhibition of the response once protein folding homeostasis has been achieved (67, 75).

1.3.2 Regulon

Early work proposed a role for the Cpx response in the regulation of envelope-localized chaperones and proteases, supporting the idea that the Cpx response is associated with protein folding at this location. Subsequent studies found that the Cpx response represses the expression of envelope-spanning macromolecular protein complexes, including the flagella, type three secretion system, and several pili. While the reason for this regulation is not clear, it has been proposed that preventing the expression of non-essential envelope proteins may reduce the burden on the protein-folding machinery and/or conserve cellular resources during stress (84). It is now known that the Cpx regulon in *E. coli* includes over 100 genes that are involved in several cellular processes (64, 85). In this section, a description of the classic Cpx regulon is provided and recent developments on this topic are discussed.

1.3.2.1 Envelope protein folding

The most strongly regulated genes by the Cpx response are those encoding envelope-localized chaperones and proteases (65). The chaperone/inhibitor CpxP is transcribed from a divergent promoter located upstream of the *cpxRA* locus, and transcription of *cpxP* is directly activated by CpxR (54, 67). Expression of *cpxP* is almost exclusively dependent on CpxR, and transcription is drastically reduced in *cpxR* mutants (54).CpxR also activates transcription of the CpxP homologue Spy (53, 86). As a putative CpxR binding site has been identified in the *spy* promoter, it is possible that CpxR regulates *spy* transcription directly (87). The mechanism by which Spy facilitates protein folding has been recently described (88). Initial interaction between Spy and its

substrate are guided by the positively charged concave surface in Spy and exposed negative charges in the unfolded protein. This interaction is stabilized by hydrophobic contacts between Spy and the substrate protein. As the substrate protein folds and the hydrophobic residues are buried, the interaction between Spy and the substrate becomes unstable and the folded substrate is eventually released. It remains to be determined whether the chaperone activity of CpxP operates through a similar mechanism.

The chaperone/protease *degP* was the first identified member of the Cpx regulon (50). Subsequent studies have shown that CpxR directly activates transcription of *degP* (51, 63, 68). Transcription of *degP* is reduced in the absence of the Cpx response, suggesting that basal levels of Cpx pathway activity stimulate *degP* expression (68). A stress-protective role for DegP in the Cpx response was first suggested by the finding that increased expression of *degP* in a Cpx-activated mutant reduces the toxicity of the secreted LamB-LacZ-PhoA fusion protein (50). However, it was observed that the Cpx-activated mutant was partially resistant to the LamB-LacZ-PhoA fusion protein in the absence of *degP*, suggesting that other Cpx-regulated factors promote resistance (50). Furthermore, it has been shown that DegP is involved in the degradation of misfolded pilin proteins and that *degP* mutants are sensitive to aminoglycoside antibiotics (82, 89). Accordingly, regulation of *degP* by the Cpx pathway may be an important aspect of the adaptive response generated to mitigate protein-folding stress.

Like *cpxP, spy,* and *degP*, transcription of the oxidoreductase *dsbA* is directly activated by CpxR (51, 52). DsbA promotes the formation of disulfide bonds in the oxidizing environment of the periplasm (90). Electrons from DsbA are transferred to the inner membrane protein DsbB, which subsequently transfers the electrons to the quinone pool of the electron transport chain (90).

The Cpx response also regulates the expression of the peptidyl-prolyl isomerase *ppiA* and the chaperone *ppiD* (63, 91). While *ppiD* was previously classified as a peptidyl-prolyl isomerase, it has been shown that the protein domain involved in peptidyl-prolyl isomerase activity is dispensable for PpiD chaperone function (92). Cumulative evidence suggests that PpiD may be involved in the folding of secreted proteins as they are released from the Sec translocon (92, 93).

Finally, transcription of the inner membrane zinc metalloprotease *htpX* is activated by the Cpx response (24). As described above, HtpX is thought to function as a non-specific protease that is redundant to FtsH (24, 94). Notably, activation of the Cpx

response prevents degradation of the FtsH substrate SecY (95). While the mechanism by which the Cpx response influences activity of FtsH is unknown, it has been shown that CpxR activates the transcription of the FstH regulator YccA (65).

While transcription of several envelope protein folding and degrading factors is activated by the Cpx response, expression of the outer membrane protein chaperone *skp* is repressed (96). A study has shown that outer membrane porin proteins are mislocalized to the inner membrane when Skp is overproduced, which may potentially disrupt the PMF (96). Accordingly, activation of the Cpx response could prevent damage to the inner membrane by decreasing expression of *skp*.

1.3.2.2 Envelope-localized macromolecular protein complexes

The expression of several multiprotein complexes located within the envelope is repressed when the Cpx response is activated, and the molecular mechanism by which this repression occurs can vary. One of the first phenotypes associated with the Cpx response was the reduced transfer of the conjugative F-plasmid, which was attributed to decreased expression of the conjugative pilus (35). Subsequent studies have found that activation of the Cpx response reduces abundance of TraJ, the transcriptional activator of the genes that encode the conjugative pilus (97). The Cpx response does not affect the transcription of *traJ*, rather, proteolysis of TraJ is increased when the Cpx response is activated (98, 99).

Another envelope-spanning multiprotein machine regulated by the Cpx response is the flagellum, which is involved in motility. A role for the Cpx response in the regulation of the flagellum was first identified in a study investigating the extent of the Cpx regulon by screening for genes with a potential CpxR binding site (100). In this study, it was found that CpxR binds to the *motABcheAW* promoter, which encodes structural components of the flagella (MotAB) and regulators of flagellar rotation (CheAW) (100). In support of this finding, activation of the Cpx response reduced swarming motility (100). Intriguingly, swarming motility was strongly increased in the *cpxR* mutant despite little change in transcription of the *motABcheAW* operon in this strain, suggesting that additional factors regulated by the Cpx response influence motility (100). Recent microarray data has found that expression of the flagellar master regulator FlhC is downregulated when the Cpx response is activated by NlpE overexpression (64). The flagellum may also be regulated by the Cpx response at the post-transcriptional level, as swarming motility is not as strongly reduced when the Cpx response is

activated in a *degP* mutant (101). Likewise, several chaperones and proteases that are regulated by the Cpx response influence swarming motility (101). Together these results suggest that activation of the Cpx response decreases expression of the flagella through several mechanisms.

The Cpx response also represses the expression of the curli fimbriae cellular appendages involved in biofilm formation (102). Like other envelope protein structures, regulation of curli expression may occur at both the transcriptional and post-transcriptional level. The Cpx response directly represses the transcription of the *csgAB* operon, which encode the major and minor subunits of curli, respectively (103). Furthermore, CpxR directly represses the expression of *csgD*, which is a transcriptional activator of the *csgAB* operon (104, 105). The Cpx response also directly activates transcription of the small RNA (sRNA) *rprA*, which represses translation of *csgD* (106, 107). Together, these regulatory mechanisms lead to decreased expression of curli when the Cpx response is active. Indeed, Cpx-mediated repression of curli production has been shown to reduce biofilm formation in the presence of envelope stress (108).

The Cpx response also represses expression of several envelope complexes involved in virulence, including the type three secretion system and bundle-forming pilus in enteropathogenic *E. coli*, and the toxin co-regulated pilus in *V. cholerae*. Transcription of the genes encoding the type three secretion system is indirectly repressed by the Cpx response (109). The Cpx response further regulates expression of the type three secretion system at the post transcriptional level, as activation of the Cpx response increases proteolysis of the type three secretion apparatus in a DegP-dependent manner (101).

CpxR also indirectly represses transcription of the *bfp* gene cluster encoding the bundle-forming pilus (110). Intriguingly, EPEC lacking *cpxR* display defects in bundle-forming pilus dependent adherence to tissue culture cells despite no change in transcription of the *bfp* gene cluster in this strain (56, 110). Subsequent studies found that expression of the bundle-forming pilus proteins is decreased in the *cpxR* mutant likely due to decreased expression of *cpxP, degP*, and *dsbA* in this strain (110). Furthermore, functional bundle-forming pili are produced in *E. coli* K-12 strain MC4100 only when the Cpx response is constitutively active (56). Accordingly, it has been concluded that the Cpx response both activates and inhibits the expression of the bundle-forming pilus (110).

It has recently been shown that activation of the Cpx response in *V. cholerae* decreases expression of the toxin co-regulated pilus (111). Like the type three secretion system and bundle-forming pilus in EPEC, regulation of the toxin co-regulated pilus is indirectly mediated by the Cpx response. Regulation of the toxin co-regulated pilus by the Cpx response is partially mediated by cAMP receptor protein (CRP). As activation of the Cpx response does not alter expression of *crp*, the mechanism by which CRP represses expression of the toxin co-regulated pilus when the Cpx response is activated is unclear.

Overall, these studies highlight the many mechanisms by which the Cpx response decreases expression of envelope-localized macromolecular protein complexes. As such, expression of these protein complexes is likely toxic during periods of protein-folding stress.

1.3.2.3 Additional regulon members

While the research presented above highlights a role for the Cpx response in regulating protein folding and the biogenesis of multiprotein complexes within the envelope, it is now known that the Cpx response leads to changes in the expression of genes involved in metal homeostasis, peptidoglycan modification, solute transport, and respiration (64, 85, 112). As the majority of the processes affected by the Cpx response are associated with the inner membrane, it has been proposed that the Cpx response is attuned to the status of inner membrane proteins (64, 112).

The Cpx regulon in *V. cholerae* is enriched for genes involved in iron metabolism and acquisition (113). Expression of genes involved in heme utilization, biosynthesis and transport of the siderophore vibriobactin, ferrichrome transport, and iron storage is increased when the Cpx response has been activated. Likewise, the Cpx response regulates the expression of several proteins with iron-containing cofactors. Intriguingly, the Cpx response is required for adaptation to low iron conditions when growing on non-fermentable carbon sources, suggesting that the Cpx response facilitates adaptation to respiratory-dependent stress produced during iron deprivation (113). Proteins involved in iron metabolism and acquisition are also regulated by the Cpx response in *E. coli*. The *E. coli* Cpx response directly activates the transcription of the ferritin-like protein FtnB, which is involved in iron storage and detoxification (65, 87, 114). Transcription of the *efeUOB* operon, which encodes an iron transporter, is repressed by the Cpx response

(65). In addition to iron, the Cpx response has been associated with copper and zinc. The finding that the *cpxRA* mutant is hypersensitive to copper suggests that members of the Cpx regulon are involved in adapting to the stress imposed by excess copper (87). However, the Cpx regulon members involved in this process have not yet been identified. Our group has shown that mutations affecting Cpx pathway activity also alter resistance to zinc (JL Wong and TL Raivio, unpublished observation).

The Cpx response regulates several genes that encode enzymes involved in peptidoglycan modification, including the amidases AmiA and AmiC, the L,D-transpeptidase LdtD, and the transglycosylase Slt70 (115, 116). Likewise, activation of the Cpx response modifies the composition of the peptidoglycan layer, alters cell morphology, and disrupts cell division (74, 116). Furthermore, the Cpx response promotes resistance to antibiotics that inhibit cell division or inactivate enzymes involved in peptidoglycan biogenesis (74). Together, these results suggest that the Cpx response modifies the peptidoglycan layer in response to envelope stress.

The Cpx regulon in *E. coli* is enriched for genes encoding inner membrane transporters, most of which are downregulated (64, 85). Several carbohydrate transporters are repressed by the Cpx response. CpxR directly represses the transcription of *glpT* and *uhpT*, which encode transporters of glycerol-3-phosphate and glucose-6-phosphate, respectively (117). The Cpx response also represses expression of transporters involved in uptake of C4-dicarboxylic acids, galactose, glucarate, and maltose (64). Furthermore, the Cpx response regulates expression of the sodium/proton antiporters NhaB and ChaA (64). Regulation of NhaB by the Cpx response has been proposed to maintain the PMF during periods of envelope stress (118). Finally, several peptide transporters are regulated by the Cpx response. Expression of the peptide transporters TppB and DppC is downregulated by the Cpx pathway, while expression of SbmA is activated (64, 85). In regards to the outer membrane, the Cpx response regulates the expression of the non-specific porins OmpC and OmpF, and the maltose-specific porin LamB (64, 119). Overall, these results suggest that the Cpx response is intimately associated with transport across the inner membrane.

Finally, preliminary microarray data suggest that the Cpx response represses the expression of several complexes of the aerobic electron transport chain, including NADH dehydrogenase I (NDH-I), succinate dehydrogenase, cytochrome bo_3, and cytochrome *bd*-I (64). Expression of anaerobic respiratory complexes was not altered by the Cpx response under the growth conditions used for the collection of the microarray data (64).

These data are reminiscent of the first phenotypes associated with mutations at the Cpx locus that linked the Cpx response to respiration.

1.3.2.4 Communication with other regulatory pathways

To facilitate a highly adaptive response, the Cpx pathway stimulates a network of regulatory pathways (84, 120). The connector protein MzrA functions to link the EnvZ-OmpR and CpxA-CpxR two-component systems (121). The EnvZ-OmpR two-component system functions to sense changes in environmental osmolarity and respond by regulating the expression of certain outer membrane porins (122). Upon Cpx pathway activation, CpxR directly upregulates the expression of MzrA (121). MzrA, a small 127 amino acid inner membrane protein, then directly interacts with the histidine kinase EnvZ and stimulates the EnvZ-OmpR regulon (121, 123). Regulatory connections between the CpxRA and EnvZ-OmpR two-component systems allow the Cpx response to regulate additional outer membrane proteins during stress, which may prevent the influx of stressors and facilitate adaptation.

The Cpx response represses transcription of the *rpoErseABC* operon encoding the σ^E envelope stress response (65). Some of the genes upregulated by the Cpx pathway are downregulated upon σ^E induction (84). Thus, repression of the *rpoErseABC* operon may increase expression of these genes under Cpx inducing conditions (65). Alternatively, it is possible that some members of the σ^E regulon have adverse effects on adaptation to inner membrane stress (65, 84).

1.4 The Electron Transport Chain of *Escherichia coli*

The electron transport chain, or respiratory chain, facilitates the transfer of electrons released during the catabolism of carbon to a terminal electron acceptor. The energy released during this process may be conserved through the generation of an electrochemical gradient known as the proton motive force (PMF), which can be used to drive ATP synthesis, the transport of nutrients, and flagellar rotation. The electron transport chain is comprised of primary dehydrogenases and terminal oxidases/reductases that are connected by lipid electron carriers known as quinones. *E. coli* possess at least fifteen primary dehydrogenases and ten terminal oxidases/reductases that are assembled into the electron transport chain according to the terminal electron acceptor that is available (124). The terminal electron acceptor also

influences the ratio of the three quinones that are present within the *E. coli* electron transport chain, which are ubiquinone, menaquinone, or demethylmenaquinone (124). *E. coli* are able to respire aerobically or anaerobically using a wide range of terminal electron acceptors, including oxygen, nitrate, fumarate, dimethylsulfoxide, or trimethylamine N-oxide (124). The major respiratory complexes present in the *E. coli* aerobic electron transport chain are NADH dehydrogenase I (NDH-I), NADH dehydrogenase II (NDH-II), succinate dehydrogenase, cytochrome bo_3, cytochrome *bd-I*, and cytochrome *bd-II* (Figure 1-3) (124).

The NADH dehydrogenase isozymes catalyze the transfer of electrons from NADH to ubiquinone or menaquinone. NDH-I is a large multiprotein membrane complex that couples the oxidation of NADH to the translocation of protons across the inner membrane, while NDH-II is a single polypeptide with a mass of 47kDa and is not involved in proton translocation (125, 126). A more detailed description of NDH-I is provided in section 1.4.1. Expression of NDH-I and NDH-II is attuned to the metabolic needs of the cell (124, 127, 128). NDH-I is preferentially expressed under energy limiting conditions, as this enzyme is able to conserve energy by generating a PMF. However, the NADH oxidase activity of NDH-I may be inhibited if the PMF is too great (129). Under these conditions, NDH-II functions to maintain the $NADH/NAD^+$ ratio.

Succinate dehydrogenase is a membrane-bound enzyme of the tricarboxylic acid cycle that catalyzes the oxidation of succinate and the subsequent transfer of electrons to ubiquinone. Succinate dehydrogenase is comprised of four subunits that are organized into two domains (130). Subunits SdhA and SdhB comprise the cytoplasmic catalytic domain that contains the flavin adenine dinucleotide and iron-sulfur (Fe-S) cluster cofactors involved in electron transport. SdhC and SdhD make up the membrane-anchor domain that contains the ubiquinone binding site and the heme *b* cofactor that is also involved in electron transport.

Cytochrome bo_3, cytochrome *bd*-I, and cytochrome *bd*-II are the three terminal oxidases produced by *E. coli* during aerobic growth. These enzymes catalyze the transfer of electrons from reduced ubiquinone (ubiquinol) to oxygen. Cytochrome bo_3 is the main terminal oxidase expressed under high oxygen tensions owing to its low affinity for oxygen (131). The reduction of oxygen by cytochrome bo_3 is coupled to the net transfer of two protons across the membrane (132). A detailed description of cytochrome bo_3 is provided in section 1.4.2. Cytochrome *bd*-I and cytochrome *bd*-II are expressed under low oxygen tensions and at least cytochrome *bd*-I has a higher affinity for oxygen

than cytochrome *bo*$_3$ (131). Each enzyme contributes to the proton motive force through the net transfer of one proton for each electron used to reduce oxygen (131, 133).

1.4.1 NADH dehydrogenase I

NDH-I couples the transfer of two electrons from NADH to quinone to the translocation of four protons across the inner membrane. The NDH-I complex is transcribed from the *nuo* (NADH:ubiquinone oxidoreductase) operon, which consists of fourteen genes named *nuoA-N* (134). In *E. coli*, genes *nuoC* and *nuoD* are fused and encode a single NuoCD subunit (135). NDH-I is one of the largest protein complexes in the *E. coli* inner membrane with a molecular mass of approximately 550kDa (136, 137). The subunits of NDH-I are organized into two perpendicular arms: a hydrophobic membrane arm that is embedded within the inner membrane and a peripheral arm that protrudes into the cytoplasm (138-141). The peripheral arm is involved in electron transfer while the membrane arm participates in the translocation of protons. Bacterial NDH-I is structurally and functionally similar to complex I in higher organisms, and represents the core-structure required for enzyme function (142).

The peripheral arm is made up of the subunits NuoB-G and NuoI and contains the flavin mononucleotide (FMN) and iron-sulfur (Fe-S) cluster cofactors that are involved in electron transfer (141, 143-145). The two electrons released upon the oxidation of NADH are transferred as a hydride ion to the FMN cofactor within the NuoF subunit. These electrons are then transferred to quinone through a chain of seven Fe-S clusters. A single electron from FMN is transferred to the Fe-S cluster N3, which is also located within the NuoF subunit. The electron is then transferred to N1b, N4, then N5 within NuoG, N6a then N6b within NuoI, and finally to N2 within NuoB. Additional Fe-S clusters are found in NDH-I that do not directly participate in electron transfer to quinone. The conserved N1a Fe-S cluster may function as an antioxidant (146). After a single electron has been transferred to N3 from FMN, the second electron may be temporarily transferred to N1a to prevent the formation of reactive oxygen species by the flavosemiquinone intermediate. However, it was recently shown that cluster N1a is important for complex stability, and its role as an antioxidant has been debated (147). The NuoG subunit contains a fourth Fe-S cluster, however its location suggests that it is not involved in electron transfer (146). Instead, this cofactor is thought to play a role in complex stability (148).

Quinone binds within a hydrophilic pocket located at the intersection between the peripheral arm and the membrane arm of NDH-I (140, 145, 149). Conformational changes that occur upon reduction of quinone, and to a lesser extent the Fe-S cluster N2, are thought to drive conformational changes in the membrane arm of NDH-I that stimulate proton translocation (140, 145). In *E. coli*, the membrane arm is comprised of the subunits NuoA, NuoH, and NuoJ-N that together form 64 transmembrane helices (149, 150). Subunits NuoL, NuoM, and NuoN are homologous to monovalent cation/proton antiporters, and likely pump a single proton each (140, 151, 152). The fourth proton channel may be formed by subunits NuoH, NuoJ, and NuoK (140). The NuoL subunit contains an amphipathic helix that runs parallel to the membrane and spans across NuoL, NuoM, and NuoN (140, 149, 150). This helix is thought to help coordinate the movement of the proton channels. Alternatively, or in addition, it may function as a clamp that holds the transporter subunits together.

Phylogenetic analysis suggests that NDH-I evolved by the fusion of preexisting protein complexes involved in electron transport and proton translocation (151, 153-158). NuoE, NuoF, and NuoG comprise the NADH dehydrogenase module that is homologous to NAD^+ reducing hydrogenases and [FeFe] hydogenases. Subunits NuoB, NuoCD, NuoH, NuoI, and possibly NuoN, form the amphipathic hydrogenase/connecting module that shares homology to membranous [NiFe] hydrogenases. Finally, subunits NuoL, NuoM, and NuoK form the transporter module. It is believed that these modules represent assembly intermediates of the NDH-I complex. In support of this hypothesis, NDH-I splits into three parts in the presence of salt or the detergent triton X-100 (137). Assembly of the NADH dehydrogenase module is enhanced by iron, riboflavin, and sodium sulfide (135), suggesting that the incorporation of the FMN and Fe-S cluster cofactors may promote the correct folding of NuoE, NuoF, and NuoG (156). Furthermore, the NADH dehydrogenase module is formed by overexpression of *nuoEFG* only if *nuoBCD* are also overexpressed (135), suggesting that NuoB and NuoCD may assist in the assembly of the dehydrogenase fragment. The NADH dehydrogenase fragment is enriched in the cytoplasm of the *nuoH, nuoI, nuoJ, nuoK, nuoM* and *nuoN* single mutants (159, 160), suggesting that the membrane subunits of NDH-I are required for the dehydrogenase fragment to be anchored into the complex (156). An overall mechanism of NDH-I assembly has been proposed (156). NuoA is thought to be the scaffold on which the other subunits assemble within the inner membrane. NuoB and NuoCD interact with NuoA, which then allows for the assembly of the NADH

dehydrogenase module within the cytoplasm and its incorporation into the complex. The addition of NuoH and NuoI strengthens the interaction of the subunits of the peripheral arm. Finally, the subunits of the membrane arm are associated with the complex. It is thought that NuoL, M, and N form a subcomplex that is assembled *en bloc.* NuoJ and NuoK may strengthen the interaction between the membrane arm and the peripheral arm.

Little is known about the chaperones that assist in the assembly of the NDH-I complex. Of those that are identified, most are involved in the biogenesis of Fe-S clusters (156). NfuA, which is thought to play a role in Fe-S cluster biogenesis under stress conditions, directly interacts with NuoG (161, 162). NuoG also interacts with the chaperone YajL, which functions under oxidative stress conditions to prevent protein aggregation (163, 164). As YajiL interacts with several proteins that contain Fe-S clusters, It has been proposed that YajL maintains NuoG in an assembly competent state during insertion of Fe-S clusters (156, 163). CyaY, which is homologous to eukaryotic frataxin and may be involved in the biogenesis or repair of Fe-S clusters, has also been associated with NDH-I (165-169). Homologs of CyaY have been co-purified with the *Thermus thermophilus* NDH-I complex, and in *E. coli* deletion of *cyaY* decreases activity of NDH-I (168, 170). The MoxR ATPase chaperone RavA and its accessory protein ViaA interact with NuoA and NuoF under aerobic conditions, and NuoCD under anaerobic conditions (171). Microarray data have shown that deletion of *ravA* or of the *ravA-viaA* operon affects transcription of several genes associated with the assembly of Fe-S clusters, suggesting that interaction with the subunits of the NDH-I complex may be related to Fe-S cluster biogenesis (171). In support of this hypothesis, RavA and ViaA interact with a third protein, CadA, which is a lysine decarboxylase involved in acid resistance (172). CadA interacts with a variant form of the NDH-I complex formed in the absence of *nuoL* that lacks Fe-S cluster N2 (160). Accordingly, it has been proposed that the RavA-ViaA-CadA complex may facilitate the insertion Fe-S clusters in NDH-I (156). At least one NDH-I assembly factor that is not involved in Fe-S cluster biogenesis has been identified – the inner membrane insertase/assembly factor YidC. YidC is required for insertion of NuoK into the inner membrane (173). Intriguingly, YidC was not required for insertion of a NuoK variant in which glutamate residues within the transmembrane segments were replaced with lysine residues, suggesting that YidC may be required for the integration of proteins with less hydrophobic, more negatively charged transmembrane segments into the inner membrane (173). As glutamate

residues are present within the transmembrane segments of NuoA, it is possible that YidC is also required for insertion of NuoA (173).

1.4.2 Cytochrome *bo₃*

The terminal oxidase cytochrome *bo₃* catalyzes the transfer of four electrons from reduced ubiquinone (ubiquinol) to oxygen and uses the energy released during this reaction to translocate protons across the membrane. It is a member of the heme-copper oxidase superfamily that also includes cytochrome *c* oxidase (complex IV) of the electron transport chain in higher organisms (174). The cytochrome *bo₃* complex is composed of four subunits that are transcribed from the *cyoA-E* (cytochrome o) operon (175). *cyoA-D* encode the structural subunits, while *cyoE* encodes a heme O synthase that catalyzes the synthesis of heme *o* from heme *b* (175-178). Subunit I (CyoB) contains the heme *b*, heme o_3, and Cu_B cofactors involved in electron transport, whereas subunit II (CyoA), subunit III (CyoC), and subunit IV (CyoD) are thought to play structural roles and/or facilitate the insertion of cofactors into subunit I (CyoB) (176, 179, 180). In contrast to NDH-I, all of the subunits of cytochrome *bo₃* are embedded within the inner membrane (176). Subunit II (CyoA) contains an additional extrinsic domain that protrudes into the periplasm (176).

The mechanism of electron transfer through cytochrome *bo₃* is debated. Early evidence supported the existence of two ubiquinone binding sites within cytochrome *bo₃*, one with low affinity for ubiquinone (Q_L) and one with high affinity for ubiquinone (Q_H) (181). The proposed Q_L site is located within subunit II (CyoA), and it is thought that ubiquinone bound at the Q_L site readily exchanges with the quinone pool (181-184). The Q_H site is located within subunit I (CyoB) and in contrast to the Q_L site, ubiquinone is bound tightly within the Q_H site and either slowly exchanges or does not exchange at all with the quinone pool (182, 183). The existence of the two ubiquinone binding sites within cytochrome *bo₃* is thought to stabilize the semiquinone intermediate formed during electron transfer (185). Ubiquinol bound at the Q_L site transfers two electrons to ubiquinone bound at the Q_H site (181, 184, 186). Electrons are then transferred one at a time from the ubiquinol bound at the Q_H site to heme *b* within subunit I (CyoB), which results in the temporary formation of the semiquinone intermediate (186). Reduced heme *b* transfers one electron to the heme o_3/Cu_B binuclear center, which then transfers the electron to oxygen (131, 187). Accordingly, the ubiquinone bound at the Q_H site is proposed to function as an additional cofactor involved in electron transfer (131). While

genetic and biochemical studies have located the putative Q_H site, the location of the Q_L site has not yet been identified. Structural studies have shown that the proposed Q_L site is not located within the transmembrane helices, and as such is unlikely to bind ubiquinol (176). Several studies have attempted to identify the Q_L binding site but have had little success (183, 188, 189). Intriguingly, a recent study has proposed that the Q_L and the Q_H site may be the same (188). Nonetheless, research supports a model were electrons from ubiquinol bound at the Q_H site are transferred one at a time to heme *b*, which subsequently transfers the electrons to the heme o_3/Cu_B binuclear center. Electrons at the binuclear center are then used to reduce oxygen.

The reduction of oxygen into water by cytochrome *bo₃* results in the net transfer of eight protons across the inner membrane (131). Each electron released by ubiquinol results in the transfer of two protons (131-133), one of which is transferred by vectorial chemistry, and the other which is directly pumped from the cytoplasm to the periplasm. Vectorial, or scalar, protons are generated by reactions that release and consume protons on either side of the membrane. For cytochrome *bo₃*, oxidation of ubiquinol releases protons into the periplasm and the reduction of oxygen consumes protons within the cytoplasm, which results in a net transfer of a single proton for each electron transferred. As four electrons are required to reduce dioxygen, four protons will be transferred via vectorial chemistry (131). The mechanism by which cytochrome *bo₃* directly pumps protons is less clear. Cytochrome *bo₃* contains two half channels that are involved in transport of protons from the cytoplasm to the binuclear center, however the exit channel has yet to be identified (176). One proton is pumped across the membrane for each electron transferred to oxygen (131).

Assembly of the cytochrome *bo₃* complex proceeds in a sequential process that begins with the insertion of subunit III (CyoC) and IV (CyoD) into the inner membrane, followed by insertion of apo-subunit I (CyoB) (190). It is thought that interaction of apo-subunit I (CyoB) with the subunit III/IV (CyoC/CyoD) complex stimulates the insertion of the heme cofactors (179). Finally, subunit II (CyoA) is added (190). The mechanism by which subunit II (CyoA) is inserted into the membrane was reported by several independent studies (191-194). CyoA is composed of two domains: a N-terminal transmembrane domain and a C-terminal extrinsic domain. The N-terminal transmembrane domain is inserted into the membrane in a Sec-independent manner that requires the insertase/assembly factor YidC. After insertion of the N-terminal

domain, the C-terminal extrinsic domain is secreted into the periplasm via the Sec translocon.

1.5 The Outer Membrane Channel TolC

TolC was originally identified in a genetic screen for mutants that became tolerant to one or more colicins, which are peptide antibiotics produced by some strains of *E. coli* (195). In addition to colicin resistance, *tolC* mutants were hypersensitive to a variety of dyes, detergents, and antibiotics, as well as to the bile salt deoxycholate (195, 196). As such, it was believed that TolC was involved in the structure and function of the bacterial envelope. The finding that TolC is a minor outer membrane protein supported this conclusion (197). Several years later, a role for TolC in the secretion of the cytotoxin α-hemolysin was proposed (198). At the time, at least two other members of the α-hemolysin secretion apparatus were known – the inner membrane transporter HlyB and its associated transport protein HlyD (199-202). As α-hemolysin is secreted into the extracellular environment directly from the cytoplasm with no periplasmic intermediate (203), TolC was hypothesized to directly interact with HlyB and HlyD to secrete α-hemolysin across the outer membrane (198). Indeed, subsequent biochemical studies provided support for this hypothesis (204, 205). In an effort to better understand the role of TolC in α-hemolysin secretion, the crystal structure of TolC was solved (205). This structure revealed two domains within TolC – a larger domain that is inserted into the lipid bilayer and forms a pore through which substrates may traverse the outer membrane, and a smaller domain that protrudes into the periplasmic space. Accordingly, it was concluded that TolC constitutes the outer membrane channel of the α-hemolysin secretion system. HlyD was proposed to function as a membrane fusion protein that associates the inner membrane transporter HlyB with the outer membrane channel TolC to form a contiguous channel for α-hemolysin secretion that spans the entire bacterial envelope (204, 206). Additional studies suggest that TolC performs a similar function in the secretion of colicin V (207).

While these studies have identified a role for TolC in the secretion of cytotoxic proteins, they do not explain the original hypersensitivity phenotypes associated with *tolC* mutants. It is important to note that α-hemolysin and colicin V are unstable in the absence of TolC (198, 207), indicating that the hypersensitivity phenotypes cannot be the result of improper secretion of cytotoxic proteins. As the outer membrane presents a

significant permeability barrier, it had long been hypothesized that multidrug efflux transporters must associate with an outer membrane channel to efficiently extrude antibiotics into the external environment (208, 209). In this regard, HlyD is related to several accessory proteins that function in conjunction with inner membrane transporters to export a wide range of antimicrobials (206). Together, this work suggested that TolC might function as the outer membrane channel in multidrug efflux systems (208, 209). Indeed, the hypersensitivity phenotype of *E. coli* lacking *tolC* is not exacerbated by deletion of genes encoding the AcrAB multidrug efflux system, suggesting that these proteins act through a common mechanism to generate antimicrobial resistance (210). Subsequent studies revealed that TolC directly interacts with the AcrAB complex to form a protein channel that extrudes compounds from the periplasmic side of the inner membrane (211-219). From this work arose a model in which the outer membrane channel TolC interacts with an inner membrane transporter and its cognate membrane fusion protein to form a tripartite protein complex that enables the direct transport of various antimicrobials and peptides from the cytoplasm or periplasm into the external environment (diagramed in Figure 1-4). To date, at least nine TolC-dependent tripartite multidrug efflux systems have been identified in *E. coli* (220).

Not all efflux transporters that require TolC are thought to interact with TolC directly. Some efflux systems function as single-component pumps within the inner membrane to transport compounds from the cytoplasm into the periplasm. These “singlet” efflux systems then require a tripartite multidrug efflux system to transport the periplasmic compounds across the outer membrane (diagramed in Figure 1-4). An example of this type of efflux system is EntS, which transports the siderophore enterobactin across the inner membrane into the periplasmic space (221, 222). Once in the periplasm, enterobactin is exported into the extracellular environment through the AcrAB-TolC, AcrAD-TolC, and MdtABC-TolC tripartite multidrug efflux systems (222, 223) (see section 1.5.1 for further details).

Due to the imminent threat of clinical antibiotic resistance, studies have mainly focused on the role of TolC and TolC-dependent efflux systems in this aspect. However, accumulating evidence suggests that antibiotic efflux may not be the primary intended function of TolC-dependent efflux systems. Efflux mutants display pleiotropic phenotypes in the absence of antibiotics, suggesting that multidrug efflux systems might function in other aspects of cellular physiology. These include host colonization and virulence, biofilm formation, cell communication, oxidative and nitrosative stress resistance, toxic

waste disposal, and envelope maintenance (224-226). The roles of TolC and TolC-dependent efflux systems in toxic waste disposal and the envelope stress response are discussed in further detail below.

1.5.1 Toxic waste disposal

TolC and TolC-dependent efflux systems participate in the export of a variety of metabolites, including enterobactin, cysteine, intermediates of heme biosynthesis, and indole. A role for multidrug efflux systems in secretion of siderophores was first proposed in the early 1990's when it was found that the MexAB-OprM multidrug efflux system in *P. aeruginosa* is required for secretion of the catechol siderophore pyoverdine during iron starvation (227, 228). Subsequent studies in *E. coli* determined that TolC functions to secrete the siderophore enterobactin, evidenced by a decrease in extracellular levels of enterobactin in the *tolC* mutant (222). Furthermore, extracellular enterobactin levels were decreased in the *acrB acrD* and *mdtBC* triple mutant to the same extent as in the *tolC* single mutant (223). Deletion of additional TolC-dependent transporter genes in the *acrB acrD mdtBC* triple mutant did not substantially alter the amount of extracellular enterobactin (223), leading to the conclusion that the AcrAB-TolC, AcrAD-TolC, and MdtABC-TolC multidrug efflux systems participate in enterobactin secretion. In the absence of TolC, toxic accumulation of periplasmic enterobactin leads to growth and cell division defects, and has been proposed to disrupt respiration (229). TolC is responsible for secretion of several intermediates of heme biosynthesis, including coproporphyrin(ogen) and protoporphyrin IX. *tolC* mutants are hypersensitive to exogenous 5-aminolevulinic acid, a precursor in heme biosynthesis, due to increased periplasmic levels of coproporphyrin(ogen) (230). Furthermore, the intracellular level of protoporphyrin IX is increased in the *tolC* mutant when YfeX, a cytoplasmic dye-decoloring peroxidase that generates protoporphyrin IX (231), is overproduced (232). Protoporphyrin IX accumulates in *E. coli* lacking *macAB* (232), suggesting that the MacAB-TolC efflux pump is responsible for protoporphyrin secretion. However, *tolC* mutants accumulate more protoporphyrin IX than the *macAB* mutant, indicating that other TolC-dependent transporters participate in protoporphyrin IX export (232). *E. coli tolC* mutants are hypersensitive to exogenous cysteine and overexpression of *tolC* increased the extracellular concentration of cysteine compared to the control (233). The same study further found that *tolC* mutants are hypersensitive to the reducing agent dithiothreitol, suggesting that *tolC* may function to protect *E. coli* from reductants or may

participate in redox homeostasis. Finally, indole, a metabolite of tryptophan degradation, is exported out of *E. coli* via the AcrEF-TolC multidrug efflux system. *E. coli* lacking *acrEF* are hypersensitive to exogenous indole and have altered intra- and extracellular levels of indole compared to the wildtype (234).

Additional metabolites are likely secreted via TolC, evidenced by an increase in *acrAB* transcription in *E. coli* mutants that accumulate specific metabolites. Mutations in genes that block several metabolic pathways increase resistance to the antibiotic nalidixic acid, including *icdA*, which encodes isocitrate dehydrogenase, *cysH* of cysteine biosynthesis, *metE* of methionine biosynthesis, and *purB* of purine biosynthesis (235). Accumulation of metabolites synthesized before the block in the biochemical pathway stimulate transcription of *acrAB*, providing an explanation for increased resistance to nalidixic acid in these mutants (235). Furthermore, *acrAB* transcription is stimulated in *E. coli* lacking *acrA, acrB*, or *tolC* in a compensatory effort to restore homeostasis (236). Mutation of genes involved in gluconeogenesis or enterobactin, cysteine, or purine biosynthesis prevented *acrAB* induction in the *acrB* mutant (236). Intriguingly, the accumulated metabolites are structurally similar to antibiotics that are exported by multidrug efflux pumps, suggesting that export of antibiotics may be incidental to metabolite secretion (228, 235).

How do metabolites stimulate transcription of *acrAB*? Several studies have shown that bacterial stress responses that regulate *acrAB* expression are activated in these mutants (235-237), suggesting that accumulation of metabolites may disrupt cellular integrity. The envelope appears to be particularly susceptible, as several envelope stress responses are activated in efflux mutants (see section 1.5.2). Accordingly, it has been proposed that TolC and TolC-dependent efflux pumps function to extrude endogenously produced metabolites that may become toxic at high levels (235, 237).

1.5.2 Envelope stress response

Impaired efflux activates multiple envelope stress responses, suggesting that endogenously produced metabolites that are normally secreted by efflux pumps may compromise envelope integrity. Furthermore, envelope stress responses have been implicated in the positive regulation of efflux pump expression in several gram-negative bacteria. Together, this work suggests that multidrug efflux systems play an integral role

in combating envelope stress.

1.5.2.1 The CpxRA response

The first hint that inhibition of efflux may activate the Cpx response was in 2009 when Slamti and Waldor found that a transposon inserted into *tolC* increased the expression of a *cpxP-lacZ* fusion in *Vibrio cholerae* (238). This finding was confirmed in two independent studies investigating the role of the Cpx response in *V. cholerae* (239, 240). It has since been shown that mutations in *tolC* activate the Cpx response in multiple organisms, including *Escherichia coli* and *Sinorhizobium meliloti* (241, 242). The reason why mutation of *tolC* activates the Cpx response is unclear. It is possible that the absence of TolC may destabilize many TolC-dependent transporters, generating a protein-misfolding signal. However, when *tolC* was deleted in a strain lacking all nine *tolC*-dependent transporters in *E. coli*, Cpx activation was unchanged from the *tolC* single mutant (241). Instead, it is likely that accumulation of endogenous metabolites that are normally excreted through *tolC* accumulate in the cell when *tolC* is mutated. Mutations in four of the RND efflux transporters in *E. coli*, *acrB*, *acrD*, *mdtC,* and *mdtF*, activate a *spy-lacZ* reporter gene in a CpxA-dependent fashion to the same extent as the mutation of *tolC* (241). This occurs only when all four transporters are inactive, as a single mutation in each transporter had only a moderate effect on Cpx pathway activity (241). Likewise, inactivation of all six RND efflux transporters in *V. cholerae* induces the Cpx response. This was attributed to the inactivation of only two transport systems, VexRAB and VexGH, in this strain (240). As multiple RND efflux pumps must be inactivated to induce the Cpx response, it is possible that these transporters have redundant activities and can efflux the same metabolites. Finally, it has been shown that mutation of the membrane fusion protein *mtrC* activates the Cpx response in *Haemophilus ducreyi* (243). In the absence of MtrC, the RND efflux transporter MtrD may remain active, but not associate with MtrE, a homologue of *E. coli* TolC. This would then lead to an accumulation of toxic metabolites in the periplasm, subsequently inducing envelope stress and activation of the Cpx response (243).

The identity of the toxic metabolite or metabolites that accumulate and induce the Cpx response in the absence of RND efflux pumps remains unknown. So far, three metabolites have been ruled out – indole, acetate, and ethanol (241). Interestingly, addition of iron reduces Cpx activation in a *tolC*, *vexB*, or *vexH* mutant in *V. cholerae*

(239). Multiple iron-chelating metabolites are predicted to accumulate in the absence of TolC, including porphyrins, citrate, and enterobactin (222, 232, 235, 236). Incidentally, accumulation of iron-free enterobactin in the periplasm is predicted to disrupt multiple processes associated with the Cpx response including motility, cell wall biogenesis and respiration (229).

Activation of the Cpx stress response in *E. coli* confers resistance to β-lactam, floroquinolone, and aminoglycoside antibiotics as well as to deoxycholate, copper, indole, and the cationic antimicrobial peptide protamine as a result of increased expression of *tolC* and the multidrug efflux pumps *mdtABC* and *acrD* (244-246). Increased efflux is only partially responsible for the observed resistance to aminoglycosides, as resistance is not completely abolished in a *tolC* mutant. This result suggests that additional factors regulated by the Cpx stress response are important for aminoglycoside resistance. Notably, resistance to β-lactam and fluoroquinolone antibiotics by increased expression of *mdtABC* and *acrD* is only observed in the absence of the major efflux pump AcrB (244). It is possible that the Cpx response plays a role in resistance to these antibiotics only under certain conditions or when the AcrAB-TolC efflux system is overwhelmed. Upregulation is achieved through a regulatory network that includes the Cpx, Bae, and Mar stress responses. Hirakawa and colleagues have shown that CpxR in conjunction with the response regulator BaeR bind directly to the promoters of *acrD* and *mdtABC* to increase their expression (246). Conversely, activation of *tolC* expression appears to occur indirectly through the Mar response. The *mar* operon consists of three genes, the repressor *marR*, the activator *marA*, and *marB*. In the absence of stress, MarR binds to the *mar* promoter to keep the response off. Under inducing conditions, toxic molecules bind directly to MarR and prevent interaction with the *mar* promoter. Toxic molecules that inhibit MarR activity, including indole and copper, can also induce the Cpx response (114, 245). Yang and colleagues have proposed a model where indole inhibits MarR activity, allowing CpxR to bind directly to the *mar* promoter and activate MarA expression. MarA then increases the transcription of genes involved in resistance to a variety of antimicrobial agents, including *tolC* and *acrAB* (245).

Regulation of multidrug efflux systems by the Cpx stress response has been implicated in antimicrobial resistance in pathogenic organisms such as *Vibrio cholerae* and *Klebsiella pneumoniae*. While the Cpx response in *V. cholerae* is not required for intrinsic resistance to antimicrobial compounds, activation enhances resistance to

ampicillin, bile salts, and other detergent-like compounds (240) via increased expression of *tolC*, *vexAB,* and *vexGH* (113, 240). Expression of the multidrug efflux pumps *acrB, acrD, eefB,* and *kpnEF* in the highly drug resistant pathogen *K. pneumoniae* is reduced in a mutant lacking *cpxR* and *cpxA* (247, 248). This mutant is sensitive to bile, the disinfectant chlorhexidine, and various antibiotics. Overall, these findings suggest that regulation of multidrug efflux pumps, and the consequent resistance to various antimicrobial compounds, by the Cpx envelope stress response is conserved in distantly related γ-proteobacteria.

1.5.2.2 The AmgRS response

The AmgRS two-component system in *P. aeruginosa*, which is functionally analogous to the Cpx envelope stress response in *E. coli*, is activated upon exposure to aminoglycoside antibiotics and contributes to intrinsic aminoglycoside resistance (249, 250). AmgRS is believed to mitigate damage caused by insertion of aminoglycoside-generated mistranslated peptides into the inner membrane by increasing the expression of several proteases and proteins that regulate protease activity (251). Recently, it was shown that AmgRS activates transcription of the *mexXY* genes of the MexXY-OprM multidrug efflux system (252). While MexXY was not shown to affect intrinsic aminoglycoside resistance in *P. aeruginosa*, the increase in aminoglycoside resistance upon activation of the AmgRS response was dependent on MexXY (252). Intriguingly, aminoglycoside induced expression of *mexXY* required AmgRS regulated proteases (252), suggesting that the MexXY-OprM efflux system may be associated with protein turnover (225).

1.5.2.3 The BaeSR response

The BaeSR response is controlled by a typical two-component signal transduction system consisting of the sensor BaeS and the response regulator BaeR (253). While little is known about the inducing cue that is sensed by the BaeSR envelope stress response, activation mediates resistance to various toxic molecules through increased expression of *tolC* and associated multidrug efflux pumps. In fact, the BaeSR stress response is encoded in an operon with the multidrug efflux pump *mdtABC* in many bacterial species (254, 255). Activation of the Bae response has been shown to increase resistance to metals such as tungstate, nickel, and zinc as well as to plant

tannins and flavonoids, detergents such as deoxycholate and SDS, gallic acid, procaine, alexidine, aminopyridine, caffeine, iodonitro tetrazolium violet, thioglycerol, and multiple antibiotics (256-259). The role of MDR efflux pumps in resistance to some of these compounds has yet to be determined. However, as the BaeR regulon is primarily composed of efflux pumps (85), it is likely that activation of the Bae response reduces the intracellular concentration of these compounds to sub-lethal levels. Regulation of multidrug efflux pumps by the Bae stress response has been observed in *E. coli* (85, 255, 257, 260), *Erwinia amylovora* (254), *Acinetobacter baumannii* (261, 262), and *Salmonella enterica* serovar Typhimurium (258).

Like the Cpx response, the Bae stress response is activated in *E. coli* efflux mutants. This is evident by a Cpx and Bae dependent increase in *spy* expression in *E. coli* lacking *tolC* (241). While the nature of the Bae inducing signal in the *tolC* mutant has yet to be determined, it has been shown that indole, acetate, and ethanol are not responsible for increased expression of *spy* in the *tolC* mutant (241). Deletion of the *tolC* homologue *abuO* in *A. baumannii* results in a 14.51 and 4.67 fold increase in expression of *baeS* and *baeR,* respectively (263). Given the small size of the Bae regulon, it is possible that the Bae stress response is activated during efflux challenge to provide additional multidrug efflux systems that facilitate export of toxic substances.

1.5.2.4 The phage shock response

The phage shock response is involved in maintenance of the proton motive force in response to stresses that perturb the inner membrane (25). High levels of PspA, a component of the phage shock response, have been found in the membrane of *E. coli tolC* mutants grown to stationary phase in minimal medium (264). No increase in PspA protein was observed in *E. coli tolC* mutants during exponential phase (264). Accordingly, this suggests that the proton motive force may be dissipated in *tolC* mutants after extended periods of growth.

1.5.2.5 The σ^E response

The σ^E envelope stress response plays an important role in mediating adaptation to stresses that perturb the outer membrane (25). Expression of the peptide antibiotic transporter *sbmA* is increased in the absence of *tolC* in *E. coli* (265). Overproduction of *sbmA* under these conditions is due to increased activity of the σ^E stress response.

Interestingly, σ^E activity is decreased in *Sinorhizobium meliloti* lacking *tolC* (242). At present the reason for this discrepancy is unclear. Inhibition of the constitutively expressed SmeIJK efflux system in *Stenotrophomonas maltophilia* also activates the σ^E stress response (266). Likewise, activation of the *S. maltopholia* σ^E response increases transcription of *smeIJK* (266). AlgU, the σ^E homologue in *P. aeruginosa*, increases expression of the MexCD-OprJ multidrug efflux system in response to membrane-damaging biocides (267). Notably, increased levels of extracellular fatty acids were observed in a mutant overexpressing the MexCD-OprJ efflux system (268), suggesting that fatty acids may be a substrate for this efflux pump. As such, the MexCD-OprJ may play a role in combating stress by modifying the lipid content of the outer membrane (225).

1.6 Thesis objectives

While the Cpx envelope stress response is known to play a role in adaptation to stresses that are thought to cause protein misfolding, the molecular details of the adaptive response that is generated upon activation of the Cpx response remain to be determined. The aim of this thesis was to examine the association between the Cpx envelope stress response and the electron transport chain. The specific objectives were:

1. Regulation of the respiratory complexes NDH-I and cytochrome bo_3 by the Cpx response:
    a. To confirm the results of preliminary microarray data suggesting that the Cpx response represses transcription of the genes encoding NDH-I and cytochrome bo_3.
    b. To determine the mechanism by which the Cpx response mediates transcriptional repression of the genes encoding NDH-I and cytochrome bo_3.
    c. To determine whether the Cpx response regulates expression of NDH-I and cytochrome bo_3 at the post-transcriptional level.
    d. To determine whether regulation of NDH-I and cytochrome bo_3 facilitates adaptation to Cpx-dependent envelope stress.
2. Contribution of respiration to Cpx pathway activation
    a. To determine whether activity or expression of NDH-I and cytochrome bo_3 contribute to Cpx pathway activity in the presence or absence of stress.
3. Effects of the Δ*tolC* mutation on Cpx pathway activity

a. To identify the metabolite(s) that accumulates and activates the Cpx response.
b. To determine whether respiration contributes to activation of the Cpx response in the Δ*tolC* mutant.
c. To investigate the adaptive response generated by the Cpx response in the Δ*tolC* mutant.

1.7 Figures

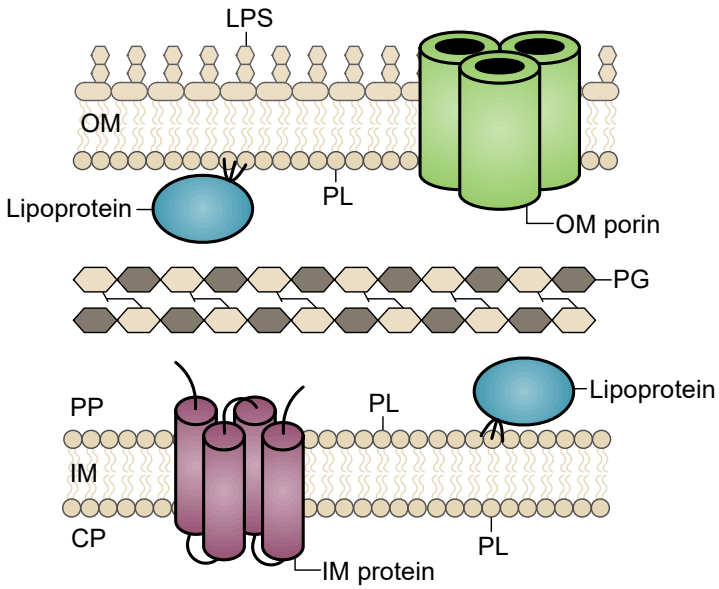

Figure 1-1. The gram-negative bacterial envelope.

The envelope in gram negative bacteria consists of the outer membrane (OM), the inner membrane (IM), and the periplasm (PP). The peptidoglycan (PG) layer is found within the periplasm. The outer membrane is an asymmetric lipid bilayer consisting of lipopolysaccharide (LPS) in the outer leaflet and phospholipids (PL) in the inner leaflet. The inner membrane is a phospholipid bilayer. Outer membrane porins and lipoproteins are found within the outer membrane. Integral inner membrane proteins with alpha helical domains and lipoproteins are found within the inner membrane. CP, cytoplasm. Figure adapted from (8).

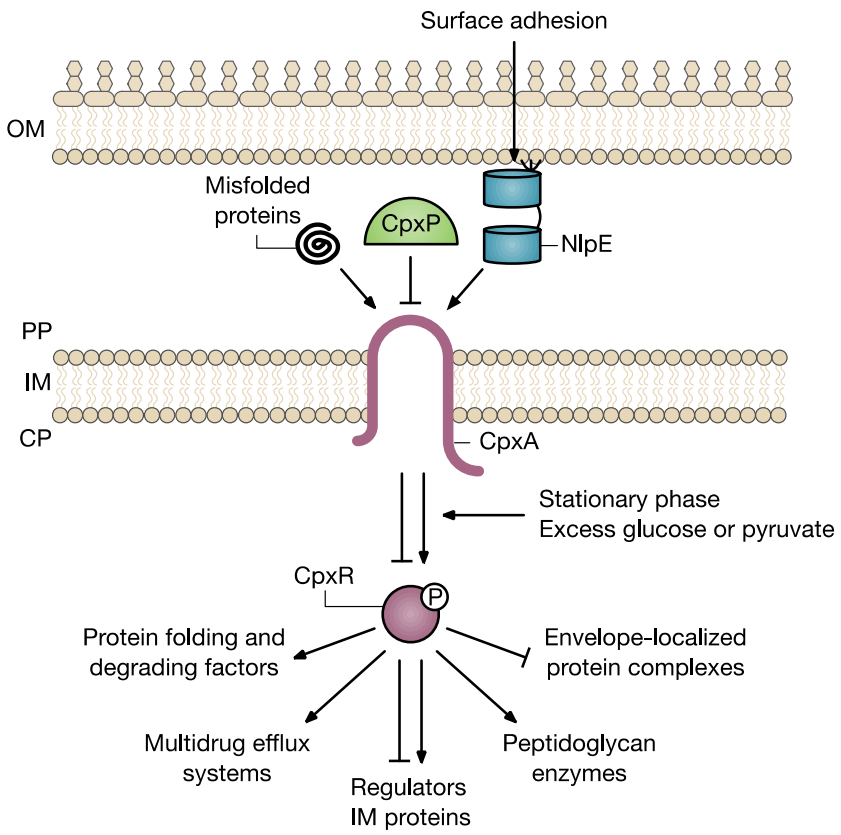

Figure 1-2 The Cpx envelope stress response.

The Cpx envelope stress response is comprised of the sensor kinase CpxA and the response regulator CpxR. In the presence of conditions that are thought to generate misfolded envelope proteins, CpxA functions as a kinase that first autophosphorylates using ATP as the phosphate donor and then transfers the phosphate to CpxR. Phosphorylated CpxR modulates the transcription of genes that encode envelope localized protein folding and degrading factors, multidrug efflux systems, regulators and inner membrane proteins, peptidoglycan modification enzymes and envelope-localized protein complexes. The auxiliary regulators CpxP and NlpE can modulate activity of CpxA. NlpE is thought to sense surface adhesion. CpxR can be phosphorylated by acetyl-phosphate and this is thought to contribute to Cpx pathway activation during stationary phase and in the presence of excess glucose of pyruvate. In the absence of stress, CpxA functions as a phosphatase to dephosphorylate CpxR. OM, outer membrane; PP, periplasm; IM, inner membrane; CP cytoplasm; P, phosphate. Figure adapted from (27, 84).

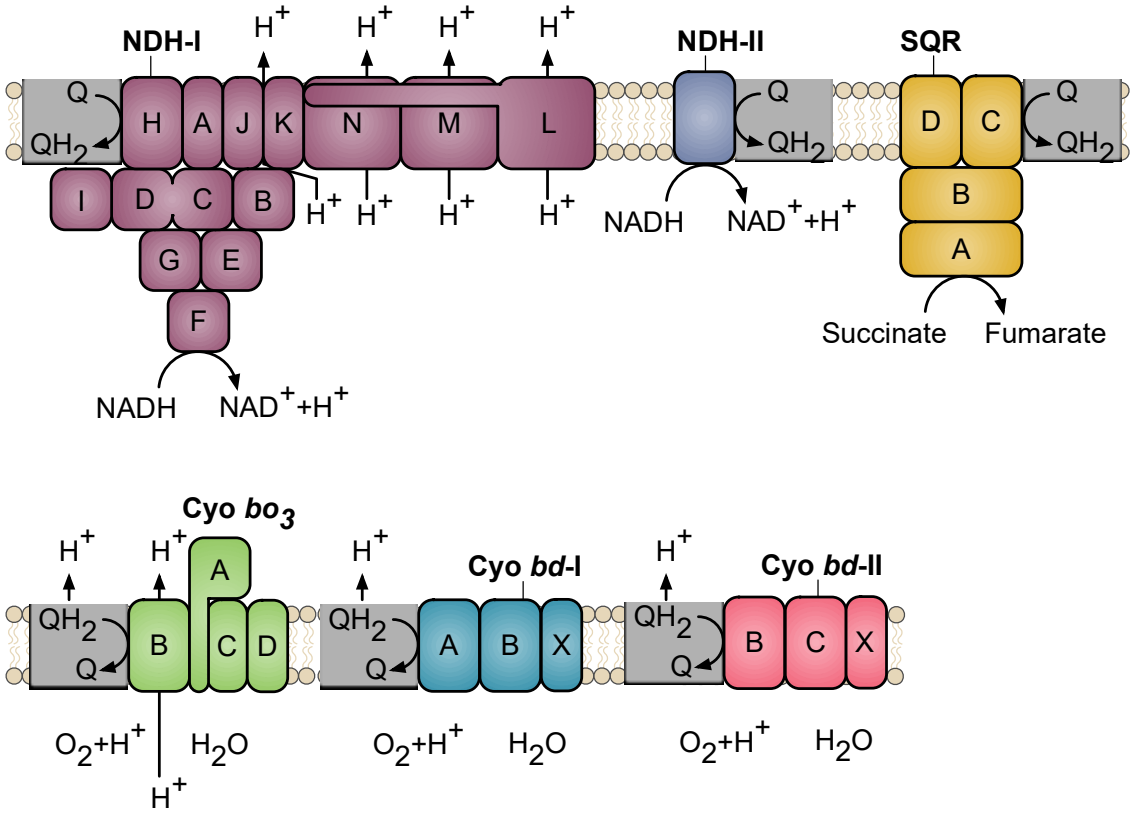

Figure 1-3 Complexes of the aerobic electron transport chain in *E. coli*.

General topology of NADH dehydrogenase I (NDH-I), NADH dehydrogenase II (NDH-II), succinate dehydrogenase (SQR), cytochrome *bo*$_3$ (Cyo *bo*$_3$), cytochrome *bd*-I (Cyo *bd*-I), and cytochrome *bd*-II. Letters indicate the subunit of the respective complex. H^+, proton; H_2O, water; NAD, nicotinamide adenine dinucleotide; O_2, molecular oxygen; Q, quinone. Information used to construct general topologies was taken from (130, 131, 145, 156)

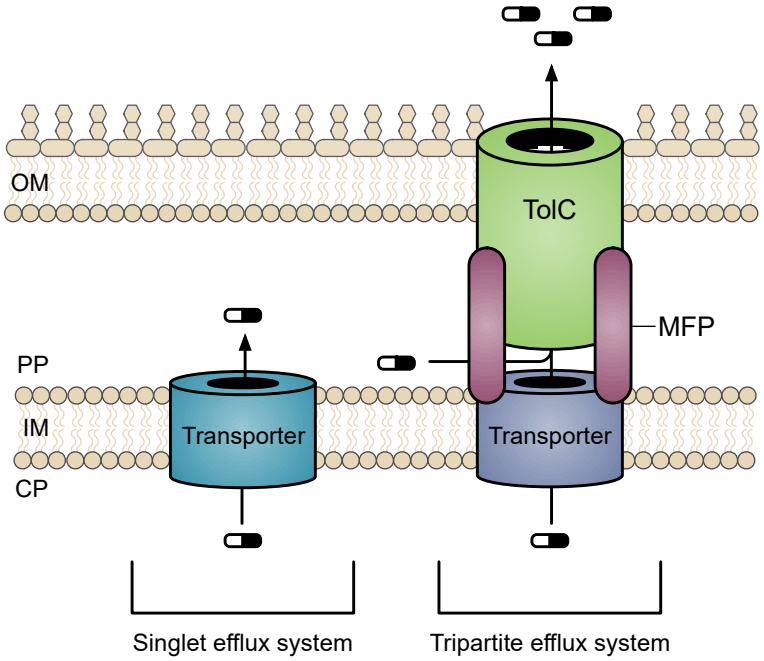

Figure 1-4 Efflux across the envelope.

Bacterial efflux pumps can function as singlet efflux systems, which are comprised of a single transporter that crosses the inner membrane, or as tripartite efflux systems that form a contiguous channel that spans the entire bacterial envelope. Tripartite efflux systems consist of an inner membrane transporter and an outer membrane channel that are linked by a membrane fusion protein (MFP). In *E. coli*, the main outer membrane channel is TolC. Compounds transported across the inner membrane by singlet efflux systems may be collected in the periplasm and transported across the outer membrane by tripartite efflux systems. OM, outer membrane; PP, periplasm; IM, inner membrane; CP, cytoplasm. Figure adapted from (269).

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CHAPTER 2

A bacterial stress response regulates expression of respiratory protein complexes to control envelope stress adaptation

Note: Junshu Wang and Julia Wong collected and analyzed the data presented in figure 2-4C. Junshu Wang assisted with writing sections 2.2.6, 2.2.7, and 2.3.4.

2.1 Introduction

Gram-negative bacteria are characterized by the structure of their cell envelope, which consists of the inner membrane (IM), outer membrane, and the peptidoglycan layer within the periplasmic space. Of these, the IM contains the greatest protein diversity (15). Proteins that reside within the IM play essential roles in energetics, metabolism, transport, and signal transduction. This membrane also serves as a selectively permeable barrier that separates the cytoplasm from the cell's environment. *Escherichia coli* encode a suite of envelope stress responses that monitor and maintain envelope integrity, one of which is the Cpx response (270). The Cpx response is controlled by a typical two-component signal transduction system that consists of the membrane-bound sensor kinase CpxA and the cytoplasmic response regulator CpxR. Under inducing conditions, CpxA autophosphorylates at a conserved histidine residue and the phosphate is then transferred to a conserved aspartate residue within CpxR (60). Once phosphorylated, CpxR alleviates envelope stress by altering the transcription of over 100 genes (64, 65, 113, 271, 272). In the absence of an inducing cue, CpxA phosphatase activity maintains CpxR in a dephosphorylated and inactive state (60). The auxiliary regulator CpxP inhibits the Cpx response through direct interaction with the sensing domain of CpxA (75, 78).

The Cpx response is thought to detect and respond to potentially lethal misfolded proteins at the bacterial IM. Several conditions predicted or known to generate misfolded IM proteins activate the Cpx response, including overexpression of the outer membrane lipoprotein NlpE, overproduction of pilin subunits in the absence of their cognate chaperones, depletion of the IM protein insertase/assembly factor YidC, mutation of the IM protease FtsH, alkaline pH, and aminoglycoside antibiotics (24, 54-56, 58, 89, 273). Upon induction, CpxR activates the expression of multiple envelope-localized protein folding and degrading factors (51, 53, 68, 84). Recently, we have shown that the Cpx regulon is enriched for genes encoding IM protein complexes, most of which are downregulated (64).

Complexes of the electron transport chain have been identified in all transcriptomic studies of the Cpx response to date (64, 113, 271, 272). Enteropathogenic *E. coli* (EPEC) microarray data indicate that the expression of the genes encoding the respiratory complexes NADH dehydrogenase I (NDH-I) and cytochrome bo_3 are among the most strongly downregulated upon activation of the Cpx

response (64). NDH-I is one of the entry points for electrons carried by NADH into the bacterial and mitochondrial electron transport chains. It is one of the largest protein complexes in the *E. coli* IM with a molecular mass of 550kDa (136, 137). It is comprised of 13 subunits that are organized into two perpendicular arms: a hydrophobic membrane arm located in the IM and a peripheral arm that protrudes into the cytoplasm (138-140). The subunits of the bacterial NDH-I represent the core structure required for the functionality of the human mitochondrial homologue (134, 142). Cytochrome bo_3 is a terminal oxidase that couples the oxidation of ubiquinone to the reduction of molecular oxygen. It is comprised of four subunits that assemble into a 144kDa complex within the bacterial IM (175, 190, 274-276). It is a member of the heme-copper oxidase superfamily that also includes cytochrome *c* oxidase found in human mitochondria (174, 276).

In this study, we tested the hypothesis that Cpx-mediated downregulation of these large protein complexes is important for adapting to protein misfolding stresses at the cytoplasmic membrane. We show that the Cpx response regulates the transcription of the genes encoding NDH-I and cytochrome bo_3, and further that basal expression of these complexes is sufficient to activate the Cpx response. Intriguingly, our data suggest that Cpx-regulated genes also impact the function, stability, and/or assembly of respiratory complexes, since aerobic respiration is diminished in a *cpxRA* mutant in spite of the fact that transcription is not altered. Cumulatively, our data suggest that the primary function of the Cpx response is to monitor and adjust the biogenesis of macromolecular IM protein complexes.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 2-1. Bacteria were routinely cultured in Lennox broth (LB; 10g/L tryptone [Difco], 5g/L yeast extract [Difco], 5g/L NaCl) at 37°C with aeration at 225 rpm, with the exception of ALN195, which was grown overnight at 30°C. ALN195 was grown at 37°C following subculture. Isopropyl-β-D-thiogalactopyranoside (IPTG, Invitrogen) was added to a concentration of 0.1mM or 1mM as indicated. Unless otherwise stated, antibiotics (Sigma) were used as required at the following concentrations: amikacin (Amk), 3μg/mL; chloramphenicol (Cam), 25μg/mL; kanamycin (Kan), 50μg/mL; streptomycin (Sm), 50μg/mL.

2.2.2 Strain and plasmid construction

All EPEC mutants were constructed by allelic exchange (277). Regions of DNA approximately 1kb upstream and downstream of the target site were amplified by PCR and joined using overlap extension PCR (primer sequences, including restriction sites, are listed in Table 2-2) (278). The full-length PCR products were digested with *Xba*I, *Kpn*I, or *Sac*I (Invitrogen) restriction endonucleases and ligated into pRE112. Constructs were transferred onto the EPEC chromosome as previously described (279). pRE112 based suicide vectors were introduced into EPEC via conjugation from strain MFDλPir. Transconjugants were selected for on LB agar containing both streptomycin and chloramphenicol. Single colonies were grown in plain LB broth to late logarithmic phase. Serial dilutions of this culture were plated on LB agar (without NaCl) containing 5% sucrose and grown for two days at room temperature. Sucrose resistant colonies were tested for chloramphenicol sensitivity, indicating loss of the suicide vector sequence. The presence of the desired allele in chloramphenicol sensitive, streptomycin resistance colonies was screened for via PCR.

Luminescent reporters of NADH dehydrogenase I and cytochrome bo_3 transcription were constructed as previously described (280). Briefly, the promoter region of each operon was amplified from the E2348/69 genome using the primers listed in Table 2-1. Gel-purified products were digested with EcoRI, BamHI, or PvuI (Invitrogen) and ligated upstream of the *luxABCDE* operon in the pJW15 plasmid. The predicted CpxR binding-site in the pJW15-P*nuo* reporter was mutated by overlap extension PCR (278). The mutated promoter DNA was digested and ligated into pJW15 as described above. PCR and DNA sequencing verified correct insertion of the promoter sequences.

2.2.3 Luminescence assay

Bacteria were grown overnight in LB at 37°C with aeration and then subcultured 1:100 into 2mL fresh LB broth in a 13x100mm glass test tube at 37°C with aeration. To measure transcription of NADH dehydrogenase I and cytochrome bo_3, 0.1mM IPTG was added at time of sub-culture to induce NlpE expression from the pCA-*nlpE* plasmid. Bacteria were grown to an OD_{600} of 0.4-0.5, at which point luminescence was measured. To determine Cpx pathway activity in EPEC, expression of *cpxP* was measured from pJW25 (Table 2-1). Bacteria were grown overnight as described above, then subcultured

at a dilution factor of 1:100 into 10mL LB in a 125mL Erlenmeyer flask and grown at 37ºC with aeration. Luminescence (counts per second [CPS]) and OD_{600} were measured every two hours for eight hours post-subculture as previously described (109). Luminescence and OD_{600} values measured from a blank well containing uncultured LB were subtracted from each sample. Luminescence values were standardized to the OD_{600} of the same culture in order to account for differences in cell numbers between samples. All luminescence assays were repeated at least twice in quintuplicate (*nuo-lux* and *cyo-lux*) or triplicate (*cpxP-lux*).

2.2.4 Oxygen consumption

Bacteria were grown overnight in 5mL of LB at 37ºC for strains E2348/69 and RG222 and 30ºC for ALN195 with shaking at 225 rpm. Overnight cultures were diluted by a factor of 1:100 into 10mL of terrific broth (12g/L tryptone [Difco], 24g/L yeast extract [Difco], 0.4% glycerol) without antibiotics in a 125mL Erlenmeyer flask and grown to an OD_{600} of 0.35 at 37ºC with shaking at 200 rpm. Cells were washed twice with phosphate-buffered saline (Sigma) and suspended in 1mL of phosphate-buffered saline at a density of ~4 x 10^7 CFU/mL in a closed 1mL microrespiration chamber (Unisense). The microrespiration chamber was placed in a 37ºC water bath for the duration of the experiment. After the baseline oxygen concentration was established, respiration was initiated by the addition of 1% terrific broth. Oxygen concentration was measured every 30 seconds for 45 minutes using an oxygen MicroOptode sensor (Unisense). Oxygen concentration at each time point was standardized to the oxygen concentration immediately before addition of terrific broth. A magnetic stirrer was used during the assay to ensure oxygen was distributed throughout the microrespiration chamber. Data shown is representative of three replicate experiments.

2.2.5 Sensitivity assays

To determine sensitivity to *nlpE* overexpression, bacteria containing the *nlpE* overexpression vector pCA-*nlpE* or the vector control pCA-24N were grown overnight in 5mL LB at 37ºC with aeration and subcultured 1:100 into 5mL fresh LB at 37ºC with aeration. 1mM IPTG was added at early exponential phase to induce expression of NlpE from pCA-*nlpE* and cultures were grown for an additional 4.5 hours. Colony forming units (CFU) were measured by serial dilution and growth on LB agar. CFU/mL was calculated

by standardizing the number of resulting colonies to the dilution factor. NlpE sensitivity assays were performed twice in triplicate.

To determine sensitivity to aminoglycoside antibiotics and alkaline pH, overnight cultures were standardized to an OD_{600} of 1, serially diluted, and plated on plain LB agar, agar containing 1.5 μg/mL amikacin, agar buffered to pH 7.0, or agar buffered to pH 9.5. Agar was buffered to pH 7.0 or 9.5 using sodium hydroxide. Plates were incubated overnight at 37ºC. Images were taken using the UVP Colony Doc-It Imaging Station. Amikacin and pH sensitivity assays shown represent at least two replicate experiments. To determine the sensitivity to increasing concentrations of amikacin, overnight cultures were standardized to an OD_{600} of 1 and serially diluted. 10μL of each dilution was plated on LB agar, or LB agar supplemented with 1.5, 3, 6, or 12 μg/mL amikacin. Cells were grown overnight at 37ºC. Images were taken as above.

2.2.6 Genetic screen

To identify genes involved in modulating activity of the Cpx response in *E.coli*, 176 Cpx-regulated envelope-localized proteins were screened based on color variation on lactose MacConkey agar using a *cpxP-lacZ* reporter. From a previously published microarray study that characterizing the Cpx regulon upon NlpE overexpression in *E.coli*, genes that were regulated at least 2-fold in expression were identified (64). The candidate pool was further narrowed down to genes encoding envelope-localized proteins by referring to their cellular localization listed in Ecocyc database (281).The genetic screen was designed and performed following previously described methodology (280). For each tested candidate, its overexpression plasmid from the ASKA library was extracted and transformed into TR50 (Table 2-1), which carries a chromosomal *cpxP-lacZ* reporter. Four single colonies of the resulting transformants along with the control TR50 (pCA-24N) were patched onto lactose MacConkey plates supplemented with 0.1 mM IPTG to induce expression from the plasmid. In comparison to TR50 (pCA-24N), brighter red colonies indicated high levels of *lacZ* transcription and pink or white colonies indicated low levels of *lacZ* expression. The observed inhibitory or activating phenotype of candidates that showed changed Cpx activity was further confirmed by β-galactosidase assay to quantify the activity of the Cpx pathway.

2.2.7 β-galactosidase assays

β-galactosidase activity was measured in microtiter plates as previously described (76). For Figure 2-4C, bacteria were grown overnight in LB at 37ºC for wild type TR50 strains and 30ºC for TR50 *cpxA* mutant strains with aeration at 225 rpm. Overnight cultures then were subcultured in 1:100 dilutions into 2mL fresh LB in a 13x100mm glass test tube with aeration. To induce protein expression from pCA-based plasmids, 0.1mM IPTG was added 1h after subculture. Bacteria were collected by centrifugation when OD_{600} reached 0.4-0.6 and resuspended in 2mL freshly prepared buffer Z (60mM $Na_2HPO_4{\cdot}7H_2O$, 40mM $NaH_2PO_4{\cdot}H_2O$, 10mM KCl, 1mM $MgSO_4{\cdot}7H_2O$; containing 270μL β-mercaptoethanol). Then, 250μL of cell mixture were transferred to a 96-well microtiter plate and the OD_{600} was read using plate reader (Perkin Elmer). The remaining cells were lysed using two drops of chloroform and one drop of 0.1% SDS for 10min and the cellular debris were removed by centrifugation. Then 50μL of 10mg/mL ONPG (o-nitrophenyl β–D-galactopyranoside) (Sigma) was added to diluted cell lysate with 5μL aliquot of lysed cell mixture and 195μL of buffer Z in 96-well plate to initiate the reaction. The A_{420} was read 20 times over approximately 30 minutes in the plate reader and Miller Units were calculated (282). Experiments were done in triplicate for three times.

For Figure 2-5A**,** overnight cultures of MC4100 containing a *cpxP-lacZ* transcriptional reporter were diluted by a factor of 1:100 into 2mL LB-Lennox broth in a 13x100mm glass test tube and grown to mid-log phase at 37ºC with aeration at 225 rpm. 100μM CCCP or an equivalent volume of DMSO was then added and cultures were grown for an additional thirty minutes. *cpxP-lacZ* expression was determined as described above.

2.2.8 Growth curve

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

2.2.9 Western blot

Overnight cultures of E2348/69 were diluted by a factor of 1:100 into 10mL LB in a 125mL Erlenmeyer flask and grown to an OD_{600} of approximately 0.5 at 37ºC with

shaking at 225 rpm. 100μM CCCP or an equivalent volume of DMSO was then added and cultures were grown for an additional thirty minutes. 1mL of culture was pelleted and resuspended in 50μL 2x SDS sample buffer (Sigma). Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). 20μg of total protein was loaded onto a SDS-polyacrylamide gel and proteins were separated by electrophoresis. Western blotting was performed as previously described (75). Primary MBP-CpxR, MBP-CpxA, and PhoA antibodies were used at a 1:10000, 1:50000, and 1:25000 dilution, respectively. Alkaline-phosphatase anti-rabbit secondary antibodies (Sigma) were used at a 1:25000 dilution. Proteins were detected using the Immun-Star alkaline phosphatase chemiluminescence kit (Bio-Rad) and the Bio-Rad ChemiDoc MP imaging system.

2.2.10 Statistical Analysis

Statistical analysis was performed using Prism version 7.0c (GraphPad Software). Activity of transcriptional reporters was compared by one-way analysis of variance followed by Sidak's multiple comparison test for experiments containing more than two strains or an unpaired *t*-test for samples containing two strains.

2.3 Results

2.3.1 Regulation of NADH dehydrogenase I and cytochrome *bo₃* by the Cpx response

Microarray data indicate that the respiratory complexes NDH-I and cytochrome *bo₃* are members of the Cpx regulon (64). To confirm these results, we examined the contribution of the Cpx response to the expression of the *nuo* and *cyo* gene clusters using luminescent transcriptional reporters. Activation of the Cpx response by NlpE overexpression resulted in a 26-fold decrease in *nuo-lux* activity compared to the vector control (Figure 2-1A). However, when NlpE was overexpressed in a Δ*cpxRA* mutant, *nuo-lux* expression was decreased less than two-fold (Figure 2-1A). These results show that overproduction of NlpE downregulates *nuo* transcription in a CpxRA-dependent manner. Notably, deletion of *cpxRA* did not completely abolish repression of *nuo-lux* activity upon NlpE overexpression, suggesting that NlpE may regulate this operon through additional signaling pathways. In the absence of stress, there was a small, but significant, increase in *nuo-lux* activity in the Δ*cpxRA* mutant relative to wildtype,

suggesting that basal *nuo* transcription is affected by loss of the Cpx response (Figure 2-1A).

A putative CpxR binding site was identified approximately 104bp upstream of the predicted *nuoA* transcription start site using Virtual Footprint (http://prodoric.tu-bs.de/vfp/vfp_promoter.php) (283) (Figure 2-1B). To determine if this DNA sequence is required for regulation of *nuo* transcription by the Cpx response, the putative upstream CpxR binding site (Figure 2-1B; -104bp) was mutated from 5'-GTAAA(N_5)GTGAA-3' to 5'-CAGTA(N_5)CAGTA-3' in the *nuo-lux* reporter. As seen previously, NlpE overexpression strongly reduced activity of the wildtype *nuo-lux* reporter. However, *nuo-lux* activity was decreased less than two-fold when the putative CpxR binding site was mutated (Figure 2-1C). These data support the conclusion that repression of *nuo* transcription upon activation of the Cpx response is mediated by the direct binding of CpxR to the *nuo* promoter region. Interestingly, we observed that basal *nuo-lux* activity was increased when the putative upstream (Figure 2-1B; -104bp) CpxR binding site was mutated in the vector control strain, although the reason for this increase is unknown (Figure 2-1C).

In accordance with microarray data, NlpE overexpression resulted in a two-fold decrease in *cyo-lux* expression compared to the vector control (Figure 2-1D). This repression was dependent on the Cpx response, as overexpression of NlpE did not reduce *cyo-lux* activity in a Δ*cpxRA* mutant (Figure 2-1D). Furthermore, basal activity of the *cyo-lux* reporter was slightly increased in the Δ*cpxRA* mutant in the absence of stress (Figure 2-1D). A putative CpxR binding site was also identified in the *cyo* promoter, which overlaps with the -35 box of the promoter. These observations suggest that CpxR may also bind at the *cyo* promoter to directly repress transcription.

It has previously been proposed that activation of the Cpx response results in changes in respiration through the ArcAB (aerobic respiratory control) two-component system (89). The authors hypothesized that upon activation of the Cpx pathway, CpxA phosphorylates the response regulator ArcA in addition to CpxR. ArcA then alters expression of genes involved in respiration. To determine whether ArcA is required for regulation of *nuo* and *cyo* by the Cpx pathway, we overexpressed NlpE in an *arcA* mutant and determined activity of the *nuo-lux* and *cyo-lux* reporters. Overexpression of NlpE in wildtype EPEC decreased *nuo-lux* activity by approximately 28-fold compared to the wildtype containing the control expression vector (Figure 2-1E). When NlpE was overexpressed in EPEC lacking ArcA, expression of *nuo-lux* was decreased by

approximately 16-fold. We also observed that deletion of *arcA* resulted in a greater than two-fold increase in *nuo-lux* activity (Figure 2-1E). This is to be expected, as ArcA is a negative regulator of *nuo* transcription (284, 285). Likewise, overexpression of NlpE in the *arcA* mutant reduced *cyoA-lux* activity by 1.98-fold, while overexpression of NlpE in wildtype EPEC decreased *cyoA-lux* expression by 2.82-fold (Figure 2-1F). These results suggest that ArcA may play a minor role in regulation of *nuo* and *cyo* transcription in the presence of the envelope stress generated by NlpE overexpression. However, as transcription of both *nuo* and *cyo* is repressed by NlpE overexpression in an ArcA-independent manner, we conclude that cross-talk between CpxA and ArcA is not the major regulatory pathway responsible for regulation of these complexes during stress. This is in line with our finding that CpxR directly regulates *nuo* transcription.

2.3.2 Cpx pathway activity affects respiration

Our results suggest that induction of the Cpx pathway would decrease activity of the aerobic electron transport chain. To confirm our findings, we compared oxygen consumption by wildtype EPEC to that of a *cpxA24* mutant, which exhibits constitutive activation of the Cpx response (60). As seen in Figure 2-2A, the rate of oxygen consumption in the *cpxA24* mutant was decreased compared to the wildtype strain. As expected, this indicates that decreased expression of respiratory complexes upon activation of the Cpx response leads to decreased activity of the aerobic electron transport chain.

We also compared oxygen consumption by wildtype EPEC to that of a Δ*cpxRA* mutant to determine the effect of loss of the Cpx response on aerobic respiration. Unexpectedly, oxygen consumption by the Δ*cpxRA* mutant was reduced compared to the wildtype strain (Figure 2-2A). In replicate experiments, we observed variable rates of respiration in the Δ*cpxRA* mutant. In some replicates, the initial rate of respiration appeared similar to that of wild-type, but in every instance (5 replicates), oxygen consumption slowed relative to wild-type over the course of the experiment, and in no case was the Δ*cpxRA* mutant ever able to consume all of the oxygen in the vial (Figure 2-2B). This result was surprising, as transcription of at least NDH-I and cytochrome bo_3 is slightly increased in this mutant (Figure 2-1). This phenotype was similar to that observed for a *cyo* mutant, lacking the cytochrome bo_3 oxidase (Figure 2-2B) and suggests that the defect in respiration observed in the Δ*cpxRA* mutant, at least under these conditions (mid-log phase cells respiring in terrific broth), is due largely to

problems with the biogenesis or function of cytochrome *bo₃* oxidase. These findings suggest that the Cpx response regulates a factor(s) that facilitates aerobic respiration.

2.3.3 Expression of respiratory complexes is toxic during envelope stress

Given the Cpx-mediated downregulation of these large protein complexes, we hypothesized that their presence must be toxic in the presence of envelope stresses. To investigate this possibility, we examined the impact of the NDH-I and cytochrome *bo₃* oxidase complexes on the toxicity of envelope stresses in a *cpxR* mutant that is not able to inhibit the expression of the *nuo* and *cyo* operons. We first established the sensitivity of wildtype EPEC and its isogenic mutant lacking *cpxR* to Cpx-sensed envelope stressors previously identified in *E. coli* K-12, including overproduction of NlpE, aminoglycoside antibiotics, and alkaline pH. NlpE overexpression in the wildtype strain had a mild deleterious effect on growth (Figure 2-3A). However, the *cpxR* mutant was approximately 100-fold more susceptible to the effects of NlpE overexpression in comparison to the wildtype (Figure 2-3A). A similar result was observed when the *cpxR* mutant was exposed to the aminoglycoside antibiotic amikacin. Wildtype EPEC was resistant to 1.5 μg/mL amikacin, however the *cpxR* mutant was susceptible to killing at this concentration (Figure 2-3B). It has previously been shown that *cpxR* is required for growth at alkaline pH in *E. coli* K-12 (54). Here, we confirmed this finding in EPEC, showing that the *cpxR* mutant has a growth defect at pH 9.5 compared to the wildtype strain (Figure 2-3D). Overall, these results show that a functional CpxR is required for EPEC to adapt to these envelope stressors.

In the absence of CpxR, expression of NDH-I and cytochrome *bo₃* is not substantially altered in the presence of envelope stress (Figure 2-1). To determine if expression of these complexes contributes to the sensitivity of the *cpxR* mutant to various stressors, we deleted the *nuo* and *cyo* operons in the wildtype and *cpxR* mutant backgrounds. We then determined if deletion of these operons could rescue the sensitivity of the *cpxR* mutant to overproduction of NlpE, amikacin, and alkaline pH. Sensitivity to NlpE overexpression in the *cpxR* mutant was unchanged when either *nuo* or *cyo* were individually deleted (Figure 2-3A). However, if both *nuo* and *cyo* were deleted, resistance of the *cpxR* mutant to NlpE overexpression was partially restored to that of EPEC containing a functional Cpx response (Figure 2-3A). Further, deletion of either *nuo* or *cyo* or both in the *cpxR* mutant restored resistance to amikacin (Figure 2-3B) and growth at alkaline pH (Figure 2-3D). Together, these results suggest that the

sensitivity of the *cpxR* mutant to multiple envelope stressors arises, in part, from the inability to downregulate NDH-I and cytochrome bo_3 and that expression of these protein complexes is toxic during envelope stress.

2.3.4 The presence of respiratory complexes contributes to envelope stress sensed by CpxA

As deletion of *nuo* and *cyo* can alleviate the toxicity of certain envelope stresses in a *cpxR* mutant strain background, we hypothesized that these complexes themselves may generate envelope stress. In order to examine this possibility, we determined Cpx pathway activity in the Δ*nuo*, Δ*cyo,* and Δ*nuo* Δ*cyo* single and double mutants by measuring activity of a *cpxP* luminescent transcriptional reporter. *cpxP* expression is commonly used as a proxy for Cpx pathway activity as it is one of the most highly transcribed genes upon activation of the Cpx response, and its expression depends almost exclusively on CpxR (54, 65, 67). As can be seen in Figure 2-4A, deletion of either *nuo* or *cyo* reduced *cpxP* expression at all stages of growth. The Δ*nuo* Δ*cyo* double mutant had lower *cpxP* expression than either the Δ*nuo* or Δ*cyo* single mutants, suggesting an additive effect on Cpx pathway activity. Notably, deletion of these complexes prevented induction of the Cpx response upon entry into stationary phase (Figure 2-4A) even though all of the strains are able to grow to stationary phase (Figure 2-4B). These results suggest that NDH-I and cytochrome bo_3 increase basal levels of Cpx pathway activity and may contribute to growth-related activation of the Cpx response.

The possibility that the Cpx pathway senses a signal associated with the NDH-I and cytochrome bo_3 protein complexes is further supported by results of an independent genetic screen performed to identify proteins that modulate the activity of the Cpx pathway. As seen in Figure 2-4C, overexpression of cytochrome bo_3 subunit II (CyoA) and subunit III (CyoC) activated the Cpx response approximately eight-fold and four-fold, respectively. When *cpxA* was mutated, activation of the Cpx pathway by overexpression of either gene was diminished to less than two-fold (Figure 2-4C). These data indicate that cytochrome bo_3 subunits II and III function as multi-copy activators of the Cpx pathway in a CpxA-dependent manner and further reinforce our finding that the Cpx response is sensitive to the presence of the cytochrome bo_3 oxidase.

2.4 Discussion

Membrane-bound respiratory complexes constitute a major part of the IM proteome (286). As such, their elaboration and function may impose a significant stress on the IM. In this study, we describe a novel role for the Cpx envelope stress response in monitoring and regulating the expression of NDH-I and cytochrome bo_3 in EPEC. We provide evidence that the Cpx response is sensitive to basal level production of these protein complexes, and that the inability to repress their expression in the presence of stress leads to lethality.

In agreement with previous microarray data, we found that the Cpx stress response regulates expression of at least two large cytoplasmic membrane complexes in EPEC: NADH dehydrogenase I and cytochrome bo_3 (Figure 2-1) (64). Repression of *nuo* and *cyo* transcription by the Cpx response is likely mediated through direct binding of CpxR within the promoter region of these operons. The putative CpxR binding site in the *cyo* promoter overlaps with the predicted -35 box. As such, CpxR likely blocks RNA polymerase from binding to the *cyo* promoter through steric hindrance (287). However, the mechanism by which CpxR directly prevents transcription at the *nuo* promoter is less clear. We identified a putative CpxR binding site approximately 104bp upstream of the *nuoA* transcription start site that is required for repression of *nuo-lux* expression upon activation of the Cpx response (Figure 2-1B and Figure 2-1C). This putative binding site is located upstream from the predicted -35 and -10 promoter elements, suggesting that CpxR does not repress *nuo* transcription through steric hindrance of RNA polymerase. Upon further investigation, we identified a second putative CpxR binding site approximately 48bp downstream of the *nuoA* transcription start site (Figure 2-1B). Thus, it is possible that interaction between CpxR bound separately at the proximal and distal binding sites prevents *nuo* transcription initiation through a looping mechanism (287). Alternatively, CpxR may work with additional regulatory pathways to repress transcription of *nuo.* Several lines of evidence point to a role for the ArcAB two-component system in control of *nuo* transcription during envelope stress. Like CpxR, the response regulator ArcA directly represses transcription of *nuo* (284, 285). However, the putative ArcA binding site within this promoter has not been reported. Inspection of the EPEC *nuo* promoter region using virtual footprint identified a putative ArcA binding site approximately 81bp upstream of the *nuoA* transcription start site (Figure 2-1B). As the putative ArcA and CpxR binding sites are close to one another, it is possible that these regulators work in synergy to repress transcription of the *nuo* operon. Furthermore, ArcA

is required for outer membrane integrity, suggesting that the ArcAB two-component system may be active during periods of envelope stress (288). However, we found that activation of the Cpx response repressed *nuo* and *cyo* expression even in the absence of *arcA* (Figure 2-1E and Figure 2-1F). While ArcA may play a minor role in regulation of *nuo* and *cyo* transcription during Cpx-dependent envelope stress, it is unlikely that ArcA is required for CpxR-mediated inhibition of *nuo* and *cyo* transcription.

As NADH dehydrogenase I and cytochrome bo_3 oxidase facilitate aerobic respiration, we examined oxygen consumption in different Cpx backgrounds to validate our findings. As expected, we found that constitutive activation of the Cpx response reduces consumption of oxygen (Figure 2-2A). Surprisingly, we found that oxygen consumption was also reduced in EPEC lacking the Cpx response (Figure 2-2A and Figure 2-2B), despite no change in transcription of the *nuo* or *cyo* gene clusters (fFigure 2-1A and Figure 2-1D). Under the conditions used here (mid-log phase cells respiring in terrific broth), the respiratory defect of the *cpxRA* mutant appears to be largely due to effects on cytochrome bo_3 oxidase (Figure 2-2B). Taking into account the demonstrated role of the Cpx response in protein folding and degradation, and that proper folding of respiratory subunits is required for their assembly into complexes (190), it is possible that in the absence of the Cpx response, respiratory complexes cannot assembly properly. As little is known about the quality control of membrane-bound respiratory complexes in either mitochondria or *E. coli*, potential Cpx-regulated factors involved in this process remain mysterious. While a small number of assembly factors involved in the biogenesis of iron-sulfur clusters in NDH-I have been identified, none of these are known to be regulated by the Cpx response (64, 156). Alternatively, or in addition, since the *cpxRA* mutant is initially able to consume oxygen at early time points in our assay (Figure 2-2), perhaps the Cpx-regulated factor(s) contribute to the stability, recycling, or maintenance of these protein complexes. At present, we cannot distinguish amongst these possibilities.

The Cpx response plays a role in the biogenesis of other macromolecular envelope complexes, including the type IV bundle-forming pilus (56, 110). *cpxR* mutants display decreased activity of the bundle-forming pilus despite no change in transcription of the *bfp* gene cluster. This was attributed to decreased expression of several Cpx-regulated protein folding factors that are required for proper folding of the pilus components. Both the elaboration and retraction of type IV pili are thought to involve the extension or retraction of pilus fibers through a platform in the IM where individual pilin

subunits are removed from, or added to, large pools that accumulate in the IM (289). Taken together with our hypothesis that the Cpx response may play a role in the biogenesis of membrane-bound respiratory complexes, these studies suggest that the Cpx response may play a general role in the biogenesis and/or quality control of abundant IM protein complexes.

Deletion of *nuo*, *cyo*, or both increases resistance of the *cpxR* mutant to several Cpx specific stresses (Figure 2-3). One possible explanation for this result is that changes in the PMF as a result of deletion of these complexes reduces the presence of stressors at the IM. NlpE is secreted into the envelope through the PMF-dependent sec translocon and aminoglycoside antibiotics require the PMF for uptake (58, 290). Furthermore, expression of both *nuo* and *cyo* is decreased at alkaline pH to maintain the cytoplasmic pH (291). However, a previous report has shown that the PMF is not substantially altered in *E. coli* lacking both NDH-I and cytochrome bo_3 (292). Therefore, changes in PMF do not likely account for the observed resistance of these mutants to envelope stress. We propose instead that defects in assembly, or irreparable damage to, these complexes in the *cpxR* mutant may increase sensitivity to envelope stressors by disrupting IM integrity. In the presence of envelope stress, EPEC with a functional Cpx response repress *de novo* synthesis of NDH-I and cytochrome bo_3 (Figure 2-1), thus reducing protein trafficking within the IM. Additional Cpx-regulated factors may assist in the biogenesis or repair of existing complexes (Figure 2-2) in order to reduce membrane damage. However in the *cpxR* mutant, transcription of NDH-I and cytochrome bo_3 is unchanged during stress (Figure 2-1). As such, newly synthesized respiratory complexes may be inserted into an already damaged membrane. In the absence of Cpx-regulated assembly factors, unassembled or misassembled respiratory subunits may further disrupt IM integrity (Figure 2-3). Therefore, deletion of *nuo* or *cyo* would reduce stress on the IM in the *cpxR* mutant. By whatever mechanism NDH-I and cytochrome bo_3 exert their toxicity, these results suggest that regulation of these complexes by the Cpx response is centrally involved in adaptation to envelope stress.

Expression of NDH-I and cytochrome bo_3 contributes to the basal activity of the Cpx stress response in EPEC, as indicated by the decrease in *cpxP* expression in bacteria lacking the *nuo* or *cyoA* operons (Figure 2-4A). At present, the mechanism by which the Cpx response might detect the presence of these protein complexes remains mysterious. One possibility is that the Cpx response detects malfunctions in these complexes that lead to the production of a Cpx activating signal. In this regard, it is

known that electron flow leads to the generation of damaging reactive oxygen species (293), which could theoretically result in damage to respiratory complexes, or other protein assemblies, and a Cpx inducing signal. In agreement with this model, Bina and coworkers recently showed in *Vibrio cholerae* that the inability to efflux the siderophore vibriobactin, as well as the oxidative stress inducing agent paraquat, resulted in Cpx pathway induction in an oxygen-dependent manner (294). Additionally, Chao and Vogel (118) recently showed that the Cpx response is activated by the protonophore CCCP in *Salmonella,* suggesting that disruption of the proton pumping activity associated with these complexes could also potentially be the source of a Cpx activating signal.

While we cannot definitively say whether the enzymatic activities and/or the assembly of these ETC complexes is responsible for inducing the Cpx response, we believe our results support the conclusion that the Cpx envelope stress response is responsive to some aspect of their biogenesis. We found that, as reported previously by Danese and Silhavy (54), the *E. coli* Cpx response is not induced by CCCP (Figure 2-5A and Figure 2-5B). Further, we observed that the ability of deletion of the *nuo* and *cyo* operons to suppress the sensitivity of a *cpxR* mutant to over-expression of NlpE, as well as amikacin, is additive (Figure 2-3A and Figure 2-3C). Similarly, the impact of these deletion mutations on Cpx pathway activity is also additive (Figure 2-4A). Finally, over-expression of individual subunits of the cytochrome bo_3 oxidase complex induces the Cpx response (Figure 2-4C), and we found this induction to occur even in a mutant lacking the *cyo* operon (J Wang and TL Raivio, unpublished observation). Cumulatively, our findings support the hypothesis that the Cpx response detects some signal related specifically to the assembly of IM protein complexes, much as the σ^E envelope stress response responds to a specific signature element present in outer membrane proteins (295).

This model is further bolstered by the fact that the Cpx response downregulates the expression of other complexes that involve IM protein assemblies that are not involved in respiration, including genes encoding pili, flagella, and the type-three secretion system (25, 100, 109, 110, 296). During normal biogenesis of complexes such as NDH-I and cytochrome bo_3, it is possible that some subunits may not assemble correctly. Such subunits may engage in nonproductive interactions that result in activation of the Cpx response. Furthermore, depletion of the IM insertase/assembly factor YidC, which is required for the assembly of NDH-I, cytochrome bo_3, and many other IM proteins, also activates the Cpx response (173, 191, 273). Finally, the

expression of inner membrane localized proteolytic factors responsible for quality control at the inner membrane, including HtpX and YccA, is under the control of the Cpx response (24, 65). These results suggest that the Cpx response monitors the biogenesis of membrane-bound protein complexes like NDH-I and cytochrome *bo*$_3$ through a signal that is specific to IM proteins. In this regard, it is of interest that Chao and Vogel have shown that the sRNA CpxQ, encoded in the 3' end of the *cpxP* mRNA, serves to down-regulate the expression of several integral IM proteins, the most strongly of which is the sodium-proton antiporter NhaB (118). Additionally, Grabowicz et al. recently showed that down-regulation of the periplasmic chaperone Skp by the CpxQ sRNA is likely needed to stem aberrant insertion of OMPs into the cytoplasmic membrane (96). Cumulatively, these results support the conclusion that the Cpx response serves as a sentinel of IM protein biogenesis.

We also observed that the presence of these complexes contributes to the activation of the Cpx response upon entry into stationary phase (Figure 2-4A). Wolfe and colleagues have shown that stationary phase activation of the Cpx response occurs by two separate processes (66). First, consumption of the amino acids present in complex medium throughout growth increases the pH of the surrounding environment. Second, catabolism through the Pta-AckA acetogenesis pathway prevents the accumulation of an unidentified inhibitory metabolite. Either of these processes may be affected by loss of NDH-I or cytochrome *bo*$_3$. *nuo* mutants have a growth defect in stationary phase in part due to their inability to catabolize multiple amino acids (297). Furthermore, inhibition of electron transport through NDH-I or cytochrome *bo*$_3$ may alter metabolism in such a way that the unidentified inhibitory metabolite is produced. Whatever happens, it is clear that activation of the Cpx response upon entry into stationary phase is associated with both NDH-I and cytochrome *bo*$_3$.

Overall, we have demonstrated a role for the Cpx response in the control of two large, abundant, IM protein complexes. It is well established that the σ^E envelope stress response serves as a sentinel for damage to prevalent β-barrel outer-membrane proteins (270), and our work suggests that the Cpx response may function in an analogous fashion at the IM, responding to stresses that impair abundant protein complexes and threaten cellular integrity. Previous studies have linked the Cpx response to diverse processes including peptidoglycan metabolism, biogenesis of virulence factors, motility, solute transport, protein export, and extrusion of waste (41, 43, 47, 56, 74, 100, 101, 109, 110, 116, 240, 244). We believe that the pleiotropic phenotypes

displayed by Cpx mutants may reflect widespread changes in the biogenesis of IM proteins, which may include altered energetics due to changes in NDH I and cytochrome bo_3 oxidase expression.

2.5 Figures and Tables

Table 2-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Bacterial strains		
E2348/69	Prototypical EPEC O127:H6 laboratory strain	(298)
ALN195	E2348/69 *cpxA24*	(109)
ALN88	E2348/69 *cpxR::kan*; Kan^R	(56)
RG139	E2348/69 Δ*cyoABCDE*	This study
RG141	ALN88 Δ*cyoABCDE*	This study
RG148	E2348/69 Δ*nuoABCDEFGHIJKLMN*	This study
RG149	E2348/69 Δ*cyoABCDE* Δ*nuoABCDEFGHIJKLMN*	This study
RG149	ALN88 Δ*cyoABCDE* Δ*nuoABCDEFGHIJKLMN*	This study
RG157	ALN88 Δ*nuoABCDEFGHIJKLMN*	This study
RG222	E2348/69 Δ*cpxRA*	This study
RG277	E2348/69 Δ*arcA*	This study
TR50	MC4100 λRS88[*cpxP'-lacZ*$^+$]	(60)
Plasmids		
pCA-24N	Vector control for ASKA library containing $P_{T5\text{-}lac}$ IPTG-inducible promoter; Cam^R	(299)
pCA-*nlpE*	IPTG-inducible *nlpE* overexpression vector from ASKA library; Cam^R	(299)
pCA-*cyoA*	IPTG-inducible *cyoA* overexpression vector from ASKA library; Cam^R	(299)
pCA-*cyoC*	IPTG-inducible *cyoC* overexpression vector from ASKA library; Cam^R	(299)
pJW25	pJW15 luminescence reporter plasmid containing *cpxP* promoter; Kan^R	(109)
pJW15-	pJW15 luminescence reporter plasmid containing	This study

P*nuo*	*nuo* operon promoter from E2348/69; Kan^R	
pJW15-Pnuo_{SUB}	pJW15-P*nuo* with base pairs –194 to –208 upstream of the *nuoA* coding region mutated to 5'-CAGTATCAGTCAGTA-3'	This study
pJW15-P*cyo*	pJW15 luminescence reporter plasmid containing *cyo* operon promoter from E2348/69; Kan^R	This study
pRE112	Suicide vector for allelic exchange; Cam^R	(277)

Table 2-2. Oligonucleotide primers used in this study

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

* Underlined sequences denote restriction endonuclease cut sites (EcoRI: GAATTC, BamHI: GGATCC, PvuI: CGATCG, KpnI: GGTACC, SacI: GAGCTC, XbaI: TCTAGA).

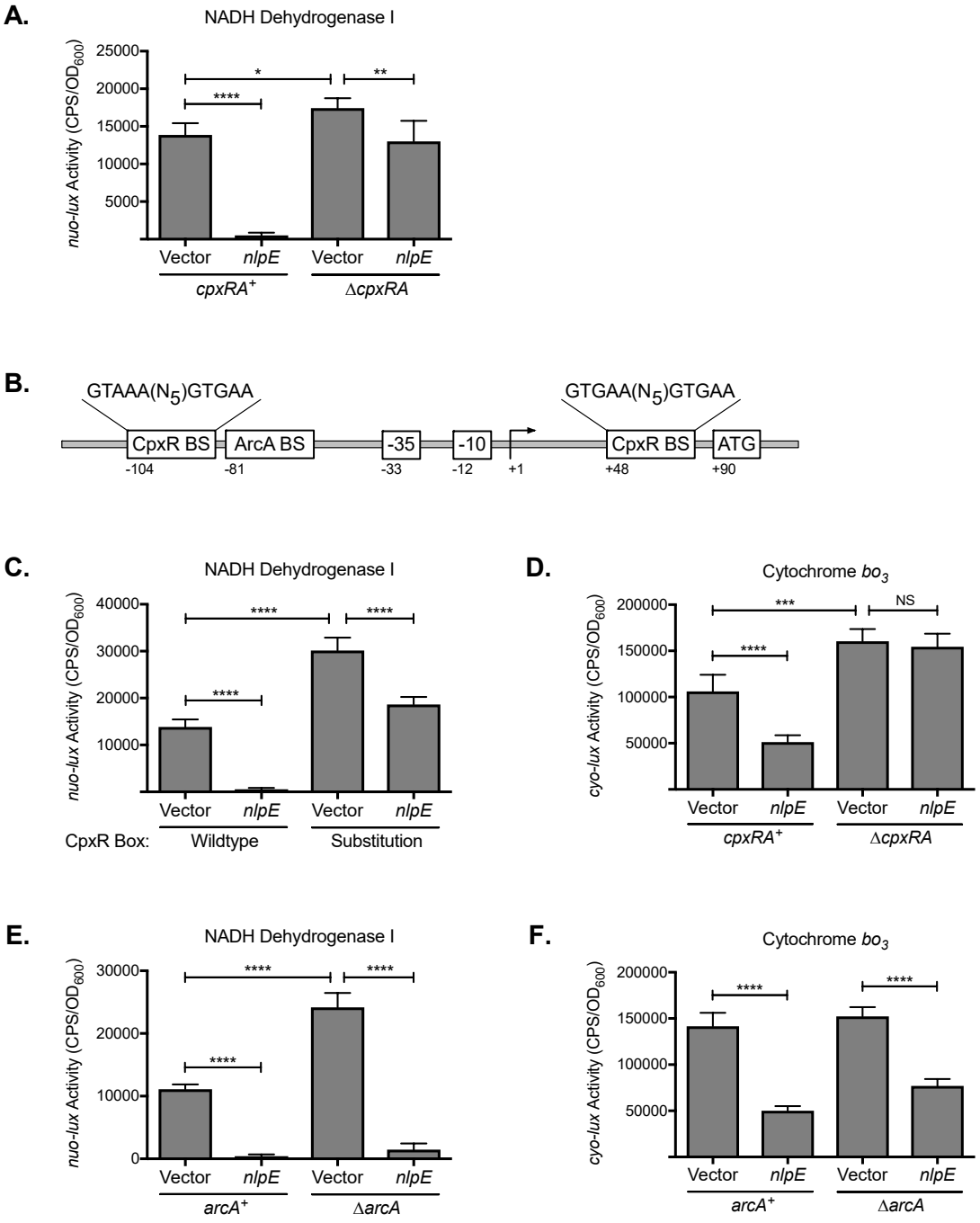

Figure 2-1 The Cpx response regulates the transcription of NADH dehydrogenase I and cytochrome *bo*$_3$

Bacteria containing the pCA-24N empty vector or the pCA-*nlpE* overexpression vector were subcultured into 2mL LB containing 0.1mM IPTG. Bacteria were grown at 37°C to an OD_{600} of approximately 0.5. 200µL of culture was transferred to a black-walled 96 well plate. Luminescence (counts per second [CPS]) and OD_{600} were measured and

standardized as described in section 2.2.3. (A) *nuo-lux* expression in wildtype and Δ*cpxRA* EPEC. (B) Schematic representation of the *nuo* promoter region indicating the location of the putative CpxR and ArcA binding sites. Numbers indicate distance from the transcription start site in base pairs. -, upstream; +, downstream; BS, binding site. (C) Activity of *nuo-lux* reporters with a wildtype or mutant (substitution) CpxR binding site in wildtype EPEC. (D) *cyo-lux* expression in wildtype and Δ*cpxRA* EPEC. (E) *nuo-lux* expression in wildtype and Δ*arcA* EPEC. (F) *cyo-lux* expression in wildtype and Δ*arcA* EPEC. Data represent the means and standard deviations of five replicate cultures. Asterisks (*) indicate a statistically significant difference from the relevant vector control (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$, one way ANOVA with Sidak's post hoc test). NS indicates no statistically significant difference in reporter activity.

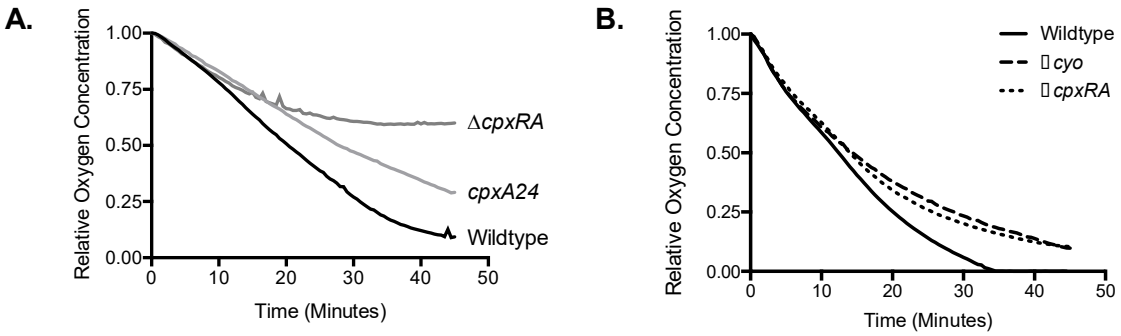

Figure 2-2 Oxygen consumption is reduced by activation and inhibition of the Cpx response

Bacteria were subcultured into 10mL terrific broth and grown to an OD_{600} of 0.35. Bacteria were pelleted by centrifugation, washed once in phosphate-buffered saline and diluted to a concentration of 4 x 10^7 cells mL^{-1} in 1mL of phosphate-buffered saline in a 1mL microrespiration chamber. Oxygen concentration was measured every 30 seconds for 45 minutes at 37ºC. The oxygen concentration at each time point was divided by the oxygen concentration at zero seconds. (A) Rate of oxygen consumption in wildtype EPEC, and the *cpxA24* (Cpx ON) and Δ*cpxRA* (Cpx OFF) mutants. Data are representative of three independent experiments. (B) Rate of oxygen consumption in wildtype EPEC, and the Δ*cyoA-E* and Δ*cpxRA* mutants. Data are representative of two independent experiments.

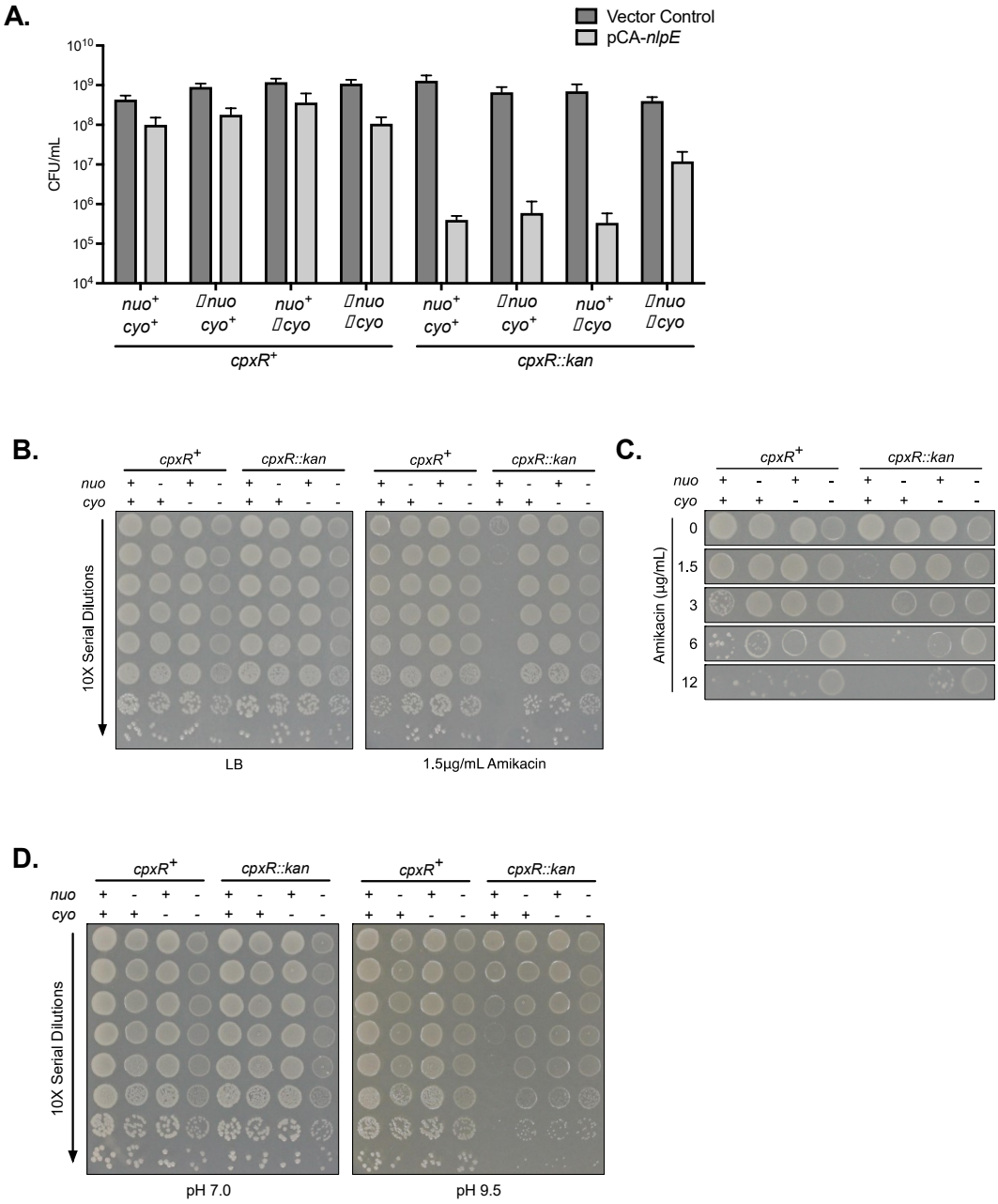

Figure 2-3 Deletion of the *nuo* and *cyo* operons in a *cpxR* mutant restores resistance to envelope stress

(A) Bacteria were subcultured from overnight growth into 5mL LB and grown at 37°C to early exponential phase. IPTG was added to all cultures to 1mM to induce expression of NlpE and bacteria were grown for an additional 4.5 hours at 37°C. To determine the number of viable cells, cultures were serially diluted, plated onto LB agar, and grown overnight at 37°C. Colony forming units (CFUs) were counted from dilutions with single, well-isolated colonies. CFU mL^{-1} was calculated by standardizing the number of CFUs to

the dilution. Data represent the means and standard deviations of three replicate cultures. (B and C) After growth overnight in LB, the OD_{600} of the EPEC cultures were standardized to 1.0. Cultures were then serially diluted by a factor of 10. 10μL of each dilution was spotted on (B) plain LB or LB supplemented with 1.5 μg/mL amikacin and (C) plain LB or LB supplemented with 1.5, 3, 6, or 12μg/mL amikacin. Bacteria were grown overnight at 37°C. Data displayed in (C) are growth of strains at the 10^{-3} dilution. (D) The OD_{600} of bacteria grown overnight in LB was standardized to 1.0 and cultures were serially diluted by a factor of 10. 10μL of each dilution was spotted on LB buffered to pH 7.0 or 9.5. Bacteria were grown overnight at 37°C. Data in B-D are representative of at least two independent experiments. The strains shown are wildtype EPEC, Δ*nuo,* Δ*cyo*, and *cpxR::kan* single mutants, Δ*nuo* Δ*cyo,* Δ*nuo cpxR::kan,* and Δ*cyo cpxR::kan* double mutants, and the Δ*nuo* Δ*cyo cpxR::kan* triple mutant. +, presence of an operon; -, deletion of an operon.

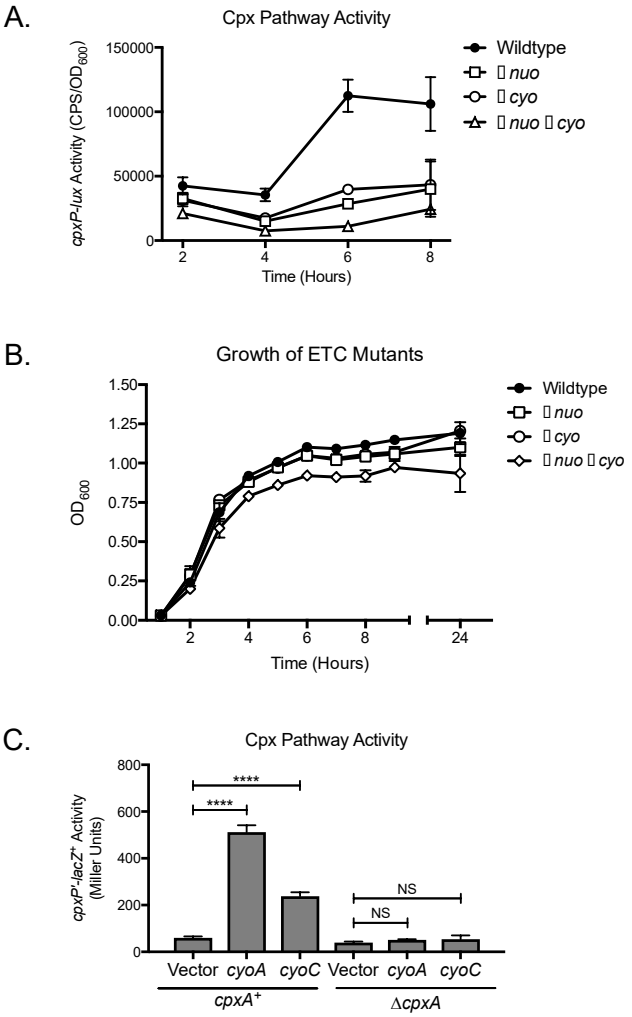

Figure 2-4 Expression of NDH-I and cytochrome *bo*$_3$ alters Cpx pathway activity

(A) Wildtype EPEC, the Δ*nuo* and Δ*cyo* single mutants, and the Δ*nuo* Δ*cyo* double mutant were each subcultured from overnight growth in LB into in 10mL LB and grown at 37ºC as described in section 2.2.3. Every 2 hours for 8 hours, bacteria were transferred to a black-walled 96 well microtiter plate and luminescence (counts per second [CPS]) and OD_{600} were measured. CPS was standardized to the culture OD_{600} to account for variation in cell number. Data represent the means and standard deviations of three replicate cultures. (B) After overnight growth in 2mL LB, the OD_{600} of each culture was standardized. Bacteria were then subcultured into 200µL of LB in a 96 well microtiter plate and grown at 37ºC. OD_{600} was measured every hour for 8 hours, and again 24 hours after subculture. For more details, please see section 2.2.8. Data represent the means and standard deviations of five cultures. (C) *cpxP-lacZ* expression in wildtype and Δ*cpxA E. coli* MC4100 carrying the vector control pCA24N or the overexpression

vectors pCA-*cyoA* or pCA-*cyoC*. Bacteria were subcultured into 2mL LB and grown for 1 hour. At this time, 0.1mM of IPTG was added to induce expression of CyoA and CyoC. *cpxP-lacZ* expression was determined once bacteria grew to an OD_{600} of 0.4-0.6 as described in section 2.2.7. Data represent the means and standard deviations of three biological replicates. Asterisks (*) indicate a statistically significant difference from the relevant vector control (****, $P \leq 0.0001$ [one way ANOVA with Sidak's post hoc test]). NS indicates no statistically significant difference in reporter activity.

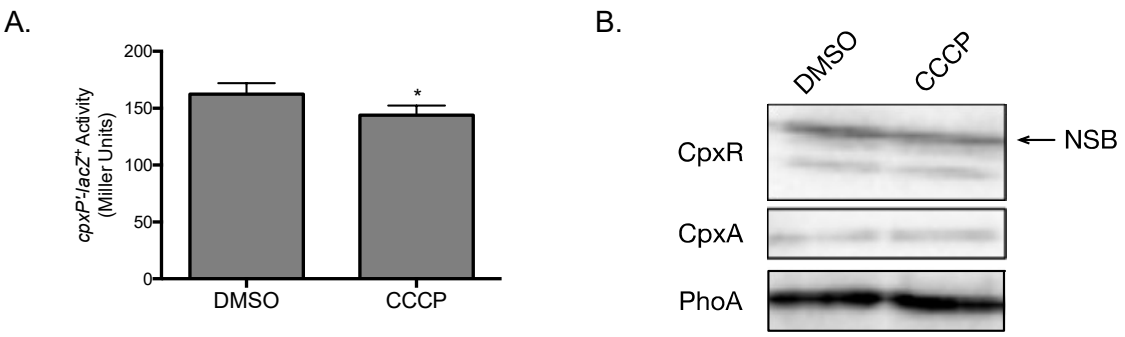

Figure 2-5 The *E. coli* Cpx response is not strongly affected by CCCP

(A) *E. coli* strain MC4100 carrying the *cpxP-lacZ* transcriptional reporter were subcultured after overnight growth in LB into 2mL of LB and grown to mid-log phase at 37ºC. Cells were then treated with 100µM CCCP or an equal volume of DMSO for 30 minutes. *cpxP-lacZ* expression was determined as described in section 2.2.7. Data represent the means and standard deviations of three biological replicates. The asterisk (*) indicates a statistically significant difference from the DMSO control (P = 0.0124 [unpaired t-test]) (B) Affect of CCCP on CpxR and CpxA protein levels in wildtype EPEC. After growth overnight in LB, EPEC were subcultured into 10mL LB and grown to an OD_{600} of approximately 0.5 at 37ºC. 100µM CCCP or an equal volume of DMSO was added and bacteria were grown for an additional 30 minutes. CpxR and CpxA protein levels were determined by western blot as described in section 2.2.9. NSB, nonspecific band.

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CHAPTER 3

Post-transcriptional regulation of respiratory complexes by the Cpx envelope stress response

Note: Christopher Chang extracted the plasmids used in figure 3-4.

3.1 Introduction

Energy production through the electron transport chain is a fundamental component of bioenergetics. Dysfunction of the human electron transport chain has been associated with several neurodegenerative diseases including Alzheimer's and Parkinson's disease as well as Leigh syndrome (300, 301). While research has uncovered a detailed mechanism of how electrons are transported through the respiratory complexes, and how this process is coupled to energy production, little work has examined the mechanism by which these multiprotein complexes are assembled within the membrane. As the respiratory enzymes found in the *E. coli* electron transport chain are homologous to human respiratory complexes, studies on the assembly of the *E. coli* respiratory complexes may provide insight into the assembly mechanisms in higher organisms.

NADH dehydrogenase, also referred to as complex I, is the entry point for electrons carried by NADH into the human and *E. coli* electron transport chain. Complex I from bovine heart mitochondria is comprised of 45 subunits and has a molecular mass of approximately 1 MDa (302). The *E. coli* homologue, known as NADH dehydrogenase I (NDH-I), consists of 13 subunits with a combined molecular mass of 550 kDa (136, 137), and represents the minimal structure required for complex I function (142). The subunits of NDH-I are transcribed from the 15 kbp *nuoA-N* operon (134). Both enzymes display a characteristic L shaped structure that consists of a hydrophobic arm embedded within the membrane and a hydrophilic arm that protrudes into the cytoplasm of *E. coli* or the mitochondrial matrix of higher organisms (141). The peripheral arm contains the flavin mononucleotide and iron-sulfur cluster cofactors involved in transferring electrons from NADH to the lipid carrier quinone (137, 145). Assembly of the *E. coli* complex occurs through the interaction of three modules (156). Oxidation of NADH occurs in the cytoplasmic dehydrogenase module, consisting of the subunits NuoE, F, and G. This module is anchored to the inner membrane by a scaffold composed of the subunits NuoA, NuoB, NuoCD, NuoI, and NuoH. NuoB, NuoCD, NuoI, and NuoH make up the hydrogenase module that guides the electrons released upon oxidation of NADH to quinone. Finally, the membrane-bound transporter subunits NuoK, L, M, and N are associated with the complex. While NuoK is assembled as an individual subunit, NuoL, M, and N form a subcomplex that is assembled *en bloc*.

The terminal oxidase cytochrome *bo₃* in the *E. coli* electron transport chain is structurally and functionally homologous to cytochrome *c* oxidase, also referred to as complex IV, in humans (174, 176). While both enzymes reduce oxygen into water, they differ in regards to the electron donating substrate. Cytochrome *bo₃* accepts electrons from quinol, while cytochrome *c* oxidase indirectly accepts electrons from quinol through cytochrome *c* (174). The *E. coli* cytochrome *bo₃* complex is comprised of four subunits encoded by the *cyoABCDE* operon (175). Subunit I, encoded by *cyoB*, is the main catalytic domain and contains the quinone-binding site, the redox centers heme *b*, heme *o*, and Cu_B, and the proton transfer pathways (175, 176). Subunits II, III and IV, encoded by *cyoA, C,* and D respectively, may play a role in the incorporation of the redox centers into subunit I (179, 180, 303). The *cyoE* gene encodes for a farnesyl transferase that is involved in heme *o* synthesis (177, 178). During cytochrome *bo₃* assembly, the apo-form of subunit I (CyoB) interacts with subunits III (CyoC) and IV (CyoD) in the inner-membrane (179). Once the heme cofactors have been inserted, subunit II (CyoA) then associates with the complex (179).

We have previously found that the expression and/or activity of NDH-I and cytochrome *bo₃* is regulated by the CpxRA envelope stress response in *E. coli* (chapter 2). The Cpx response is believed to mediate adaptation to stresses that affect protein folding within the inner membrane of the gram-negative bacterial envelope (84). CpxR and CpxA comprise a typical two-component signal transduction system, with CpxA functioning as the sensor kinase and CpxR functioning as the response regulator (31-34). Conditions predicted or known to generate misfolded proteins stimulate autophosphorylation of CpxA, which then transfers the phosphate to CpxR (60). Once phosphorylated, CpxR acts as a transcription factor to activate or repress gene transcription (84). To clear the envelope of misfolded proteins, CpxR activates the expression of several envelope-localized protein folding and degrading factors including the chaperones *cpxP*, *spy,* and *ppiD* , proteases *degP* and *htpX*, the disulfide bond oxidoreductase *dsbA* and the peptidyl-prolyl isomerase *ppiA* (24, 51-54, 65, 91). CpxR represses the expression of several macromolecular protein complexes with extensive inner membrane domains that may disrupt inner membrane integrity (100, 109, 110, 296), chapter 2). In the previous chapter (304), we provide evidence to suggest that activation of the Cpx response directly represses the transcription of the *nuo* and *cyo* operons. Furthermore, in the absence of the Cpx response transcription of these operons is slightly increased, suggesting that basal levels of Cpx pathway activation are

sufficient to mediate transcriptional repression. However, activity of the aerobic electron transport chain is substantially decreased in *E. coli* lacking the Cpx response. The discord between the transcription of the *nuo* and *cyo* operons and the activity of the electron transport chain in the *cpxRA* mutant suggest that the Cpx response may decrease the expression and/or activity of these complexes at the post-transcriptional level.

In this chapter, we analyzed the protein expression of the NuoA and CyoA subunits of the NDH-I and cytochrome bo_3 complex, respectively. We have found that activation of the Cpx response decreases NuoA and CyoA protein levels. However, NuoA and CyoA expression is not substantially affected by loss of the Cpx response. We provide evidence to suggest that several Cpx-regulated protein-folding and degrading factors that are associated with the non-specific protease FtsH alter the production of the NuoA protein. We also assessed the effect of these genes on aminoglycoside antibiotic resistance. Finally, we show that neither NDH-I nor cytochrome bo_3 significantly contribute to activation of the Cpx response by mutation of the protease *htpX* or NlpE overexpression.

3.2 Materials and Methods

3.2.1 Bacterial strains, media, and reagents

Bacterial strains and plasmids used in this study are listed in table 3-1. Bacteria were routinely cultured in Lennox Broth (LB; 10g/L tryptone [Difco], 5g/L yeast extract [Difco], 5g/L NaCl) at 37°C with shaking at 225 rpm. Strain ALN195 was grown at 30°C overnight, but was grown at 37°C after subculture. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.1mM to induce gene expression from pMPM-K3 and pCA-24N based vectors, unless otherwise indicated. Unless otherwise stated, antibiotics were added as necessary to the following concentrations: amikacin (Amk), 3μg mL^{-1}; ampicillin (Amp), 100μg mL^{-1}; chloramphenicol (Cam), 25μg mL^{-1}; kanamycin (Kan), 50μg mL^{-1}; and streptomycin (Str), 50μg mL^{-1}. All reagents were purchased from Sigma-Aldrich, unless otherwise indicated.

3.2.2 Strain construction

Plasmids were transformed into BW25113 strains via chemical competency (305). After overnight growth, *E. coli* were subcultured into 5mL fresh LB at a 1:100

dilution and grown at 37°C with shaking for 1 hour. Cells were pelleted by centrifugation at 2880 x g for 10 minutes, resuspended in 1mL cold magic formula (0.1mM MOPS [pH 6.5], 0.1mM $CaCl_2$), and incubated on ice for 30 minutes. Cells were once again pelleted by centrifugation at 2880 x g for 10 minutes. The pellet was resuspended in 200µL cold magic formula. Following the addition of 1-5µL of plasmid DNA, cells were incubated on ice for 10 minutes. Bacteria were heat shocked at 42°C for 30 seconds and briefly returned to ice. 1mL of LB was added and bacteria were recovered at 37°C for 45 minutes. 100µL of culture was transferred to a selective agar medium while the remaining bacteria were pelleted by centrifugation at 2880 x g. The pellet was resuspended in approximately 100µL of LB and plated on a selective agar medium. Transformants were grown overnight at 37°C.

Plasmids were transformed into E2348/69 strains via electroporation. After overnight growth, EPEC were subcultured into 50mL LB in a 250mL Erlenmeyer flask at a dilution factor of 1:100 and grown at 37°C with shaking to an OD_{600} of 0.5. Bacteria were pelleted by centrifugation at 2880 x g for 10 minutes, resuspended in 2mL of LB, and incubated in a 50°C water bath for 30 minutes. After cooling on ice, EPEC were pelleted by centrifugation at 21,130 x g for 1 minute and resuspended in 1mL cold 10% glycerol. This step was repeated three times, however EPEC were resuspended in 500µL, then 200µL, then 200µL cold 10% glycerol rather than 1mL cold 10% glycerol. 50µL of washed EPEC were transferred to a 0.6mL microfuge tube and 1-5µL of plasmid DNA was added. This was then transferred to a cold 0.2cm electroportation cuvette (BioRad) and incubated on ice for 2 minutes. Plasmid DNA was then electroporated into bacteria via the MicroPulser™ electroportator (BioRad) on the EC2 setting (2.50kV, 5ms). Bacteria were immediately resuspended in 1mL LB, transferred to a 13x100mm test tube, and recovered at 37°C with shaking for 2 hours. ALN195 strains were recovered at 30°C. 100µL of bacteria were plated on a selective agar medium. The remaining bacteria were pelleted by centrifugation at 2880 x g for 10 minutes, resuspended in the residual medium left after pouring off the supernatant, and plated on a selective agar medium. Transformants were grown overnight at 37°C, with the exception of ALN195 strain backgrounds, in which transformants were grown at 30°C overnight. pMPM-K3 based vectors were passed though EPEC strain NH4 prior to transformation into other EPEC strains (306).

To generate BW25113 Δ*nuoA*, the FRT-flanked kanamycin resistance cassette within the *nuoA* gene was removed from strain JW2283 of the keio library (307) as

previously described (308). The site-specific excision vector pFLP2, which expresses the FLP recombinase from a temperature sensitive promoter, was transformed into JW2283 according to the protocol described above with the notable exception that bacteria were recovered at 30°C after heat shock. Transformants were selected for on LB agar containing ampicillin at 37°C, which induces the expression of the FLP recombinase from the pFLP2 vector. Transformants were screened for kanamycin sensitivity, indicating loss of the kanamycin resistance cassette. The resulting construct contains the start codon and the six C-terminal codons of *nuoA* separated by an in-frame scar sequence. To cure the resulting BW25113 Δ*nuoA* strain of pFLP2, cells were grown for six hours in 2mL liquid LB at 37°C with shaking at 225 rpm. 100µL of a 10^{-2}, 10^{-3}, and 10^{-4} dilution of this culture were each plated on LB agar lacking NaCl and containing 5% sucrose. 100µL of the 10^{-4} dilution was also plated on plain LB agar as a control to confirm that sucrose selection worked properly. Sucrose resistant colonies were screened for ampicillin sensitivity, indicating loss of the pFLP2 plasmid. BW25112 Δ*cyoA* was constructed from JW0422 of the keio library (307) as described above.

Deletion of the *nuoABCDEFGHIJKLMN* and *cyoABCDE* operons in TR50 was performed by lamba-red recombinase as previously described (309). Primer sequences were obtained from (307) (primer sequences, including restriction endonuclease cut sites, are listed in table 3-2). The DNA sequence of the K12nuoKOF primer corresponds to the 5' primer used to delete *nuoA* in (307), while sequence of the K12nuoKOR primer corresponds to the 3' primer used to delete *nuoN* (307). PCR was performed using high-fidelity phusion DNA polymerase (ThermoFisher) according to the manufacturers specifications with the addition of 20% betaine. K12nuoKOF and K12nuoKOR were used to amplify the FRT-flanked kanamycin resistance cassette from the keio library (307). DNA was separated by electrophoresis on a 1% agarose gel. A DNA fragment approximately the size of the kanamycin resistance cassette with 50bp flanking regions homologous to the *nuo* or *cyo* operon was extracted and cleaned using the GeneJet gel purification kit (Fermentas). DNA concentration was determined using the NanoDrop 2000c spectrophotometer. This DNA fragment was used to delete the *nuoA-N* locus in *E. coli* strain DY378, which encodes the lambda-red recombinase system from a temperature sensitive promoter (310). Briefly, DY378 was grown to an OD_{600} of 0.4-0.5 in 35mL of LB in a 250mL Erlenmeyer flask at 30°C with shaking at 225 rpm. Half of this culture was then transferred to a 125mL flask and incubated in a 42°C shaking water bath for 15 minutes, while the other half was incubated at 30°C as before. Cells were

washed three times in sequentially lower volumes of ice-cold distilled water, terminating with cells resuspended in 200µL ice-cold distilled water. 100ng or 300ng of purified kanamycin resistance cassette DNA with *nuo* or *cyo* homologous ends was electroportated into DY378 and cells were recovered at 30ºC with shaking for 2 hours. Recombinants were selected for on LB agar supplemented with kanamycin. Presence of the kanamycin resistance cassette was confirmed by PCR using primers PnuoEMSAF and K12nuo(del)R (Table 3-3), which flank the *nuo* operon and can only generate a product if the *nuo* operon has been deleted. Recombinant taq was used according to the manufacturers instructions, with the addition of 10% betaine, for PCR. PCR cycle conditions were are follows: denaturing stage, 96ºC for 30 seconds; annealing stage, 60ºC for 45 seconds; extending stage, 72ºC for 1 minute and 45 seconds.

A similar strategy was used to delete the *cyo* operon. Primer K12-cyoKOF corresponds to the 5' primer used to delete *cyoA* in (307), and primer K12-cyoKOR corresponds to the 3' primer used to delete *cyoE* (307). All subsequent steps are the same as for deletion of the *nuo* operon described above, with the exception of the confirmation PCR. To confirm the presence of the kanamycin resistance cassette within the *cyo* operon three separate PCR reactions were performed. All confirmation PCR reactions were performed with recombinant taq according to the manufacturers specifications with the addition of 10% betaine. The first PCR used primers cyoF and cyoR (Table 3-3) that flank the *cyo* operon. The second confirmation PCR used primers cyoF and K1 (Table 3-3). As K1 binds within the kanamycin resistance cassette, a product will be formed only if the kanamycin resistance cassette has replaced the majority of the *cyo* operon. The third confirmation PCR used primers K2 and cyoR (Table 3-3). Like K1, primer K2 binds within the kanamycin resistance cassette. Sequences of the K1 and K2 primers were obtained from (311). PCR reaction conditions were as follows: denaturing stage, 96ºC for 30 seconds; annealing stage, 60ºC for 45 seconds; extending stage, 72ºC for 2 minute and 10 seconds.

P1 transduction was used to move the *nuo::kan*, *cyo::kan*, and *htpX::kan* constructs into the desired strains as previously described (305). *htpX::kan* was obtained from the keio library (307). After overnight growth in LB broth at 37ºC for strain JW1818 and 30ºC for RG383 and RG397, bacteria were diluted into LB supplemented with 0.2% glucose and 5mM $CaCl_2$. Bacteria were grown at 37ºC or 30ºC for 30 minutes, at which point 100µL of P1*vir* lysate was added to the culture. Bacteria were then incubated at 37ºC or 30ºC until the culture had cleared. Intact cells were pelleted by centrifugation at

2880 x g for 10 minutes and the supernatant was filtered though a 0.22µm syringe-tip filter. An overnight culture of the recipient bacteria was centrifuged at 2880 x g and bacteria were resuspended in 10mM $MgCl_2$ containing 5mM $CaCl_2$. 100µL of the recipient bacteria were combined with 100µL of P1*vir* phage lysate grown from the donor strain and incubated at 30°C statically for 30 minutes. Following the addition of 1mL LB supplemented with 10mM sodium citrate, bacteria were recovered at 37°C statically for 45 minutes. Bacteria were pelleted by centrifugation at 2880 x g for 10 minutes and bacteria were resuspended in the residual medium left over after the supernatant was removed. Transductants were selected for on LB agar supplemented with 30µg mL^{-1} kanamycin. The kanamycin resistance cassette was removed as necessary using the FLP-site specific recombinase as described above. After removing the kanamycin resistance cassette from the *nuo* and *cyo* mutants, the locus contains the start codon of *nuoA* or *cyoA* and the six C-terminal codons of *nuoN* or *cyoE*, respectively, separated by an in-frame scar sequence.

3.2.3 Construction of FLAG-tagged fusion proteins

NuoA was triple FLAG-tagged at its C-terminus. *nuoA* was amplified from the E2348/69 chromosome via PCR using primers nuoAFLAGFwd and nuoAFLAGrev. Primer nuoAFLAGfwd binds directly upstream of the *nuoA* start codon to include the putative NuoA Shine-Dalgarno sequence. Primer nuoAFLAGrev contains the nucleotide sequence to insert a triple FLAG-tag directly upstream of the *nuoA* stop codon. PCR was performed using high-fidelity phusion DNA polymerase (ThermoFisher) according to the manufacturers protocol with the addition of 10% betaine. PCR cycle conditions were as follows: denaturing stage, 96°C for 30 seconds; annealing stage, 62°C for 45 seconds; extending stage, 72°C for 35 seconds. DNA was separated by electrophoresis on a 1% agarose gel and the band corresponding to the *nuoA*-3xFLAG DNA was extracted and cleaned using the GeneJet gel purification kit (Fermentas). Both the *nuoA*-3xFLAG and pMPM-K3 DNA were digested with the HindIII and XbaI restriction endonucleases (Invitrogen) according to the manufacturers protocol. The *nuoA*-3xFLAG DNA was then ligated downstream of an IPTG inducible promoter in the pMPM-K3 vector, and transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen) as per the manufacturers protocol. Transformants were selected for on LB agar supplemented with kanamycin and 40µg mL-1 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plasmids from white colonies were extracted using the GenElute plasmid DNA miniprep

kit (Sigma-Aldrich) as per the manufacturers instructions. PCR and DNA sequencing were used to confirm the presence of the *nuoA*-3xFLAG fragment within pMPM-K3. The confirmation PCR was performed using recombinant taq (Invitrogen) according to the manufacturers specifications with the addition of 10% betaine. Primers M13F and M13R, which bind within the LacZ α-fragment that flanks the pMPM-K3 multiple cloning site, were used. PCR cycle conditions were as follows: denaturing stage, 96°C for 30 seconds; annealing stage, 57°C for 45 seconds; extending stage, 72°C for 35 seconds. The University of Alberta Molecular Biology Service Unit (MBSU) performed DNA sequencing.

A similar strategy was used to generate the CyoA-3xFLAG construct. *cyoA* was amplified from the E2348/69 chromosome using primers cyoAFLAGfwd and cyoAFLAGrev. All subsequent steps, including PCR cycle conditions and restriction enzymes used, are the same as described for generation of pMPM-*nuoA*-3xFLAG.

3.2.4 Complementation assay

For Figure 3-1A, wildtype BW25113 or the Δ*nuoA* mutant containing either the pMPM-K3 empty vector or pMPM-*nuoA*-3xFLAG were grown overnight in 5mL LB at 37°C with shaking at 225 rpm. The following day, 1mL of culture was pelleted by centrifugation at 21,130 x g for 1 minute. The pellet was resuspended in 1mL 1x concentrated phosphate-buffered saline and pelleted by centrifugation as before. Again, the pellet was resuspended in 1mL 1x concentrated phosphate-buffered saline. The OD_{600} of each strain was standardized to 1.0 and then cultures were serially diluted by a factor of 10. 10µL of each of the 10^{0}-10^{-7} dilutions were spotted on M9 minimal medium (Difco) agar containing 0.4% glucose or 0.4% malic acid, pH 7.0 (malate). Bacteria were grown for 24 hours at 37°C. Images were taken using the UVP Colony Doc-It Imaging Station.

For Figure 3-1B, wildtype BW25113 or the Δ*cyoA* mutant containing either the pMPM-K3 empty vector or pMPM-*cyoA*-3xFLAG were streaked out onto LB agar from frozen glycerol stocks and grown overnight at 37°C. Single, isolated colonies were then streaked onto M9 minimal medium (Difco) agar containing 0.4% glucose or 0.4% malic acid, pH 7.0 (malate). Bacteria were grown for 24 hours at 37°C. Images were taken as above.

3.2.5 Western blotting

For Figure 3-2 and Figure 3-3A, bacteria were subcultured into 25mL LB in a 250mL Erlenmeyer flask at a dilution factor of 1:100 after overnight growth in 5mL LB at 37°C with shaking. Bacteria were grown at 37°C with shaking to an OD_{600} of approximately 0.35. IPTG was added to a concentration of 0.1mM and bacteria were grown for an additional 30 minutes as before. 2 x 1mL samples were collected. Cells were pelleted by centrifugation at 21,130 x g for 1 minute. One sample was resuspended in 50µL 2x laemmli sample buffer (Sigma) and the other sample was resuspended in 50µL 1x concentrated phosphate-buffered saline. Protein concentration was determined from the sample resuspended in phosphate-buffered saline using the Pierce BCA protein assay kit (ThermoFisher) according to the manufacturers protocol. Samples were incubated in a boiling water bath for 5 minutes. 20µg of protein was loaded onto a 10% (α-CyoA-3xFLAG and α-PhoA) or 15% (α-NuoA-3xFLAG) SDS polyacrylamide gel.

For Figure 3-4C and Figure 3-4D, EPEC were subcultured into 10mL LB containing 0.1mM IPTG in a 10mL Erlenmeyer flask at a dilution factor of 1:100 and were grown to an OD_{600} of 0.5 at 37°C with shaking at 225 rpm. Approximately 1 x 10^9 cells mL^{-1} were collected, pelleted by centrifugation at 21,130 x g for 1 minute, and resuspended in 50µL 2x Laemmli sample buffer. 10µL of each sample was loaded onto a 15% SDS polyacrylamide gel.

Samples were electrophoresed at 110V for 1.5 hours in Tris-glycine running buffer (10% SDS, 250mM unbuffered Tris, 1.2M glycine). Proteins were transferred onto a nitrocellulose membrane via the trans-blot semi-dry transfer system (Bio-Rad) at 10V for 30 minutes using semi-dry transfer buffer (78mM glycine, 1.3mM SDS, 20% methanol). Membranes were blocked in 2.5% MTS (2.5% skim milk powder, 154mM NaCl, 1mM unbuffered Tris) for 1.5 hours at room temperature with shaking at approximately 100 rpm. Primary α-FLAG (Sigma) and α-PhoA antibodies were diluted by a factor of 1:5000 and 1:25000 into 2.5% MTS, respectively. Membranes were incubated with the primary antibody for 1 hour at either room temperature with shaking at approximately 100 rpm (α-PhoA) or 30°C with shaking at 225 rpm (α-FLAG). Following incubation with the primary antibody, membranes were washed for 20 minutes in wash solution (154mM NaCl, 1mM unbuffered Tris, 0.5% Tween 20) three times. Membranes were then incubated with the secondary antibody for 1 hour at room temperature with shaking at approximately 100 rpm. Alkaline-phosphatase anti-rabbit secondary antibodies (Sigma) were diluted at a factor of 1:25000 in 2.5% MTS, and were used to recognize the α-PhoA primary antibody. Alkaline-phosphatase anti-mouse secondary

antibodies (Sigma) were used at a 1:10000 dilution in 2.5% MTS, and were used to recognize the α-FLAG primary antibody. Membranes were washed following incubation with the secondary antibody as before. Proteins were detected using the Immun-Star alkaline phosphatase chemiluminescence kit (Bio-Rad) and the Bio-Rad ChemiDoc MP imaging system. Quantification of each band was compared to the wildtype using the Bio-Rad ChemiDoc MP imaging software.

3.2.6 Protein stability assay

Bacteria were grown overnight in 5mL LB at 37°C with shaking. The following day, bacteria were subcultured at a 1:100 dilution into 25mL LB in a 250mL Erlenmeyer flask and grown at 37°C with shaking to an OD_{600} of approximately 0.5. IPTG was then added to a final concentration of 0.1mM and bacteria were grown as before to an OD_{600} of 1. Approximately 1x 10^9 cells mL^{-1} (corresponding to an OD_{600} of 1) were collected and cells were pelleted by centrifugation at 21,130 x g for 1 minute. After the supernatant was removed, the pelleted bacteria were resuspended in 50µL 2x Laemmli sample buffer. Immediately after the sample was removed, the protein synthesis inhibitor chloramphenicol was added to the remaining culture to a concentration of 100µg mL^{-1}. The culture was returned to the 37°C incubator and shaken at 225 rpm. 1 x 10^9 cells mL^{-1} were collected 1, 5, 10, 20, 30, 45, 90, and 120 minute(s) after the addition of chloramphenicol. Cells were collected, pelleted by centrifugation, and resuspended in 50µL 2x Laemmli sample buffer. 10µL of each sample was loaded onto a 15% SDS polyacrylamide gel. Incubation with primary and secondary antibodies, washes and detection were performed as described in section 3.2.5. The relative amount of protein compared to the zero time point was determined using the Bio-Rad ChemiDoc MP imaging software. Relative quantification data were fit with a one-phase exponential decay curve to determine protein half-life using the Prism v7.0c (GraphPad) software.

3.2.7 Dot blotting

Strain RG480 harboring overexpression plasmids from the ASKA library, pTrc99A, or pCpxP were grown overnight in 5mL LB at 37°C with shaking at 225 rpm. The following day, bacteria were subcultured into 10mL LB in a 125mL Erlenmeyer flask at a dilution factor of 1:100 and grown at 37°C with shaking to exponential phase. IPTG was added to a final concentration of 1mM to induce gene expression and bacteria were grown to an OD_{600} of approximately 1. Approximately 1 x 10^9 cells mL^{-1} (corresponding

to an OD_{600} of 1) were collected and cells were pelleted by centrifugation at 21,130 x g for 1 minute. Cells were resuspended in 50µL 2 x sample buffer lacking dye (125mM Tris [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 6% SDS). Cells were incubated in a boiling water bath for 5 minutes, followed by cooling at room temperature for 10 minutes. 3µL of sample was spotted onto a nitrocellulose membrane and spots were allowed to dry. Incubation with primary and secondary antibodies, washes, detection, and quantification were performed as described in section 3.2.5. Dots corresponding to strains containing the empty pMPM-K3 vector were not detected by the α-FLAG antibody (data not shown). The candidate gene list was derived from previous publications (64, 65), as well as unpublished RNA-Seq data (data not shown).

3.2.8. Efficiency of plating assay

The indicated bacteria were grown overnight in 5mL LB at 37°C with shaking at 225 rpm. The next day, the OD_{600} of each culture was standardized to 1 and this was serially diluted by a factor of 10. 10µL of each of the 10^{0}-10^{-7} dilutions was spotted on LB agar or LB agar supplemented with 1.5µg mL^{-1} amikacin. All LB agar contained 0.1mM IPTG to induce gene expression. Bacteria were grown overnight at 37°C. Images were taken using the UVP Colony Doc-It Imaging Station.

3.2.9 β-galactosidase assay

Bacteria were grown overnight in 2mL LB at 37°C with shaking at 225 rpm, subcultured into 2mL fresh LB at a 1:100 dilution, and grown at 37°C with shaking for 6 hours. The β-galactosidase assay was performed as described in (76). Cells were pelleted by centrifugation at 2880 x g for 10 minutes. The supernatant was removed and cells were resuspended in 2mL of 1x concentrated Z-buffer (10mL 10x concentrated Z-buffer [600mM $Na_2HPO_4•7H_2O$, 400mM $NaH_2PO_4•H_2O$, 100mM KCl, 10mM $MgSO_4•7H_2O$], 90mL distilled water, 270µL β-mercaptoethanol). 250µL of culture was then transferred to a 96-well microtiter plate and the OD_{600} was read using the PerkinElmer Wallac Victor^2 1420 plate reader. Cells were then lysed with chlorophorm and SDS. 5µL of sample was diluted into 195µL of 1x concentrated Z-buffer. 50µL of 10mg/mL *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added. The absorbance at 420nm (A_{420}) was read to measure hydrolysis of ONPG. A_{420} was measured 20 times with a 45 second break between each measurement. β-galactosidase activity was

normalized to growth (OD_{600}) to account for slight differences in cell number between samples.

3.2.10 Luminescence assay

Wildtype EPEC, the *nuo* and *cyo* single mutants, and the *nuo cyo* double mutants containing the pCA-*nlpE* vector or its vector control (Table 3-1) were grown overnight in 2mL LB at 37°C with shaking. The following day, bacteria were subcultured into 2mL LB at a dilution of 1:100 and grown at 37°C with shaking for 2 hours. IPTG was added to a final concentration of 0.1mM and 200µL of culture was transferred into a black-walled 96 well microtiter plate. Bacteria were then grown in the 96 well plate for an additional 2 hours at 37°C with shaking at 225 rpm. Luminescence (expressed as counts per second [CPS]) and OD_{600} were measured using the PerkinElmer Wallac Victor^2 1420 plate reader. Luminescence and OD_{600} values measured from a blank well containing uncultured LB were subtracted from each sample. CPS was standardized to the OD_{600} to correct for differences in cell numbers between samples.

3.2.10 Statistical analysis

Statistical analysis was performed using Prism version 7.0c (GraphPad Software). Activity of transcriptional reporters was compared by two-way analysis of variance followed by Sidak's multiple comparison test.

3.3 Results

3.3.1 FLAG-tagged CyoA and NuoA fusion proteins are functional

To monitor the effect of the Cpx response on NDH-I protein levels, we generated a plasmid vector that expresses triple FLAG-tagged NuoA. NuoA is part of the membrane arm of NDH-I and is thought to be the scaffold on which NDH-I assembles within the inner membrane (156). While NuoA is not part of a NDH-I functional module (156), loss of NuoA decreases NADH oxidase activity (160). The triple FLAG-tag was inserted directly upstream of the *nuoA* stop codon via PCR. Transcription of *nuoA* is initiated from an IPTG inducible promoter in the pMPM-K3 vector that is not thought to be regulated by the Cpx pathway. As such, any changes observed in NuoA-3xFLAG protein levels are due to regulation at the post-transcriptional level.

To ensure that the NuoA-3xFLAG fusion protein is functional, we carried out a complementation assay to determine if NuoA-3xFLAG can rescue the growth defect of the *nuoA* mutant on minimal medium containing malate, which is a non-fermentable carbon source. While the reason *nuo* mutants cannot grow using malate as the sole carbon source is unknown, it is has been hypothesized that *nuo* mutants cannot generate sufficient energy to support growth under these conditions (312). As expected, the *nuoA* mutant containing the empty pMPM-K3 vector has a growth defect on malate minimal medium compared to the wildtype harboring the empty vector (Figure 3-1A). Expression of the NuoA-3xFLAG restores growth of the *nuoA* mutant on malate minimal medium (Figure 3-1A), suggesting that the NuoA-3xFLAG fusion protein is functional. All strains are capable of growth on glucose minimal medium (Figure 3-1A).

We employed a similar strategy to construct a C-terminal triple FLAG-tagged CyoA expression vector to monitor cytochrome bo_3 protein levels. CyoA is thought to stabilize the catalytic subunit CyoB (176). The proposed low affinity quinone-binding site is thought to be located in CyoA (176). Furthermore, overexpression of CyoA activates the Cpx response (304) (section 2.3.4). As with NuoA-3xFLAG, we confirmed that the CyoA-3xFLAG fusion protein is functional by complementing the growth defect of the *cyoA* mutant on minimal medium containing malate. The *cyoA* mutant harboring the empty vector grew poorly on malate minimal medium in comparison to the wildtype, but was capable of growth on glucose minimal medium (Figure 3-1B). Expression of the CyoA-3xFLAG protein rescued the growth defect of the *cyoA* mutant on malate minimal medium (Figure 3-1B), suggesting that the fusion protein is functional. As both NuoA-3xFLAG and CyoA-3xFLAG encode functional proteins, these constructs can be used to determine whether the Cpx response alters NuoA or CyoA protein levels, respectively.

3.3.2 Activation of the Cpx response regulates CyoA protein levels

To determine whether the Cpx response regulates CyoA protein expression at the post-transcriptional level, we determined expression of CyoA-3xFLAG in wildtype EPEC and the EPEC *cpxA24* mutant in which the Cpx response is constitutively active. Expression of CyoA-3xFLAG in the *cpxA24* mutant was 36% that of CyoA-3xFLAG expression in wildtype EPEC (Figure 3-2). As the CyoA-3xFLAG construct is expressed from an IPTG inducible promoter, this effect is not likely due to changes in transcription. As such, these results suggest that activation of the Cpx response decreases CyoA protein levels in a post-transcriptional manner. Given that the respiration rate of the

cpxRA mutant is similar to EPEC lacking cytochrome bo_3 (304) (section 2.3.2), we hypothesized that inhibition of the Cpx response would also decrease CyoA-3xFLAG expression. However, levels of CyoA-3xFLAG protein were nearly identical in the wildtype and *cpxRA* mutant (Figure 3-2). These data suggest that inhibition of the Cpx response does not decrease activity of the cytochrome bo_3 complex by disrupting CyoA protein levels.

3.3.3 Activation of the Cpx response regulates NuoA protein levels

We also wished to determine whether activation of the Cpx response exerts post-transcriptional effects on NuoA of NDH-I. As with transcription of the *nuo* and *cyo* operons (304) (section 2.3.1), we observed a greater decrease in NuoA-3xFLAG levels than CyoA-3xFLAG levels when the Cpx response was activated. Expression of NuoA-3xFLAG in the *cpxA24* mutant was 8% that of NuoA-3xFLAG expression in the wildtype (Figure 3-3A). NuoA-3xFLAG levels were increased by 14% in the *cpxRA* mutant compared to the wildtype (Figure 3-3A). Together, these results suggest that the Cpx response post-transcriptionally regulates NuoA.

There are two possible explanations for decreased levels of CyoA-3xFLAG and NuoA-3xFLAG in the *cpxA24* mutant. The Cpx response regulates several factors that affect translation, and as such, activation of the Cpx response may decrease translation of *cyoA*-3xFLAG and *nuoA*-3xFLAG mRNA. Alternatively, proteases regulated by the Cpx response may degrade existing CyoA-3xFLAG and NuoA-3xFLAG proteins. To test the latter possibility, we performed a protein stability assay to determine the rate at which NuoA-3xFLAG is degraded once translation has been inhibited by the addition of the protein synthesis inhibitor chloramphenicol. Through this method, we were able to determine that the half-life of NuoA-3xFLAG in wildtype EPEC is approximately 4.00 minutes (Figure 3-3B and Figure 3-3C). In the *cpxRA* mutant, NuoA-3xFLAG half-life is increased to 8.14 minutes (Figure 3-3B and Figure 3-3C). Unexpectedly, the half-life of NuoA-3xFLAG is increased in the *cpxA24* mutant to 12.87 minutes. However, the total amount of NuoA-3xFLAG degraded in the *cpxA24* mutant over the course of the experiment is greater than in the wildtype (Figure 3-3B and Figure 3-3C). After 120 minutes, approximately 35% of the NuoA-3xFLAG protein is left remaining in the wildtype (Figure 3-3B and Figure 3-3C). At this same time point, only 7% of the NuoA-3xFLAG remains in the *cpxA24* mutant (Figure 3-3B and Figure 3-3C). This experiment may be complicated by the fact that chloramphenicol inhibits total protein synthesis.

Nonetheless, we believe that these data support the hypothesis that proteases regulated by the Cpx response degrade NuoA-3xFLAG. It is important to note that addition of the FLAG-tag may alter the specificity of NuoA for proteases regulated by the Cpx response. At this point in time, we are unable to rule out this possibility.

3.3.4 Cpx-regulated protein folding and degrading factors affect NuoA protein levels

As activation of the Cpx response decreases abundance of the NuoA protein, we hypothesized that Cpx-regulated protein folding and degrading factors may affect NuoA protein levels. To test this, we overexpressed several Cpx-activated genes in *E. coli* strain BW25113 Δ*nuoA* containing the NuoA-3xFLAG expression vector and analyzed NuoA-3xFLAG protein levels by dot blot. A description of the proteins overproduced in this study can be found in table 3-3. Overproduction of several Cpx-regulated protein folding and degrading factors altered NuoA-3xFLAG protein levels compared to the vector control (Figure 3-4A). Overexpression of DegP, HtpX, PpiD, and YccA increased NuoA-3xFLAG levels by a factor of 3.33, 17.75, 3.07, 2.59, respectively, in comparison to the vector control (Figure 3-4A). Overexpression of Spy increased NuoA-3xFLAG protein levels less than two-fold (Figure 3-4A). Furthermore, expression of NuoA-3xFLAG was 0.49 and 0.59 that of the vector control when FtsH or HflC were overexpressed, respectively (Figure 3-4A). CpxP, which was expressed from a different plasmid than the other genes tested, decreased NuoA-3xFLAG protein levels to 0.33 that of the vector control (Figure 3-4B).

We also tested this hypothesis in EPEC by analyzing the steady-state level of NuoA-3xFLAG in strains overexpressing DegP, DsbA, HtpX, Spy, YccA, and CpxP by western blot. Expression of NuoA-3xFLAG was also analyzed in EPEC overexpressing YebE, which was not included in the above screen. As observed in BW25113, overexpression of DegP, HtpX, and YccA increased NuoA-3xFLAG protein levels compared to the vector control in EPEC (Figure 3-4C). In contrast to the previous results, NuoA-3xFLAG levels were unaffected by overexpression of CpxP (Figure 3-4D). Overall, these results demonstrate that several Cpx-regulated protein folding and degrading factors affect abundance of the NuoA protein and support the hypothesis that the Cpx response regulates NuoA at the post-translational level.

3.3.5 Cpx-regulated factors affect amikacin resistance

Previously, we have shown that improper regulation of NDH-I and cytochrome *bo*$_3$ in the *cpxRA* mutant decreases resistance to the aminoglycoside antibiotic amikacin (304) (section 2.3.3). As such, we hypothesized that Cpx-regulated factors that decrease NDH-I and/or cytochrome *bo*$_3$ activity would increase resistance of the *cpxRA* mutant to aminoglycoside antibiotics. To test this, we overproduced several Cpx-regulated proteins in the *cpxRA* mutant and measured sensitivity by an efficiency of plating assay in the presence of 1.5μg mL^{-1} amikacin. Cpx-regulated proteins overexpressed include several chaperones and proteases, NuoF, which is the NADH binding component of NDH-I and inhibits the Cpx response when overexpressed (134, 143) (JL Wong, J Wang, and TL Raivio, unpublished observation), YceI, which is thought to bind to the isoprenoid chain of quinones (313), YceJ, which encodes a putative cytochrome, and YqaE, which is homologous to proton leak proteins in yeast (314). Wildtype EPEC expressing the empty vector grew at all dilutions, while the cpxRA mutant grew to the 10^{-3} dilution (Figure 3-5A). Overexpression of CpxP, YccA, NuoF, and YebE in the *cpxRA* mutant increased efficiency of plating by four logs compared to the *cpxRA* mutant expressing the control vector (Figure 3-5A). Furthermore, overexpression of PpiA, YceI, and YqaE in the *cpxRA* mutant increased efficiency of plating by 1-2 logs (Figure 3-5A). All strains grew to the 10^{-7} dilution on plain LB agar (Figure 3-5B), indicating that bacteria are capable of growth in the absence of stress. It is noteworthy that overexpression of HtpX or DegP did not increase resistance to amikacin, as these proteins affect NuoA-3xFLAG levels. Likewise, overexpression of NuoF, YebE, and YqaE increased resistance to amikacin, but did not affect expression of the NuoA-3XFLAG protein (Figure 3-4A). The effect of YceI on NuoA-3XFLAG protein levels remains to be determined. Nonetheless, these results demonstrate that several Cpx-regulated proteins promote resistance to the aminoglycoside antibiotic amikacin, potentially by influencing expression or activity of respiratory complexes.

3.3.6 Induction of the Cpx response by envelope stress is not dependent on NDH-I or cytochrome *bo*$_3$

A previous study found that deletion of *htpX* activates the Cpx response (24). As overexpression of HtpX substantially increases NuoA-3xFLAG levels (Figure 3-4A and Figure 3-4C), we wondered whether improper expression or assembly of the NDH-I complex in the *htpX* mutant is responsible for Cpx pathway activation. To determine Cpx pathway activity, we monitored the expression of a *cpxP-lacZ* transcriptional reporter.

Cpx pathway activity is increased approximately two-fold in the *htpX* mutant compared to the wildtype strain (Figure 3-6A). However, a two-fold increase in Cpx pathway activity was still observed in the *htpX* mutant lacking the entire *nuo* operon (Figure 3-6A), suggesting that *htpX*-mediated activation does not require NDH-I. Given that cytochrome bo_3 influences Cpx pathway activity (304) (section 2.3.4), we also determined whether cytochrome bo_3 contributes to pathway activation in the *htpX* mutant. However, a two-fold increase in pathway activity was still observed in the *htpX cyo* double mutant and the *htpX cyo nuo* triple mutant in comparison to the *cyo* single mutant and *cyo nuo* double mutant, respectively (Figure 3-6A).

We wondered whether NDH-I or cytochrome bo_3 contribute to Cpx pathway activation upon overexpression of the outer-membrane lipoprotein NlpE in EPEC. Cpx pathway activity was monitored by measuring activity of a *cpxP* luminescent transcriptional reporter. Overexpression of NlpE caused a 237-fold increase in *cpxP-lux* activity compared to the vector control in wildtype EPEC (Figure 3-6B). Overexpression of NlpE in the *nuo, cyo*, or *nuo cyo* mutants resulted in a 198-, 216-, and 358-fold increase in *cpxP-lux* activity, respectively, in comparison to the same mutant containing the control vector (Figure 3-6B). Overall, these results suggest that NDH-I and cytochrome bo_3 do not substantially contribute to Cpx pathway activation under conditions of envelope stress caused by deletion of *htpX* or overexpression of NlpE.

3.4 Discussion

Activation of the Cpx response is believed to mediate adaptation to stresses that generate misfolded proteins within the envelope by facilitating their degradation. However, the Cpx response is known to affect the stability of relatively few proteins, including subunits of the bundle-forming pilus (110), the P pilus (315), and the type three secretion system (101). In this study, we have determined that activation of the Cpx response decreases NuoA and CyoA protein levels. We have identified several Cpx-regulated proteins that affect expression of the NuoA protein, some of which also promote resistance to the aminoglycoside antibiotic amikacin. One of these, HtpX, activates the Cpx response in a manner that does not require NDH-I or cytochrome bo_3.

Decreased abundance of the NuoA-3xFLAG and CyoA-3xFLAG proteins in the *cpxA24* mutant is not likely due to decreased translation. Two lines of evidence support this conclusion. First, mutational activation of the Cpx response does not alter production

of alkaline phosphatase (PhoA) (Figure 3-2 and Figure 3-3A), suggesting that the Cpx response does not decrease translation in general. Second, less NuoA-3xFLAG is present in the *cpxA24* mutant than in wildtype EPEC 120 minutes after translation has been chemically inhibited (Figure 3-2B and Figure 3-2C). Together, these results support the conclusion that activation of the Cpx response increases proteolysis of NuoA and CyoA.

We found that overexpression of several Cpx-regulated proteins alters NuoA-3xFLAG protein levels, including DegP, YccA, HtpX, HflC, and PpiD. Intriguingly, all of these proteins have been associated with the zinc metalloprotease FtsH (diagramed in Figure 3-7). HflC, YccA, and HtpX are part of the FtsH interactome and are thought to modulate FtsH activity or substrate recognition (20). DegP degrades another member of the FtsH interactome, HflK (316). While overexpression of HflK did not substantially impact NuoA-3xFLAG protein levels (Figure 3-4A), it is possible that in its absence FtsH activity is altered. Finally, FtsH degrades the chaperone PpiD (317). Accordingly, we propose that FtsH mediates degradation of NuoA upon activation of the Cpx response. In support of this hypothesis, overexpression of FtsH decreased NuoA-3xFLAG protein levels (Figure 3-4A). However, as *ftsH* is essential in *E. coli* (318), we were unable to determine if FtsH is required for Cpx-mediated degradation of NuoA-3xFLAG. While YccA, HtpX, HflC, DegP, and PpiD affect NuoA expression to different extents when individually overexpressed, it is possible that their combined activities during Cpx pathway activation stimulate degradation of the NuoA subunit of NDH-I by FtsH.

As a secondary approach to identify Cpx-regulated proteins that affect NDH-I and/or cytochrome bo_3 expression, we overexpressed several genes whose expression is activated by the Cpx response and screened for increased resistance to aminoglycoside in the *cpxRA* mutant. We found that overexpression of CpxP, NuoF, YebE, PpiA, YceI, and YqaE increased amikacin resistance in the *cpxRA* mutant to some extent (Figure 3-5). As these factors did not alter NuoA-3xFLAG protein levels (Figure 3-4A, Figure 3-4C, and Figure 3-4D) we are not able to determine whether these proteins promote amikacin resistance by altering activity of NDH-I. As aminoglycoside antibiotics are predicted to generate misfolded proteins (319), it is possible that overexpression of the chaperones CpxP and PpiA reduces protein-folding stress. In this regard, however, it is unclear why overexpression of the chaperone/protease DegP did not impact amikacin resistance. NuoF is the NADH-binding subunit of the dehydrogenase module in NDH-I (134, 143). As such, it is possible that overexpression

of NuoF titrates NADH from the NDH-I complex, thus reducing activity of NDH-I. The function of YebE, YceI, and YqaE is unknown. YqaE is homologous to the Pmp3 protein in yeast (unpublished observation), which is predicted to function as a proton leak (314). Overexpression of YqaE may dissipate the proton motive force and reduce uptake of amikacin. While we did not test whether YceI alters NuoA-3xFLAG protein levels, YceI may promote amikacin resistance by binding to isoprenoid chains, such as those found in quinones (313). Overexpression of YceI may influence the metabolism, transport, or storage of isoprenoid quinones (313), which could alter activity of the electron transport chain. Overall, we have identified several Cpx-regulated proteins that may contribute to amikacin resistance. Whether this occurs due to changes in the activity of respiratory complexes remains to be determined.

Several components of the FtsH interactome have previously been shown to promote resistance to aminoglycoside antibiotics in *Pseudomonas aeruginosa*, including YccA, HtpX, HflC, HflK, and FtsH (249, 251). While we found that overexpression of YccA in the *E. coli cpxRA* mutant increased amikacin resistance, overexpression of HtpX did not (Figure 3-5). Nonetheless, the data presented in this study suggest that the FtsH interactome may contribute to aminoglycoside resistance by degrading subunits of the NDH-I complex.

We have previously shown that inhibition of the Cpx response reduces oxygen consumption despite increased transcription of the genes encoding NDH-I and cytochrome bo_3 (304) (section 2.3.1 and 2.3.2). Furthermore, oxygen consumption in the *cpxRA* mutant is similar to that of the *cyo* mutant, suggesting that decreased aerobic respiration in EPEC lacking CpxR and CpxA may be due to decreased activity of cytochrome bo_3 (304) (section 2.3.2). As such, we hypothesized that post-transcriptional events in the *cpxRA* mutant may decrease the abundance of cytochrome bo_3 proteins. However, CyoA-3xFLAG protein levels are not substantially changed in *E. coli* lacking *cpxRA*. It is possible that loss of the Cpx response impacts subunits other than CyoA. Alternatively, the Cpx response may facilitate the assembly of the cytochrome bo_3 subunits into a functional multiprotein complex. While the mechanism by which loss of the Cpx response inhibits respiration remains unknown, we have ruled out decreased levels of CyoA as the cause.

We have previously proposed that defects in assembly of the NDH-I and cytochrome bo_3 complexes activate the Cpx response (304) (section 2.4). However, activation of the Cpx response by mutation of *htpX* is not affected by loss of NDH-I

and/or cytochrome *bo*$_3$. It is possible that several proteins are affected by the loss of the protease HtpX, and the combined stress activates the Cpx response. As such, loss of NDH-I and cytochrome *bo*$_3$ under these conditions would not substantially reduce protein-folding stress. Alternatively, NDH-I, cytochrome *bo*$_3$, and deletion of *htpX* may generate envelope stress though independent pathways. Activation of the Cpx response by overexpression of NlpE does not require NDH-I or cytochrome *bo*$_3$. This is in agreement with the hypothesis that CpxA directly senses misfolded NlpE subunits.

In conclusion, we have provided evidence to suggest that that the Cpx response regulates NDH-I and cytochrome *bo*$_3$ expression at the post-translational level. Several Cpx-regulated proteins affect expression of NuoA, potentially by changing the activity of the zinc metalloprotease FtsH. FtsH has been proposed to degrade membrane proteins that fail to assemble into multisubunit complexes (320), suggesting that FtsH may function to degrade NuoA subunits that are not properly assembled within the NDH-I complex. As homologues of the FtsH interactome are present in human mitochondria (20), subunits of NADH dehydrogenase may be conserved substrates of FtsH. The results presented in this study provide support for the hypothesis that the Cpx response affects the biogenesis of respiratory complexes.

3.5 Tables and Figures

Table 3-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Bacterial strains		
E2348/69	Prototypical EPEC O127:H6 laboratory strain; Str^R	(298)
MC4100	F' *traD36 lacI^q Δ(lacZ) M15 proA$^+$B$^+$*/ e14 (McrA$^-$) *Δ(lac-proAB) thi gyrA96* (Nal^r) *endA1 hsdR17*(r_k^- m_k^+) *relA1 supE44 recA1*; Str^R	(321)
TR50	MC4100 λRS88[*cpxP'-lacZ*$^+$]; Str^R	(60)
BW25113	F$^-$ *lacI^q rrnB$_{T14}$* Δ*lacZ$_{WJ16}$ hsdR514* Δ*araBAD$_{AH33}$* Δ*rhaBAD$_{LD78}$*	(322) (311)
JW2283	BW25113 *nuoA::*kan; Kan^R	(307)
JW0422	BW25113 *cyoA::kan*; Kan^R	(307)
JW1818	BW25113 *htpX::*kan; Kan^R	(307)
DY378	W3110 λcI857 Δ(cro-bioA)	(310)
RG383	DY378 *nuoABCDEFGHIJKLMN::kan*; Kan^R	This study
RG397	DY378 *cyoABCDE::kan*; Kan^R	This study
ALN195	E2348/69 *cpxA24*; Str^R, Amk^R	(109)
RG139	E2348/69 Δ*cyoABCDE*	(304), this study
RG148	E2348/69 Δ*nuoABCDEFGHIJKLMN*	(304), this study
RG149	E2348/69 Δ*cyoABCDE* Δ*nuoABCDEFGHIJKLMN*	(304), this study
RG222	E2348/69 Δ*cpxRA*; Str^R	(304), this study
RG411	TR50 Δ*nuoABCDEFGHIJKLMN*; Str^R	This study
RG448	TR50 Δ*cyoABCDE*; Str^R	This study

RG450	TR50 Δ*nuoABCDEFGHIJKLMN* Δ*cyoABCDE*; Str^R	This study
RG463	TR50 *htpX::kan*; Str^R Kan^R	This study
RG464	TR50 Δ*nuoABCDEFGHIJKLMN htpX::kan*; Str^R Kan^R	This study
RG465	TR50 Δ*cyoABCDE htpX::kan*; Str^R Kan^R	This study
RG466	TR50 Δ*nuoABCDEFGHIJKLMN* Δ*cyoABCDE htpX::kan*; Str^R Kan^R	This study
RG480	BW25113 Δ*nuoA* derivative of JW2283	This study
RG481	BW25113 Δ*cyoA* derivative of JW0422	This study
Plasmids		
pCA-24N	Vector control for ASKA library containing the $P_{T5\text{-}lac}$ IPTG-inducible promoter; Cam^R	(299)
pCA-*degP*	IPTG-inducible *degP* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*dsbA*	IPTG-inducible *dsbA* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*efeB*	IPTG-inducible *efeB* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*ftsH*	IPTG-inducible *ftsH* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*hflC*	IPTG-inducible *hflC* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*hflK*	IPTG-inducible *hflK* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*htpX*	IPTG-inducible *htpX* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*nlpE*	IPTG-inducible *nlpE* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*nuoF*	IPTG-inducible *nuoF* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*ppiA*	IPTG-inducible *ppiA* overexpression vector from the ASKA library; Cam^R	(299)

pCA-*ppiD*	IPTG-inducible *ppiD* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*spy*	IPTG-inducible *spy* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*yccA*	IPTG-inducible *yccA* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*yceI*	IPTG-inducible *yceI* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*yceJ*	IPTG-inducible *yceJ* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*ydeH*	IPTG-inducible *ydeH* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*yebE*	IPTG-inducible *yebE* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*yncJ*	IPTG-inducible *yncJ* overexpression vector from the ASKA library; Cam^R	(299)
pCA-*yqaE*	IPTG-inducible *yqaE* overexpression vector from the ASKA library; Cam^R	(299)
pFLP2	Broad host-range plasmid expressing the FLP recombinase from a temperature sensitive promoter	(308)
pFLP2	Broad host-range plasmid expressing the FLP recombinase from a temperature sensitive promoter	(308)
pJW25	pJW15 luminescence reporter plasmid containing *cpxP* promoter; Kan^R	(109)
pMPM-K3	Low copy-number cloning vector derived from pACYC184 and pBluescript; Kan^R	(323)
pMPM-CyoA-3xFLAG	pMPM-K3 derived *cyoA*-3xFLAG expression vector; Kan^R	This study
pMPM-NuoA-3xFLAG	pMPM-K3 derived *nuoA*-3xFLAG expression vector; Kan^R	This study
pTrc99A	High copy-number expression vector with IPTG inducible promoter; Amp^R	Pharmacia

pCpxP	pTrc99A derived *cpxP* expression vector; Amp^R	(75)

Table 3-2. Oligonucleotide primers used in this study

Primer name	Sequence
cyoAFLAGFwd	5'-TTTTAAGCTTGGAATTGAGGTCG-3'
cyoAFLAGRev	5'-TTTTTCTAGATTATTTATCATCATCATCTTTATAATCAATATC ATGATCTTTATAATCGCCATCATGATCTTTATAATCSTGGGCGGA TTCCGCGTGGC-3'
cyoF	5'-GAGCTCAGCCAAATCCAGGTAACAGG-3'
cyoR	5'-TCT AGA ATC GTT CAT TAC CAC CTC TG-3'
K1	5'-CAGTCATAGCCGAATAGCCT-3'
K12-cyoKOF	5'-CCACACACTTTAAACGCCACCAGATCCCGTGGAATTGAGG TCGTTAAATGATTCCGGGGATCCGTC-3'
K12-cyoKOR	5'-CGTAGCACCTTTTTAATAGAGAGGTTTTGTTACCACACAGCA GCCAGCAGTGTAGGCTGGAGCTGC-3'
K12nuo(del)R	5'-GCTCTGCGTCTATCTACTATCC-3'
K12nuoKOF	5'-CTGCCGTGAAGAGCAGTGAATCTGGCGCTACTTTTGATGAGT AAGCAATGATTCCGGGGATCCGTC-3'
K12nuoKOR	5'-GGCGGCTTTCTGACTTACAAAGTAACAGATTACATCAGCGGC ATTGCCAATGTAGGCTGGAGCTGC-3'
K2	5'-CGGTGCCCTGAATGAACTGC-3'
M13F	5'-GTTTTCCCAGTCACGAC-3'
M13R	5'-AACAGCTATGACCATG-3'
nuoAFLAGFwd	5'-TTTTAAGCTTCTTTTGATGAGTA-3'
nuoAFLAGRev	5'-TTTTTCTAGATTATTTATCATCATCATCTTTATAATCAATATCAT GATCTTTATAATCGCCATCATGATCTTTATAATCGCGTTGACGATT AGCGATAC-3'
PnuoEMSAF	5'-CACAACGGACACGATTCAAC-3'

* Underlined sequences denote restriction endonuclease cut sites (AAGCTT, XbaI: TCTAGA).

Table 3-3. Summary of the Cpx regulated proteins overexpressed in this study

Protein	Cellular location*	Description*
CpxP	Periplasm	Protein chaperone involved in degradation of pilins. Inhibitor of the Cpx response.
DegP	Periplasm, inner membrane	Dual function chaperone and protease involved in biogenesis of outer membrane proteins and pilins
DsbA	Periplasm	Disulfide bond oxidoreductase
FtsH	Inner membrane	ATP-dependent zinc metallopeptidase
HflC	Inner membrane	Regulator of FtsH activity
HflK	Inner membrane	Regulator of FtsH activity
HtpX	Inner membrane	Zinc endopeptidase potentially involved in degrading misfolded proteins
NuoF	Cytoplasm	Subunit of the NADH dehydrogenase module of NADH dehydrogenase I. Contains flavin mononucleotide cofactor. NADH binding site.
PpiA	Periplasm, outer membrane	Protein chaperone involved in isomerization of prolyl residues.
PpiD	Inner membrane	Protein chaperone involved in outer membrane protein biogenesis
Spy	Periplasm	ATP-independent chaperone that prevents aggregation of misfolded proteins and assists in protein folding
YccA	Inner membrane	Substrate and modulator of FtsH proteolysis
YceI	Periplasm	Function unknown. Expression is increased at alkaline pH and under osmotic stress.
YceJ	Inner membrane	Putative cytochrome
YdeH	Cytoplasm	Diguanylate cyclase involved in biofilm formation
YebE	Inner membrane	Function unknown
YncJ	Inner membrane	Function unknown
YqaE	Inner membrane	Function unknown

*Cellular location and protein descriptions were obtained from the Ecocyc database

(281) (https://ecocyc.org).

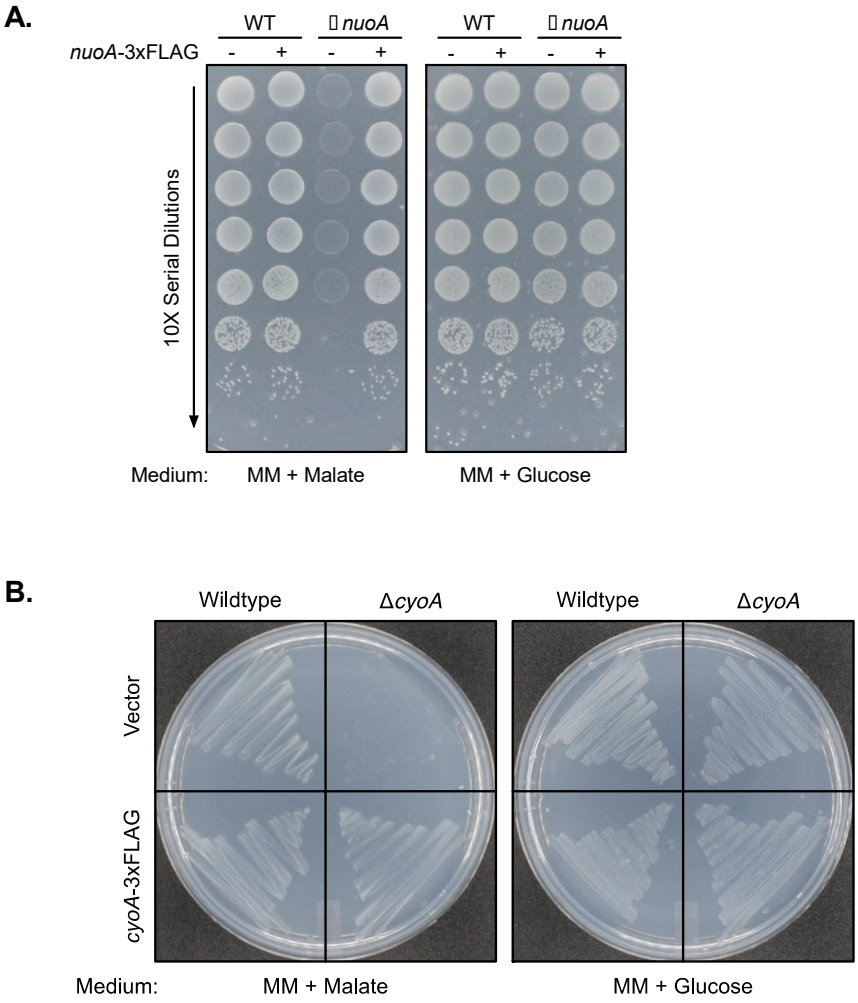

Figure 3-1 NuoA-3xFLAG and CyoA-3xFLAG constructs are functional

(A) Wildtype BW25113 and the Δ*nuoA* mutant containing either the empty pMPM-K3 vector (-) or pMPM-*nuoA*-3xFLAG (+) were grown overnight in LB medium, washed once in phosphate-buffered saline, and diluted to an OD_{600} of 1 in phosphate buffered saline. 10µL of 10-fold serial dilutions were spotted onto minimal medium (MM) agar containing 0.4% malic acid, pH 7.0 (malate) or 0.4% glucose and bacteria were grown overnight at 37°C. (B) Single colonies of wildtype BW25113 and the Δ*cyoA* mutant containing either the pMPM-K3 vector or *cyoA*-3xFLAG grown on LB agar were streaked onto minimal medium agar containing 0.4% malic acid, pH 7.0 (malate) or 0.4% glucose. Bacteria were grown overnight at 37°C. WT, wildtype

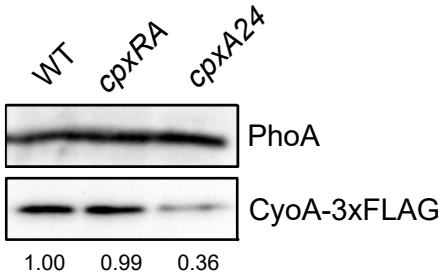

Figure 3-2 Activation of the Cpx response decreases CyoA-3xFLAG protein levels

Wildtype EPEC and the *cpxA24* and *cpxRA* mutants were subcultured into 25mL of LB broth and grown to an OD_{600} of 0.35 at 37°C. 0.1mM IPTG was added and bacteria were grown for an additional 30 minutes at 37°C. 20µg of total protein was loaded onto a 10% SDS polyacrylamide gel. CyoA-3xFLAG and PhoA protein levels were determined via western blotting as described in section 3.2.5. PhoA protein levels were determined as a loading control. Data are representative of two independent experiments. WT, wildtype

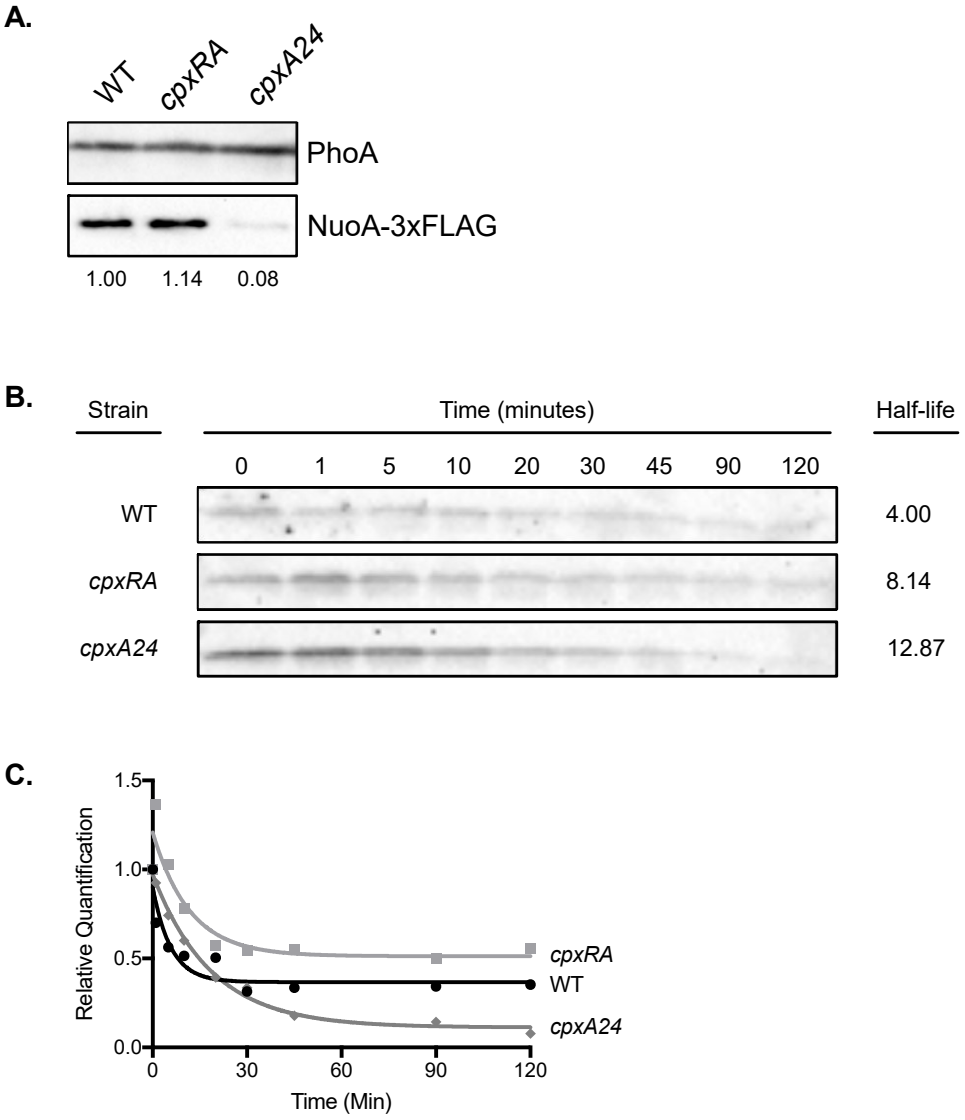

Figure 3-3 Activation of the Cpx response decreases NuoA-3xFLAG protein levels

(A) Wildtype EPEC and the *cpxA24* or *cpxRA* mutants containing the pMPM-*nuoA*-3xFLAG expression vector were grown in 25mL LB at 37ºC to an OD_{600} of 0.35. 0.1mM IPTG was added to induce *nuoA*-3xFLAG transcription and bacteria were grown for an additional 30 minutes. 20µg of total protein was loaded onto a 15% SDS polyacrylamide gel. NuoA-3xFLAG and PhoA protein levels were determined via western blot as described in section 3.2.5. Data are representative of two independent experiments. PhoA protein levels were determined as a loading control. (B) Bacteria were grown in 25mL LB at 37ºC to an OD_{600} of 0.5 at which point IPTG was added to a final concentration of 0.1mM. After addition of IPTG, bacteria were grown to an OD_{600} of approximately 1. Approximately 1 x 10^9 cells mL^{-1} were collected, pelleted by

centrifugation, and resuspended in 50µL 2x Laemmli sample buffer. 100µg mL^{-1} of the protein synthesis inhibitor chloramphenicol was added to the remaining bacterial culture. Approximately 1 x 10^9 cells mL^{-1} were collected 1, 5, 10, 20, 30, 45, 90, and 120 minutes after the addition of chloramphenicol. Proteins were separated by electrophoresis on a 15% SDS polyacrylamide gel. NuoA-3xFLAG was detected via western blot as described in section 3.2.5. Data are representative of a single experiment. Protein half-life was calculated as described in section 3.2.6. (C) One-phase exponential decay curve based on the relative amount of NuoA-3xFLAG present at each time point compared to the zero time point in (B). WT, wildtype.

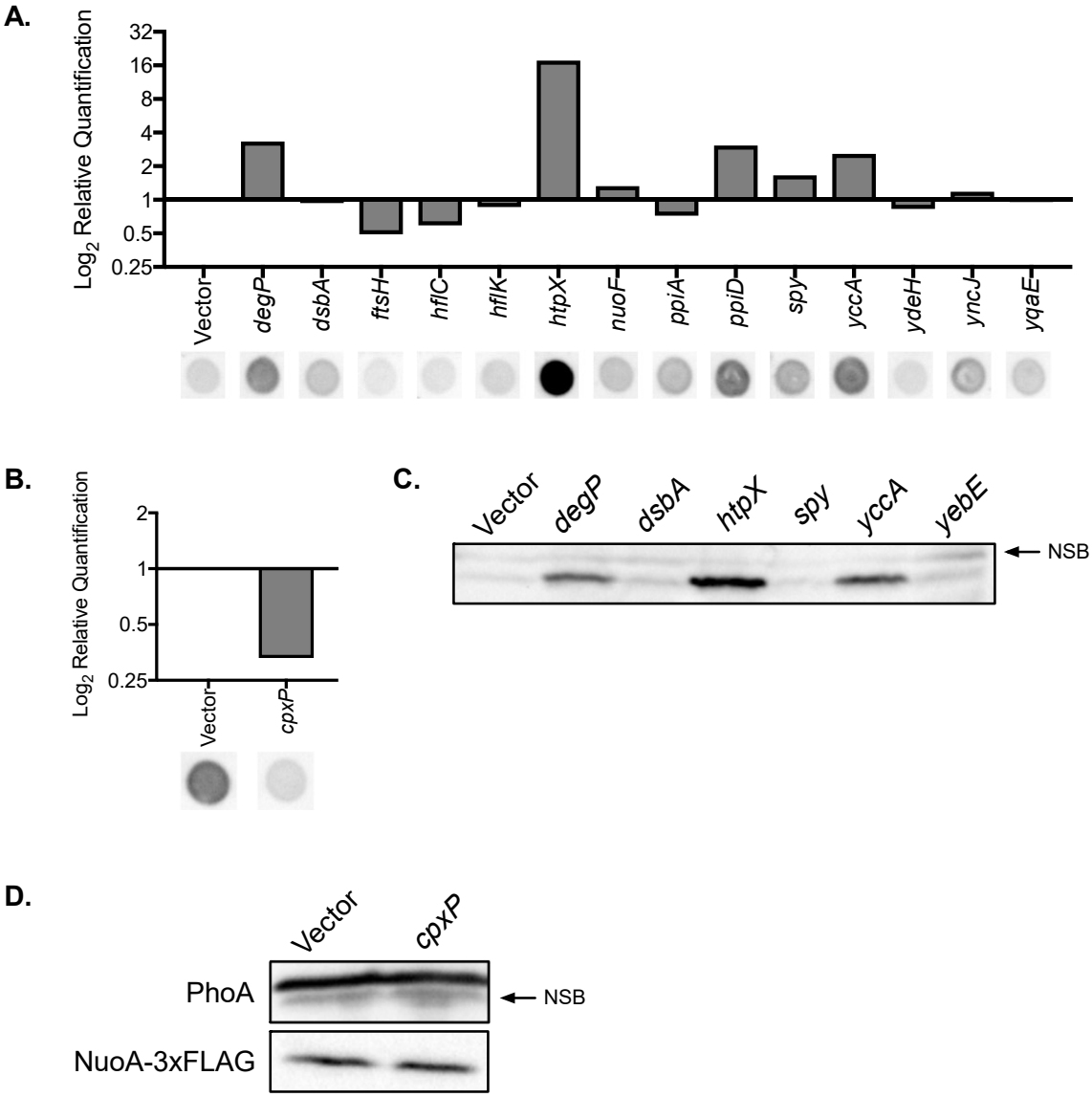

Figure 3-4 NuoA-3xFLAG protein levels are altered by overexpression of several Cpx-regulated genes

(A and B) Bacteria were subcultured from overnight growth into 10mL LB and grown to an OD_{600} of 0.5. 1mM IPTG was added to induce transcription from both the pMPM-*nuoA*-3xFLAG vector and the gene overexpression vector, and bacteria were grown to an OD_{600} of approximately 1. Approximately 1 x 10^9 cells mL^{-1} were collected, pelleted by centrifugation, and resuspended in 50µL 2x sample buffer. Samples were boiled in a water bath and cooled at room temperature. 3µL of sample was spotted onto a nitrocellulose membrane, and samples were allowed to dry. NuoA-3xFLAG protein levels were determined as described in section 3.2.7. Graphs indicate the relative amount of

NuoA-3xFLAG in the dot blot compared to the empty vector control. Results of the dot blot are displayed below the gene name. (A) Expression of the indicated gene from the pCA-24N based vector. (B) Expression of *cpxP* from the pTrc99A based vector. (C and D) EPEC containing the overexpression vector for the indicated gene were subcultured into 10mL LB containing 0.1mM IPTG and grown to an OD_{600} of 0.5. Approximately 1 x 10^9 cells mL^{-1} were collected, pelleted by centrifugation, and resuspended in 2x Laemmli sample buffer. Proteins were separated by electrophoresis on a 15% SDS polyacrylamide gel. NuoA-3xFLAG was detected as described in section 3.2.5. (C) Expression of the indicated gene from the pCA-24N based vector. (D) Expression of *cpxP* from the pTrc99A based vector. All data are representative of a single experiment. NSB, nonspecific band.

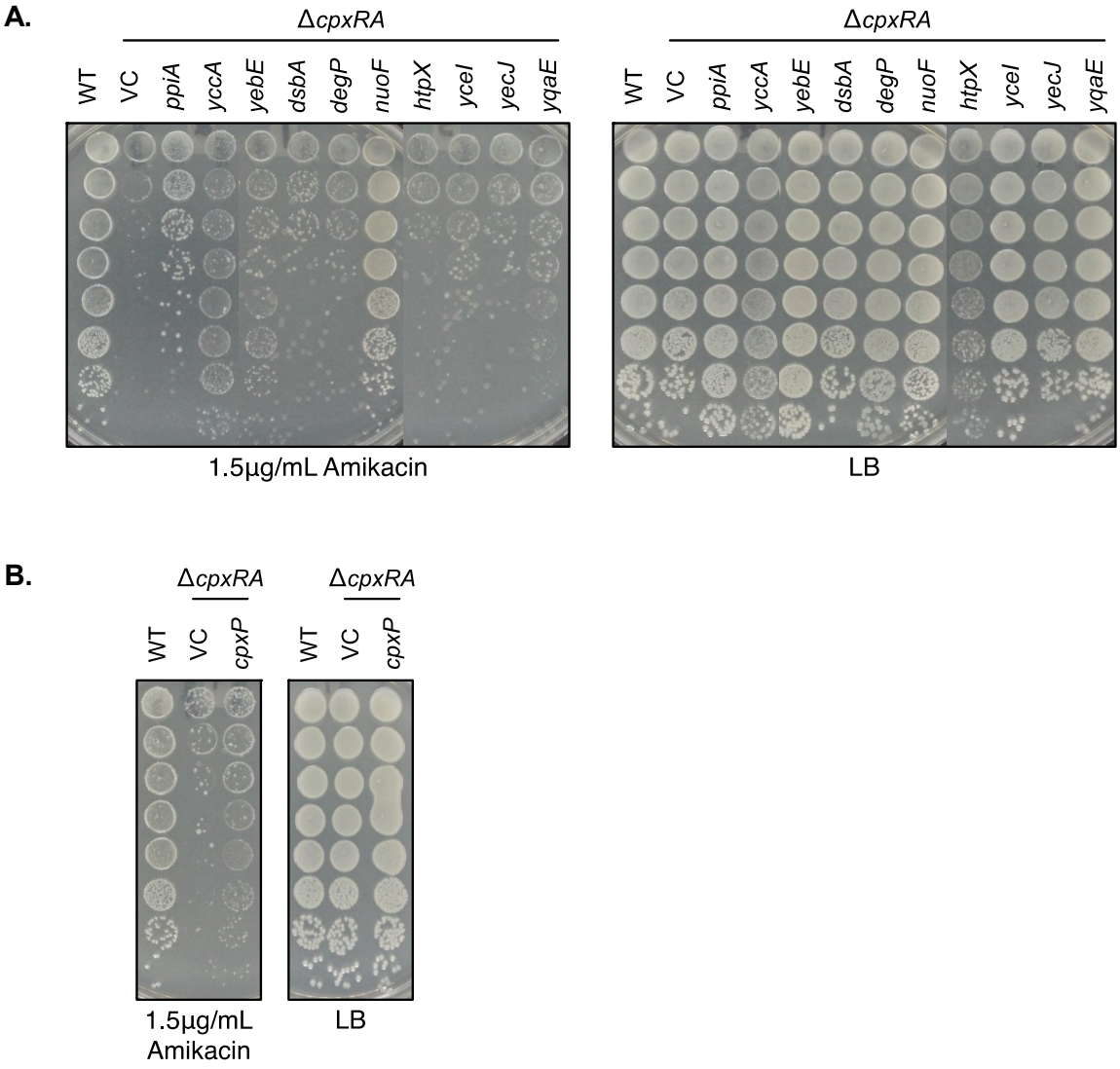

Figure 3-5 Overexpression of Cpx-regulated genes increases amikacin resistance in the *cpxRA* mutant

Wildtype EPEC containing the empty expression vector and the Δ*cpxRA* mutant EPEC carrying the indicated gene expression vector were grown overnight in LB at 37°C and diluted to an OD_{600} of 1. Diluted culture was serially diluted by a factor of 10. 10µL of each dilution was spotted onto LB containing LB containing 1.5µg mL^{-1} amikacin. Bacteria were grown at 37°C. All media contained 0.1mM IPTG to induce transcription of the indicated gene from the (A) pCA-24N based expression vector or (B) pTrc99A based expression vector. Data are representative of a single experiment. WT, wildtype; VC, vector control.

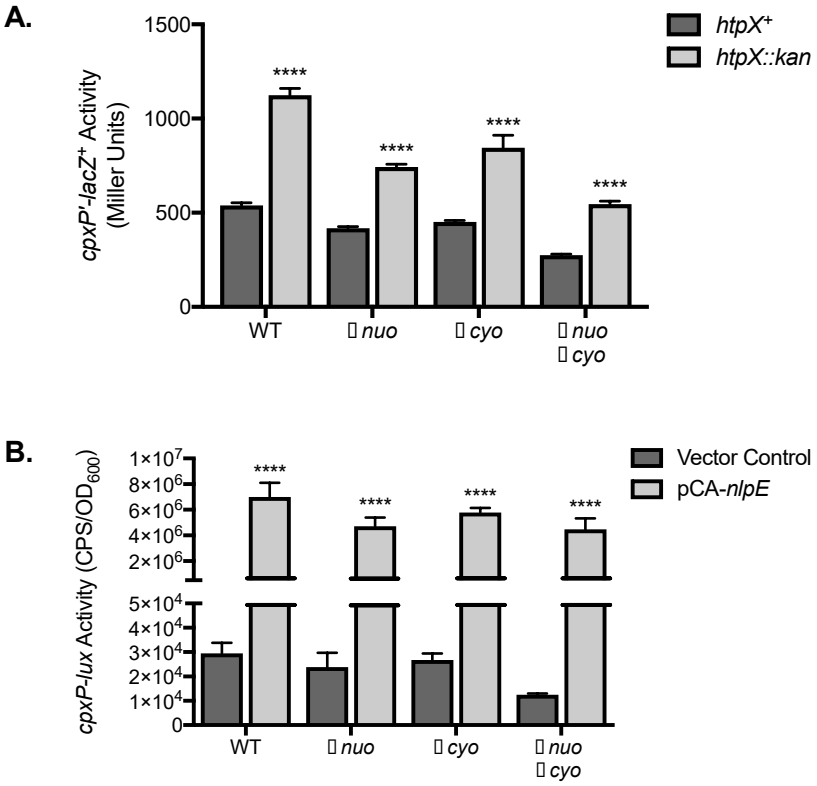

Figure 3-6 NADH dehydrogenase I and cytochrome bo3 are not required for activation of the Cpx response by mutation of htpX or overproduction of NlpE

(A) Bacteria containing a chromosomal *cpxP-lacZ* transcriptional reporter were subcultured into 2mL LB and grown for 6 hours at 37°C. Cells were pelleted by centrifugation and resuspended in 1x Z buffer. 250µL of sample was transferred to a 96 well plate and the OD_{600} was read. Cells were lysed with chloroform and SDS. 5µL of sample was diluted into 195µL of 1x Z buffer. β-galatosidase activity was measured as described in section 3.2.9. β-galatosidase was standardized to the OD_{600} to account for differences in cell number. The strains shown are TR50, the TR50 Δ*nuo,* Δ*cyo*, and *htpX::kan* single mutants, Δ*nuo* Δ*cyo,* Δ*nuo htpX::kan,* and Δ*cyo htpX::kan* double mutants, and the Δ*nuo* Δ*cyo htpX::kan* triple mutant. Data represent the means and standard deviations of three biological replicates. Asterisks (*) indicate a statistically significant difference from the control strain containing a wildtype copy of *htpX* (*htpX*$^+$) (****, P ≤ 0.0001 [one way ANOVA with Sidak's post hoc test]) (B). Bacteria containing a *cpxP* luminescent transcriptional reporter on the pJW15 plasmid were subcultured into 2mL LB and grown for 2 hours at 37°C. 0.1mM IPTG was added to induce transcription of *nlpE* and 200µL of culture was transferred to a black-walled 96 well microtiter plate. 0.1mM IPTG was also added to cultures containing the empty vector control. Bacteria

were grown for an additional two hours at which point luminescence (counts per second [CPS]) and OD_{600} were measured. Luminescence was standardized to the OD_{600} to account for differences in cell number. Data represent the means and standard deviations of five replicate cultures. Asterisks (*) indicate a statistically significant difference from the relevant vector control (****, $P \leq 0.0001$ [two-way ANOVA with Sidak's post hoc test]). WT, wildtype.

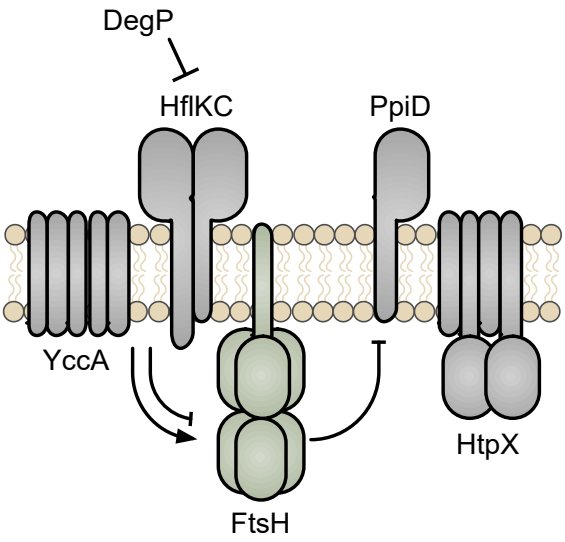

Figure 3-7. Model of the interactions with the zinc metalloprotease FtsH.

YccA, HflK, and HflC modulate activity of the zinc metalloprotease FtsH (20). The periplasmic protease DegP has been shown to degrade HflK (316). PpiD is subject to degradation by FtsH (317). HtpX is a non-specific protease and functions in conjunction with FtsH to degrade membrane or cytoplasmic proteins (20).

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CHAPTER 4

Improper efflux of siderophores induces envelope stress in gram-negative bacteria

Note: Emily Court collected and analyzed the data presented in figure 4-1A and 4-2A, and generated strains EC3 and EC4. Kiersten Schock collected and analyzed the data presented in figure 4-1C. Shargeel Hyatt generated strain SH11. Jayne Waldon collected and analyzed the data presented in figure 4-6A, 4-6B, and 4-6D.

4.1 Introduction

The envelope of gram-negative bacteria is structurally complex, consisting of the outer membrane, the inner membrane, and the intervening periplasmic space that contains the peptidoglycan layer. Given the essential functions performed by the envelope and envelope-localized proteins, gram-negative bacteria possess several regulatory systems that monitor and maintain envelope integrity. One such system is the Cpx envelope stress response, which regulates gene transcription in response to stresses that are predicted to affect the biogenesis of inner membrane proteins or protein complexes (112, 304) . The Cpx response is controlled by a typical two-component signal transduction system consisting of the inner membrane bound sensor CpxA and the cytoplasmic response regulator CpxR (33, 34). In the presence of an inducing signal, CpxA first autophosphorylates and then transfers the phosphate to CpxR (60). Once phosphorylated, CpxR functions as a transcription factor to activate the expression of genes associated with protein biogenesis and inner membrane integrity (51-54, 64, 65, 68), and repress the expression of genes associated with macromolecular envelope-localized protein complexes (35, 103, 109-111, 296, 304). Once homeostasis is achieved, CpxA functions as a phosphatase to dephosphorylate CpxR and attenuate the response (60).

Accumulating evidence suggests that multidrug efflux pumps function as components of envelope stress responses to extrude noxious compounds that threaten envelope integrity (225). Many multidrug efflux pumps interact with an outer membrane channel and periplasmic membrane fusion protein to form a tripartite protein complex that can directly transport toxic molecules from the cytoplasm or periplasm to the external environment (269). *Escherichia coli* and *Vibrio cholerae* possess several multidrug efflux systems, many of which use the same outer membrane channel, TolC (269). Numerous cellular stress responses are activated in bacteria lacking *tolC* (27, 237), including the Cpx response. Mutation of *tolC* has been shown to activate the Cpx response in several gram-negative bacteria, including *E. coli* (241), and *V. cholerae* (113, 238, 240). While we were carrying out this work, Bina and colleagues demonstrated Cpx activation in the *V. cholerae tolC* mutant is due to accumulation of the catechol siderophore vibriobactin (294). However, the reason why inhibition of *tolC* activates the Cpx response in *E. coli* is unknown. It is possible that in the absence of TolC, efflux pump and membrane fusion proteins that normally interact with TolC are unstable and generate a Cpx inducing signal (241). However, the Cpx response is still

activated in an *E. coli tolC* mutant lacking all nine TolC-dependent efflux pumps (241). As such, it is hypothesized that the signaling molecule is a noxious compound that is normally expelled through TolC (241).

In this regard, several studies have provided evidence to suggest that multidrug efflux systems secrete endogenously produced metabolites. Indeed, intra- and extracellular concentrations of cysteine, porphyrins, and enterobactin are affected by loss of TolC or TolC-dependent efflux pumps (222, 223, 230, 233). Consequently, bacteria lacking *tolC* are hypersensitive to cysteine, enterobactin, and intermediates of heme biosynthesis (229, 230, 233). Accumulation of several metabolites increases expression of the TolC-dependent AcrAB multidrug efflux system and confers resistance to nalidixic acid (235, 236). Accordingly, it has been proposed that in the absence of a functional TolC protein, a toxic level of an unidentified metabolite(s) disrupts inner membrane integrity and generates a Cpx response (241).

In this study, we sought to identify the metabolite or metabolites responsible for activation of the Cpx response by deletion of *tolC*. We show that the Cpx response is activated in *E. coli* lacking *tolC* during iron deprivation due to the accumulation of the siderophore enterobactin. We also confirmed that accumulation of the siderophore vibriobactin activates the Cpx response in the *V. cholerae tolC* mutant. While this has recently been demonstrated elsewhere (294), our findings suggest that the envelope damage inflicted by siderophore accumulation is a conserved Cpx inducing signal. We show that while enterobactin accumulation decreases activity of NADH dehydrogenase I of the electron transport chain, NADH dehydrogenase I is not required for Cpx pathway activation in the *tolC* mutant. Finally, we provide evidence to suggest that activation of the Cpx response facilitates adaptation to toxic envelope stresses such as enterobactin accumulation by down-regulating transcription of genes involved in enterobactin biosynthesis.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in the course of this study are listed in table 4-1. Bacteria were grown in either Lennox broth (LB, 10g/L bactotryptone [Difco], 5g/L yeast extract [Difco], and 5g/L NaCl) or M9 minimal medium (Difco) containing 0.4% glucose at 37^{o}C with shaking at 225 rpm. Bacteria were grown at 30^{o}C for experiments

that included strain TR10 or ALN195. Antibiotics were added as necessary to the following concentrations: amikacin (Amk), 3μg mL^{-1}; chloramphenicol (Cam), 25μg mL^{-1}; kanamycin (Kan), 50μg mL^{-1}; streptomycin (Str), 50μg mL^{-1} (for *E. coli* strains) or 100 μg mL^{-1} (for *V. cholerae* strains). All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

4.2.2 Strain and plasmid construction

Strains EC3, EC4, and RG244 were constructed by P1 transduction (305). Donor strains, in which the *tolC* or *entC* open-reading frame was replaced with the kanamycin resistance cassette, were obtained from the Keio library (307). The kanamycin resistance cassette in the *tolC* gene was removed by FLP/FRT mediated recombination (308) to produce an in-frame, markerless deletion as described in section 3.2.2.
The *V. cholerae* VC0773 (*vibC*) mutant used in this study was obtained from a TnFGL3 transposon library (324). To generate a markerless mutant, the kanamycin resistant cassette encoded within the transposon inserted into VC0773 was removed using FLP/FRT mediated recombination (325). Following this recombination event, a 192bp scar region is left within the VC0773 gene (324).

Strains RG270 and RG271 were constructed by allelic exchange (277). DNA approximately 1kb upstream and 1kb downstream of the *V. cholerae* C6706 VC2435 (*tolC*) locus was amplified by PCR using primers tolCDelUpFVc and tolCDelUpRVc (upstream fragment) and tolCDelDnFVc and tolCDelDnRVc (downstream fragment) (all primers sequences, including restriction endonuclease cut sites, are listed in table 4-2). The upstream and downstream fragments were amplified from the C6706 chromosome using high fidelity Phusion DNA polymerase (ThermoFisher) according to the manufacturer's protocol, with the addition of 10% betaine. PCR cycling conditions were as follows: denaturing cycle, 96°C for 30 seconds; annealing cycle, 62°C for 45 seconds; extending cycle, 72°C for 35 seconds. The two PCR products were then joined by overlap extension PCR using primers tolCDelUpFVc and tolCDelDnRVc. The 2kb product was amplified using the same conditions as for each 1kb fragment, with the exception of the extending cycle which occurred at 72°C for 1 minute and 15 seconds. The PCR product was gel-extracted using the GeneJet Gel Extraction kit (Fermentas), digested with XbaI and SacI restriction endonucleases (Invitrogen), and ligated into the suicide vector pRE112. The Δ*tolC* construct was then moved onto the *V. cholerae* chromosome as described in (279) with slight modifications. pRE112-Δ$tolC_{VC}$ was

introduced into *V. cholerae* strains C6706 and SH11 via conjugation from strain MFDλPir. Transconjugants were selected for on LB agar containing both streptomycin and chloramphenicol. Single colonies were grown in plain LB broth to late logarithmic phase. Serial dilutions of this culture were plated on LB agar (without NaCl) containing 5% sucrose and grown for two days at room temperature. Sucrose resistant colonies were tested for chloramphenicol sensitivity, indicating loss of the suicide vector sequence. To determine which of the sucrose resistant, chloramphenicol sensitive colonies contained the Δ*tolC* allele, colonies were screened for lack of growth on MacConkey agar, which contains bile. The presence of the Δ*tolC* allele in colonies that could not grow on MacConkey agar, but could grow on LB agar, was confirmed by PCR.

Luminescent transcriptional reporters of *entCEBA* expression were constructed as previously described (280). Briefly, the promoter region of the *entCEBA* operon was amplified from E2348/69 or MC4100 using the primers PentCluxF and PentCluxR and the high fidelity Phusion DNA polymerase (ThermoFisher) according to the manufacturer's protocol with the addition of 10% betaine. PCR cycling conditions were as follows: denaturing cycle, 96°C for 30 seconds; annealing cycle, 52-62°C for 45 seconds; extending cycle, 72°C for 25 seconds. As the PCR reaction resulted in multiple bands, a gradient of annealing temperatures was used. DNA was separated by electrophoresis on a 1% agarose gel. DNA bands corresponding to the size of the MC4100 *entCEBA* promoter (476bp) and the E2348/69 *entCEBA* promoter (276bp) were gel-purified using the GeneJet Gel Purification kit (Fermentas), digested with BamHI and EcoRI (Invitrogen), and ligated upstream of the *luxABCDE* operon in the pJW15 plasmid. PCR and DNA sequencing verified correct insertion of the promoter sequences. To ensure that the reporters reflect accurate expression of the *entCEBA* operon, luminescence was determined under iron-replete and iron-deplete conditions. In accordance with published observations, luminescence was reduced in the presence of iron (data not shown). DNA sequencing was performed by the University of Alberta Molecular Biology Services Unit.

4.2.3 β-galactosidase assay

Bacteria were grown overnight in LB at 37°C with shaking at 225 rpm. The following day, strains were subcultured at a dilution of 1:100 into fresh LB broth or M9 minimal medium (Difco) and grown for twenty hours at 37°C with shaking at 225 RPM. Where indicated, $FeSO_4$ and enterobactin were added to a final concentration of 80μM

and 10μM, respectively. As enterobactin is dissolved in 42% DMSO, an equivalent volume of 42% DMSO was added to the enterobactin control cultures. β-galactosidase activity was measured as previously described (76). Bacteria were pelleted by centrifugation at 2880 x g for 10 minutes. The supernatant was removed, and bacteria were resuspended in 2mL of 1 x Z buffer [600mM $Na_2HPO_4•7H_2O$, 400mM $NaH_2PO_4•H_2O$, 100mM KCl, 10mM $MgSO_4•7H_2O$], 90mL distilled water, 270μL β-mercaptoethanol). 250μL of sample was transferred to a 96 well plate and OD_{600} was measured using the PerkinElmer Wallac Victor^2 1420 plate reader. Chloroform and SDS were used to lyse the remaining cells. 5μL of sample was added to 195μL of 1 x Z buffer in a 96 well plate. 50μL of 10mg/mL *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added, and hydrolysis of ONPG was measured at an absorbance of 420nm (A_{420}). A_{420} was read 20 times with 45 seconds between each reading.

For Figure 4-4, bacteria were grown overnight in LB at 37°C with shaking at 225 rpm. Bacteria were pelleted by centrifugation at 2880 x g for 10 minutes, washed once in 1mL phosphate-buffered saline, and resuspended in 2mL phosphate buffered saline. 10μL of washed bacteria were spotted onto M9 minimal medium agar containing 0.4% glucose and grown for 24 hours at 37°C. Bacteria were then scraped off the plate using plastic inoculating loops and resuspended in 2mL 1 x Z buffer. β-galactosidase activity was measured as described above.

4.2.4 Oxygen consumption

After growth overnight in 5mL LB broth with shaking at 225 rpm, bacteria were diluted by a factor of 1:100 into 5mL M9 minimal medium (Difco) containing 0.4% glucose and grown for 20 hours at 37°C. Bacteria were pelleted by centrifugation at 2880 x g for 10 minutes and the pellet was resuspended in 1mL of cold 50mM 4-morpholineethanesulfonic acid (MES) buffer, pH 6.0. Bacteria were pelleted again by centrifugation at 21,130 x g for 1 minute. The supernatant was removed and the wet weight of the bacteria was determined. Bacteria were resuspended in 1mL cold 50mM MES buffer, pH 6.0 and 25μL of protease inhibitor was added for every 100mg of wet cell weight. Bacteria were then lysed by sonication with the Branson Sonifier 450 sonicator. Intact cells were removed by centrifugation at 10,000 x g for 30 minutes at 4°C. 100μL of sample was added to 890μL of pre-warmed 50mM MES buffer, pH 6.0 in a 1mL microrespiration chamber and covered with 150μL of light mineral oil to prevent oxygen from dissolving into the medium. The microrespiration chamber was placed in a

30°C water bath for 5 minutes prior to the addition of β-NADH. 100μM β-NADH was added by reverse pipetting using a capillary pipet tip and oxygen concentration was measured every 30 seconds for 10-15 minutes using an oxygen MicroOptode sensor (Unisense). Oxygen concentration at each time point was standardized to the oxygen concentration just prior to the addition of β-NADH. Oxygen consumption for each sample was measured in technical duplicate. The rate of oxygen consumption (% minute^{-1}) was calculated from the linear range of the reaction. The average rate of oxygen consumption of the technical replicates was standardized to the amount of total protein added to the microrespiration chamber. Protein concentration for each sample was determined using the Pierce BCA Protein Assay kit (Thermo Scientific).

4.4.5 Luminescence assay

For Figure 4-2C and Figure 4-2D, bacteria were grown overnight in 2mL LB at 37°C with shaking at 225 rpm. The following day, bacteria were diluted to an OD_{600} of 1 and 10μL of the diluted bacterial culture was spotted onto LB agar. Bacteria were grown overnight at 37°C. Luminescence from bacteria on the plates was detected, and growth images were taken, using the Bio-Rad ChemiDoc MP imaging system. Bacteria were then removed from the plate using plastic inoculating loops and resuspended in 1mL LB. 200μL of culture was transferred to a black-walled 96 well microtiter plate. Luminescence (expressed in counts per second [CPS]) and OD_{600} were read from the microtiter plate for each sample using the PerkinElmer Wallac Victor^2 1420 plate reader. *cpxP-lux* activity was calculated by subtracting the CPS and OD_{600} values measured from a blank well containing uncultured LB from the raw CPS and OD_{600} values measured for each sample. The normalized CPS was divided by the normalized OD_{600} to account for differences in growth between samples.

For Figure 4-5, bacteria were grown overnight in 2mL LB supplemented with 50μg mL^{-1} γ-aminolevulinic acid at 37°C with shaking at 225 rpm. Bacteria were pelleted by centrifugation at 2880 x g for 10 minutes, washed twice in plain LB, and resuspended in 2mL plain LB. Bacteria were then subcultured at a 1:100 dilution in 200μL LB containing 0.4% glucose. 50μg mL^{-1} γ-aminolevulinic acid was added to the indicated cultures at the time of subculture. Glucose was added to the LB to provide a fermentable carbon source. Bacteria were grown for 2 hours at 37°C with shaking at 225 rpm. Luminescence was determined as described above.

For Figure 4-6, bacteria were grown overnight in 2mL LB at 30°C for experiments containing *E. coli* strains TR10 or ALN195 or at 37°C for experiments that did not contain TR10 or ALN195 with shaking at 225 rpm. Bacteria were then diluted at a factor of 1:100 into 2mL M9 minimal medium (Difco) containing 0.4% glucose, 5.34mM isoleucine, and 6.53mM valine and grown at 30°C with shaking. 200µL of culture was transferred to a black-walled 96 well microtiter plate 8 hours after subculture and luminescence (in counts per second [CPS]) and OD_{600} were measured as described above. *entC-lux* activity was calculated as described above for the calculation of *cpxP-lux* activity.

4.3 Results

4.3.1 Iron limitation induces the Cpx response in the *tolC* mutant

In order to confirm the results of previous studies indicating that inhibition of efflux activates the Cpx stress response, we examined Cpx pathway activity in the *E. coli tolC* mutant using a *cpxP-lacZ* transcriptional reporter. No change in *cpxP-lacZ* reporter activity was observed when *E. coli* were grown in LB (Figure 4-1A). This was surprising, as previous studies have shown that deletion of *tolC* activates *spy* expression in a CpxA-dependent manner under these growth conditions (241). However, when *E. coli* were grown in M9 minimal medium, there was an approximate 11-fold increase in *cpxP-lacZ* activity in the *tolC* mutant compared to wildtype (Figure 4-1A). We found that this activation was dependent on CpxA, as the increase in *cpxP-lacZ* activity observed in the *tolC* mutant was abolished in *E. coli* lacking *cpxA* (Figure 4-1B). Accordingly, we have found that inhibition of efflux activates the Cpx response in a CpxA-dependent manner under specific growth conditions. These results suggest that the metabolite(s) responsible for activating the Cpx response is produced in minimal medium, but not in rich medium.

Previous results from our lab have shown that inhibition of efflux activates the *Vibrio cholerae* Cpx response when iron is limiting (113). As such, we hypothesized that iron may be involved in activation of the Cpx response by inhibition of efflux in *E. coli* as well. As observed previously, mutation of *tolC* resulted in approximately an eleven-fold increase in *cpxP-lacZ* activity when *E. coli* were grown in iron-deplete M9 minimal medium (Figure 4-1C). However, when the *tolC* mutant was grown in M9 minimal medium supplemented with 80µM $FeSO_4$, activation of the Cpx response was no longer observed (Figure 4-1C). These results suggest that iron deficiency is responsible for

activation of the Cpx pathway in *E. coli* lacking TolC and indicate that the metabolite(s) that accumulates and activates the Cpx stress response in the absence of efflux is produced during iron deprivation.

4.3.2 Siderophore accumulation in the *tolC* mutant activates the Cpx response

Several lines of evidence implicate the siderophore enterobactin in activation of the Cpx response by deletion of *tolC*. First, enterobactin biosynthesis is repressed in the presence of iron by the master iron regulator Fur (326, 327). Second, enterobactin is secreted into the environment via TolC (222) and in the absence of TolC, enterobactin accumulates in the periplasm (229). To test whether accumulation of enterobactin is responsible for activation of the Cpx response by deletion of *tolC*, we constructed an *entC* mutant in which enterobactin biosynthesis is disrupted (328). Unlike the *tolC* single mutant, in which *cpxP-lacZ* activity was increased eleven-fold, there was no increase in *cpxP-lacZ* activity in the *tolC entC* double mutant (Figure 4-2A). Furthermore, addition of exogenous enterobactin to the medium restored Cpx pathway activation in the *tolC entC* double mutant, as indicated by the ten-fold increase in activity of the *cpxP-lacZ* reporter in the *tolC entC* mutant compared to the *entC* single mutant under these conditions (Figure 4-2B). Together, these results suggest that enterobactin is the metabolite responsible for activating the Cpx stress response in the *tolC* mutant.

Given that activation of the Cpx response by inhibition of efflux is associated with iron deficiency in both *E. coli* and *V. cholerae* (Figure 4-1) (113), we hypothesized that accumulation of siderophores may also stimulate Cpx pathway activity in the *V. cholerae tolC* mutant. The only known siderophore that is endogenously produced by *V. cholerae* is vibriobactin, a catechol siderophore similar to *E. coli* enterobactin (329, 330). To disrupt vibriobactin biosynthesis, we mutated the *V. cholerae entC* homologue *vibC* (329). Deletion of *tolC* in *V. cholerae* induced *cpxP-lux* activity approximately 239-fold compared to the wildtype (Figure 4-2C and Figure 4-2D). However, there was no increase in *cpxP* expression in the *tolC vibC* double mutant compared to the wildtype and *vibC* single mutant (Figure 4-2C and Figure 4-2D). As such, accumulation of siderophores in the *tolC* mutant is a conserved inducing cue of the Cpx response in *V. cholerae* and *E. coli*. These results are supported by the findings of a recent paper that also found that accumulation of vibriobactin activates the Cpx stress response in *V. cholerae* efflux mutants (294).

4.3.3 Enterobactin accumulation affects respiration

We next sought to determine whether other phenotypes associated with the *tolC* mutant are due to accumulation of enterobactin. While *tolC* is not essential for growth in rich medium, the growth rate of *tolC* deficient *E. coli* is substantially reduced in minimal medium (229, 264). This phenotype was attributed to reduced activity of NADH dehydrogenase of the electron transport chain (264), and could be suppressed by the addition of iron (229). Together, these results suggest that enterobactin may reduce NADH dehydrogenase activity. To examine this possibility, we measured activity of the aerobic electron transport chain in the *tolC, entC,* and *tolC entC* single and double mutants using β-NADH as the electron donor by measuring the rate of oxygen consumption. As expected, oxygen consumption using β-NADH is reduced in the *tolC* mutant compared to the wildtype (Figure 4-3). However, oxygen consumption in the *tolC entC* double mutant is similar to that of the *entC* single mutant (Figure 4-3). These results are consistent with the hypothesis that enterobactin is responsible for reduced NADH dehydrogenase activity in the *tolC* mutant.

4.3.4 The Cpx response is activated by enterobactin accumulation in the absence of NADH dehydrogenases

E. coli encode two NADH dehydrogenase isoenzymes, NADH dehydrogenase I (NDH-I) and NADH dehydrogenase II (NDH-II), both of which can oxidize β-NADH. Given that enterobactin accumulation in *tolC* deficient *E. coli* reduces NADH oxidase activity, we asked whether NDH-I or NDH-II contribute to activation of the Cpx response in the *tolC* mutant. As the *tolC nuo* and *tolC ndh* double mutants grow poorly in M9 minimal medium broth, we determined *cpxP-lacZ* expression from bacteria grown on agar. As seen previously, deletion of *tolC* activated the Cpx response under these conditions, evidenced by a 5.5-fold increase in *cpxP-lacZ* activity in the *tolC* mutant compared to the wildtype (Figure 4-4). No statistically significant increase in *cpxP-lacZ* expression was observed in the *tolC entC* double mutant compared to the *entC* single mutant (P >0.5 [one-way ANOVA with Sidaks post-hoc test], Figure 4-4). However, *cpxP-lacZ* activity was increased in the *tolC nuo* and *tolC ndh* double mutants by a factor of 5.7 and 5.8 compared to the control containing the wildtype *tolC* allele (Figure 4-4). Together with the results presented in Figure 4-3, we conclude that while enterobactin accumulation may reduce NADH dehydrogenase activity, NDH-I and NDH-II are not required for activation of the Cpx response under these conditions.

4.3.5. Disruption of heme biosynthesis activates the Cpx response

Several respiratory complexes contain heme cofactors that are required for their function in electron transport. In the absence of heme, it is thought that *E. coli* cannot respire and instead grow by fermentation (331). We determined whether disruption of heme biosynthesis activates the Cpx response by deleting *hemA,* which catalyzes the first step in heme biosynthesis to generate γ-aminolevulinic acid (ALA) (332), in EPEC. *cpxP-lux* activity was increased 3.83 fold in the EPEC *hemA* mutant compared to the wildtype (Figure 4-5). No difference in *cpxP-lux* activity was seen in the *hemA* mutant compared to the wildtype when heme biosynthesis was restored by the addition of exogenous ALA to the medium (Figure 4-5). These results suggest that disruption of heme biosynthesis activates the Cpx envelope stress response.

4.3.6 Regulation of enterobactin biosynthesis genes by the Cpx response

Previous microarray experiments performed to identify members of the Cpx regulon found that expression of several genes involved in enterobactin biosynthesis is decreased upon activation of the Cpx response, including *entA, entB, entC, and entE* (64). To investigate regulation of the enterobactin biosynthesis genes by the Cpx response, we constructed luminescent transcriptional reporters of EPEC and MC4100 *entCEBA* expression. Activity of each lux reporter was analyzed in wildtype EPEC or MC4100, mutants containing the *cpxA24* allele that constitutively activates the Cpx response, or in *E. coli* lacking the Cpx response. Mutational activation of the Cpx response in EPEC resulted in a 4.0-fold decrease in activity of the EPEC *entCEBA-lux* reporter (Figure 4-6A). No change in reporter activity was observed in EPEC lacking the Cpx response (Figure 4-6A), suggesting that basal expression of the enterobactin biosynthesis genes is not affected by the Cpx response. Likewise, activation of the Cpx response in MC4100 lead to a 6.2-fold decrease in activity of the MC4100 *entCEBA-lux* reporter in comparison to the wildtype (Figure 4-6B). No change in MC4100 *entCEBA-lux* activity was observed in the MC4100 *cpxR* mutant (Figure 4-6B). These data suggest that the transcription of genes involved in enterobactin biosynthesis is repressed by the Cpx response in EPEC and MC4100.

Intriguingly, we observed substantial differences in basal *entCEBA* expression between MC4100 and EPEC. Activity of the native *entCEBA-lux* reporter in MC4100 was 5.1-fold higher than activity of the native *entCEBA-lux* reporter in EPEC (Figure 4-6D).

The promoter regions of the *entCEBA* operon are substantially different between MC4100 and EPEC. In addition to several base pair substitutions, there is a large, 186bp deletion in the promoter region of the EPEC *entCEBA* operon (Figure 4-6C). Therefore, there are two possible explanations for the difference in the basal levels of *entCEBA* transcription in MC4100 and EPEC. The first possibility is that the changes in the DNA sequence of the EPEC *entCEBA* promoter decrease basal transcription of the *entCEBA* operon. If true, we would expect that activity of the EPEC *entCEBA-lux* reporter would decrease in MC4100. Furthermore, activity of the MC4100 *entCEBA-lux* reporter would increase in EPEC. The second possibility is that activity of transcription factors that regulate *entCEBA* transcription is different between EPEC and MC4100. Here, expression of the EPEC and MC4100 *entCEBA-lux* reporters would be similar in EPEC. Likewise, expression of both reporters would be similar in MC4100. Here, we found that the difference in *entCEBA* transcription is not due to differences in the DNA sequence of the *entCEBA* promoter regions as activity of the EPEC *entCEBA-lux* and MC4100 *entCEBA-lux* reporters are similar in EPEC (Figure 4-6D). Furthermore, we found activity of the EPEC *entCEBA-lux* in MC4100 is increased in comparison to activity of the MC4100 *entCEBA-lux* in MC4100 (Figure 4-6D). Accordingly, these results suggest that expression of the *entCEBA* operon is decreased in EPEC through changes in activity of transcriptional regulators.

4.4 Discussion

Multidrug efflux pumps export a wide range of antimicrobial compounds, and thus play a major role in the intrinsic resistance of gram-negative bacteria to various antibiotics. However, several studies have revealed that multidrug efflux pumps are involved in cellular processes beyond antibiotic resistance, including cell division, biofilm formation, pathogenesis, cell communication, oxidative and nitrosative stress resistance, and envelope biogenesis (224-226). Furthermore, it has been proposed that drug efflux pumps function to secrete toxic endogenous metabolites that disrupt cellular integrity (235, 237). In this study, we report that the Cpx envelope stress response is activated in *tolC* mutants due to improper efflux of siderophores. While the mechanism by which siderophores activate the Cpx response remains unknown, we propose that accumulation of periplasmic siderophores may disrupt the biogenesis of inner membrane proteins to generate a Cpx inducing signal.

In this study, we found that the Cpx response was activated by deletion of *tolC* under specific growth conditions. Activity of the *cpxP-lacZ* reporter was increased in the *tolC* mutant when *E. coli* were grown in M9 minimal medium, but not LB (Figure 4-1A). Furthermore, the Cpx response was not activated when *E. coli* were grown in M9 minimal medium supplemented with iron (Figure 4-1B). Together, these results suggest that iron starvation activates the Cpx response in the *tolC* mutant. Given that *E. coli* synthesize the siderophore enterobactin in response to iron starvation (326), and that enterobactin is secreted into the external environment via TolC (222), we hypothesized that enterobactin could be responsible for activation of the Cpx response by deletion of *tolC*. Indeed, we observed that inhibition of enterobactin biosynthesis in the *tolC* mutant significantly reduced Cpx pathway activity (Figure 4-2A), and that pathway activity could be restored in the presence of exogenous enterobactin (Figure 4-2B). Notably, basal levels of periplasmic enterobactin in *E. coli* containing a functional TolC protein do not affect activity of the Cpx response, as *cpxP-lacZ* expression was unchanged in the *entC* single mutant compared to wildtype (Figure 4-2A). Accordingly, accumulation of enterobactin in *E. coli* lacking *tolC* activates the Cpx response.

Unlike other potent inducers of the *E. coli* Cpx response, activation of the Cpx pathway by deletion of *tolC* is conserved in *V. cholerae* (113, 238, 240). In fact, inhibition of efflux activates the Cpx response in several gram-negative bacteria, including *E. coli*, *V. cholerae*, *S. meliloti*, and *Haemophilus ducreyi* (113, 238, 240-243), making this the most conserved Cpx inducing cue identified to date. Since siderophores are responsible for activation of the Cpx response in the *E. coli tolC* mutant, we hypothesized that siderophores may contribute to Cpx pathway activity in the *V. cholerae tolC* mutant. As expected, inhibition of vibriobactin biosynthesis in the *V. cholerae tolC* mutant reduced Cpx pathway activity (Figure 4-2C and Figure 4-2D). Whether siderophore accumulation activates the Cpx response in the efflux mutants of other gram-negative bacteria remains to be determined. Accordingly, the damage resultant from siderophore-mediated perturbations of the envelope is a conserved Cpx inducing cue in the distantly related γ–proteobacteria *E. coli* and *V. cholerae*.

Although we have determined that siderophore accumulation is responsible for activation of the Cpx response by deletion of *tolC*, the molecular nature of the Cpx inducing cue under these conditions remains enigmatic. One possibility is that enterobactin interferes with electron transport, which generates a Cpx inducing signal. In support of this hypothesis, a previous study found that deletion of *tolC* reduces NADH

oxidase activity in *E. coli* grown in M9 minimal medium, but not LB (264). Here, we provide evidence to suggest that this phenotype may be due to enterobactin accumulation. Activity of the aerobic electron transport chain using NADH as the electron donor is reduced in the *tolC* mutant (Figure 4-3), as evidenced by a decrease in the rate of oxygen consumption in the *tolC* mutant compared to wildtype. However, when enterobactin biosynthesis in the *tolC* mutant is disrupted, the decrease in NADH-dependent activity of the electron transport chain is no longer observed (Figure 4-3). There are two possible explanations for why enterobactin accumulation in the *tolC* mutant may reduce NADH dehydrognase activity. The first possibility is that periplasmic enterobactin damages one or both of the NADH dehydrogenase protein complexes, thus reducing their ability to oxidize NADH. The second possibility is that activation of the Cpx response, or another regulatory system, by deletion of *tolC* reduces expression of NDH-I or NDH-II. Indeed, CpxR has been shown to directly repress the transcription of the operon encoding NDH-I (304) (section 2.3.1) and increased proteolysis of the NuoA subunit of NDH-I is observed when the Cpx response is activated (section 3.3.3). At this point, we are unable to distinguish between these two possibilities.

Given that NDH-I contributes to basal activity of the Cpx response in enteropathogenic *E. coli* (304) (Figure 2-4A), and that enterobactin accumulation may disrupt NADH dehydrogenase activity (Figure 4-3), it is possible that activation of the Cpx response by deletion of *tolC* and the resultant accumulation of enterobactin may require NDH-I. However, deletion of the genes encoding NDH-I or NDH-II did not reduce *cpxP-lacZ* activity in the *tolC* mutant (Figure 4-4). Together, these results suggest that while enterobactin accumulation in the *tolC* mutant reduces NADH dehydrogenase activity, other factors besides diminished NADH dehydrogenase activity must contribute to induction of the Cpx response under these conditions. Notably, a recent study in *V. cholerae* found that succinate dehydrogenase is required for activation of the Cpx response in the *tolC* mutant (294). Unlike NDH-I or NDH-II, succinate dehydrogenase contains heme cofactors that are required for assembly of the enzyme into the cytoplasmic membrane (333, 334). Interestingly, succinate dehydrogenase is not properly assembled in *E. coli* unable to incorporate iron into protoporphyrin IX to create heme *b* (334). As such, it is possible that intracellular enterobactin interferes with assembly of succinate dehydrogenase by sequestering iron and making it unavailable for heme biosynthesis. This would suggest that enterobactin accumulation in the *tolC* mutant activates the Cpx response by disrupting heme biosynthesis. Indeed, we have

found that deletion of *hemA*, which is required for heme biosynthesis, activates the Cpx response in EPEC (Figure 4-5). Given that several Cpx inducing cues are predicted to generate misassembled inner membrane protein complexes (304), we believe that Cpx activation by deletion of *tolC* occurs due to improper assembly of heme containing respiratory complexes, such as succinate dehydrogenase and the terminal oxidases, in the inner membrane.

In agreement with previous transcriptomic data, we found that the Cpx stress response represses expression of the genes for enterobactin biosynthesis in EPEC and *E. coli* K-12 (Figure 4-6A and Figure 4-6B). We also found that basal transcription of the *entCEBA* operon is decreased in EPEC in comparison to MC4100, likely due to changes in activity of transcription factors in EPEC (Figure 4-6D). As expression of the enterobactin biosynthesis genes is regulated in response to intracellular iron concentrations (326, 327), it is possible that intracellular iron concentrations are different in EPEC and MC4100. Alternatively, it is possible that pathogens such as *E. coli* more tightly control regulation of iron metabolism, and this facilitates host colonization.

In addition to regulation of enterobactin biosynthesis, other processes regulated by the Cpx response could facilitate adaptation to the stress caused by enterobactin accumulation. Activation of the Cpx response increases expression of multidrug efflux pumps in *E. coli*, *V. cholerae*, *Pseudomonas aeruginosa*, and *Klebsilla pneumoniae* (335, 336), which could lead to increased efflux of periplasmic enterobactin. Indeed, the Cpx-regulated expression of the VexGH efflux pump, which was recently shown to be required for vibriobactin secretion, supports this hypothesis (294). Furthermore, the Cpx response in *E. coli* represses expression of several components of the electron transport chain, including heme containing respiratory complexes such as succinate dehydrogenase, cytochrome bo_3, and cytochrome *bd-I* (64, 304). As such, activation of the Cpx response would decrease expression of the target of enterobactin-mediated stress. Through decreased enterobactin biogenesis, increased efflux, and decreased expression of target respiratory complexes, activation of the Cpx response could mount an effective adaptive response to the stress exerted by enterobactin accumulation.

Several noxious compounds secreted by TolC are present in the host environment. Enteric bacteria such as *E. coli* and *V. cholerae* are exposed to host-produced factors such as bile and cationic antimicrobial peptides, as well as antibiotics produced by competing members of the intestinal microbiome (337). Furthermore, *E. coli* and *V. cholerae* likely synthesize and secrete siderophores in response to the iron-poor

environment within the host. As a large number of noxious compounds present *in vivo* require TolC for secretion, it is possible that they accumulate within the cell faster than can be effluxed through TolC. Activation of the Cpx response could provide protection against infrequent surges in periplasmic enterobactin concentrations that occur under these conditions.

4.5 Figures and Tables

Table 4-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Escherichia coli strains		
E2348/69	Prototypical EPEC O127:H6 strain; Str^R	(298)
MC4100	F' *traD36 lacI^q Δ(lacZ)M15 proA$^+$B$^+$*/ e14 (McrA$^-$) *Δ(lac-proAB) thi gyrA96* (Nal^r) *endA1 hsdR17*(r_k^- m_k^+) *relA1 supE44 recA1*; Str^R	(321)
JW0585	BW25113 *entC::kan*; Kan^R	(307)
JW1095	BW25113 *ndh::kan*; Kan^R	(307)
JW5503	BW25113 *tolC::kan*; Kan^R	(307)
TR50	MC4100 λRS88[*cpxP'-lacZ*$^+$]; Str^R	(60)
ALN195	E2348/69 *cpxA24*; Str^R Amk^R	(109)
EC3	TR50 Δ*tolC*; Str^R	This study
EC4	TR50 Δ*tolC entC::kan*; Kan^R	This study
RG205	E2348/69 Δ*hemA*; Str^R	This study
RG222	E2348/69 Δ*cpxRA*; Str^R	(304) This study
RG244	TR50 *entC::kan*; Kan^R	This study
RG249	TR50 *ndh::kan*; Kan^R	This study
RG250	TR50 Δ*tolC ndh::kan*; Kan^R	This study
RG280	TR50 *cpxA::kan*; Kan^R	This study
RG281	TR50 Δ*tolC cpxA::kan*; Kan^R	This study
RG397	DY378 *nuoABCDEFGHIJKLMN::kan*; Kan^R	This study
RG392	TR50 *nuoABCDEFGHIJKLMN::kan*; Kan^R	This study
RG479	TR50 Δ*tolC nuoABCDEFGHIJKLMN::kan*; Kan^R	This study
TR10	MC4100 *cpxA24*; Amk^R	(75)
TR51	MC4100 *cpxR::spc*; Spc^R	(75)
Vibrio cholerae strains		

C6706	*Vibrio cholerae* O1 El Tor biotype clinical isolate; Str^R	Dr. J. Mekalanos (Harvard Medical School)
EC1910	Derivative of C6706 strain carrying TnFGL3 insertion in the *vibC* gene (VC0773); Kan^R	(324)
SH11	*vibC* mutant, EC1910 markerless TnFLG3 mutant; Str^R	This study
RG270	C6706 ΔVC2435 (*tolC*); Str^R	This study
RG271	SH11 ΔVC2435; Str^R	This study
Plasmids		
pCA-24N	Vector control from ASKA library; Cam^R	(299)
pCA-*nlpE*	IPTG-inducible *nlpE* overexpression vector from ASKA library; Cam^R	(299)
pFLP2	Broad host-range plasmid expressing the FLP recombinase from a temperature sensitive promoter	(308)
pJW15-P*entCEBA*$_{K\text{-}12}$	pJW15 luminescence reporter plasmid containing the MC4100 *entCEBA* promoter; Kan^R	This study
pJW15-P*entCEBA*$_{EPEC}$	pJW15 luminescence reporter plasmid containing the E2348/69 *entCEBA* promoter; Kan^R	This study
pN3	pJW15 luminescence reporter plasmid containing the *V. cholerae cpxP* promoter; Kan^R	(113)
pRE112	Suicide vector for allelic exchange; Cam^R	(277)

Table 4-2. Oligonucleotide primers used in this study

Primer name	Sequence*
hemADelDnF	5'-GCAGACATGACCCTTCTGGAGTAGCAGTACATCAT-3'
hemADelDnR	5'-TTTTGAGCTCTATTCCTGCTCGGACAACGC-3'
hemADelUpF	5'-TTTTTCTAGAAGGTTATCTTCATGTTCCACG-3'
hemADelUpR	5'-GTACTGCTACTCCAGAAGGGTCATGTCTGCGGGAA-3'
PentFEcoRI	5'-TTTTGAATTCCTGAACTGCGGCTATTCCTG-3'
PentRBamHI	5'-TTTTGGATCCTACTTCCTCAGCCAGTGACG-3'
tolCDelDnFVc	CCGATGAAAAAACTGGCGAAGAAGTAATCCATCTC-3'
tolCDelDnRVc	5'-TTTTTCTAGACGTTAGAGCACGTACAGCAG-3'
tolCDelUpFVc	5'-TTTTGAGCTCACAGACATGGTCGGCAATGG-3'
tolCDelUpRVc	GGATTACTTCTTCGCCAGTTTTTTCATCGGTCC

*Underlining denotes a restriction enzyme sequence (BamHI: GGATCC; EcoRI: GAATTC; SacI: GAGCTC; XbaI: TCTAGA)

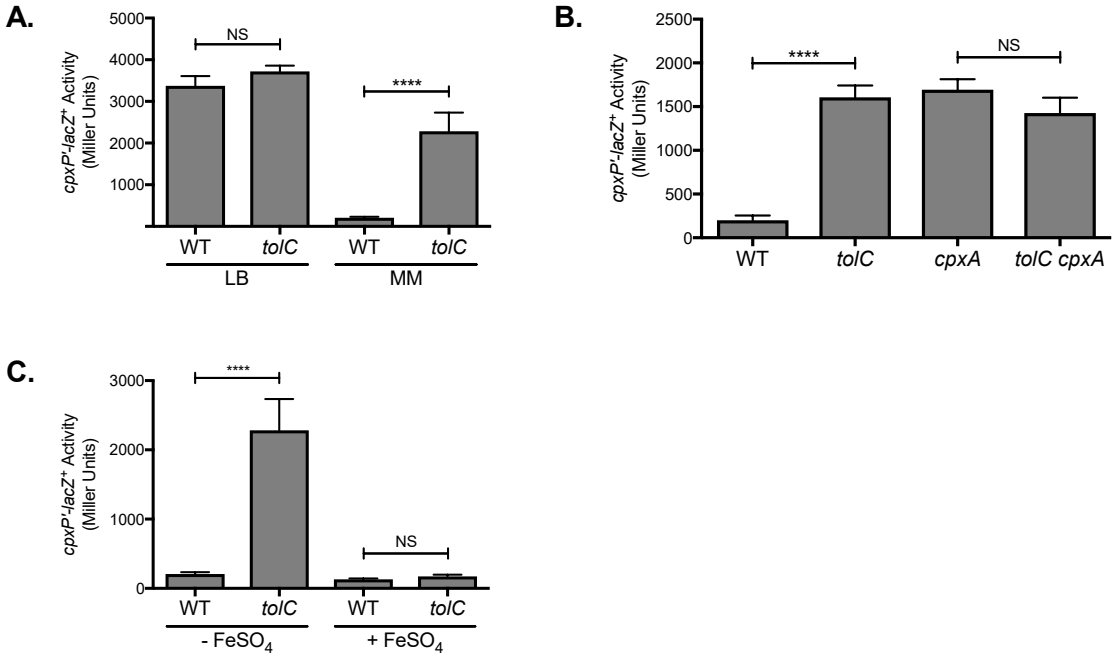


Figure 4-1 Deletion of *tolC* activates the *E. coli* Cpx response under iron-deplete conditions

(A) Wildtype and *tolC* mutant *E. coli* MC4100 strains carrying the chromosomal *cpxP-lacZ* transcriptional reporter were subcultured into Lennox broth (LB) or M9 minimal medium (MM) after overnight growth in LB medium and grown for twenty hours at 37°C. (B) Expression of the *cpxP-lacZ* transcriptional reporter in wildtype *E. coli* strain MC4100, the *tolC* and *cpxA* single mutants, and the *tolC cpxA* double mutant. Strains were subcultured into M9 minimal medium after overnight growth in LB medium, and grown for twenty hours at 37°C. (C) Expression of the *cpxP-lacZ* in wildtype and *tolC* mutant *E. coli* MC4100 strains subcultured into M9 minimal medium with (+) or without (-) 80μM $FeSO_4$ from overnight cultures grown in LB medium. Bacteria were grown for 20 hours at 37°C. To measure *cpxP-lacZ* expression, cells were lysed with chloroform and SDS, and β-galactosidase levels were measured after addition of ONPG in a 96 well plate as described in section 4.2.3. Data represent the means and standard deviations of three replicate cultures. Asterisks indicate a statistically significant difference from the indicated wildtype control (****, $P \leq 0.0001$ [one-way ANOVA with Sidak's post-hoc test]). NS indicates no statistically significant difference in *cpxP-lacZ* reporter activity. WT, wildtype

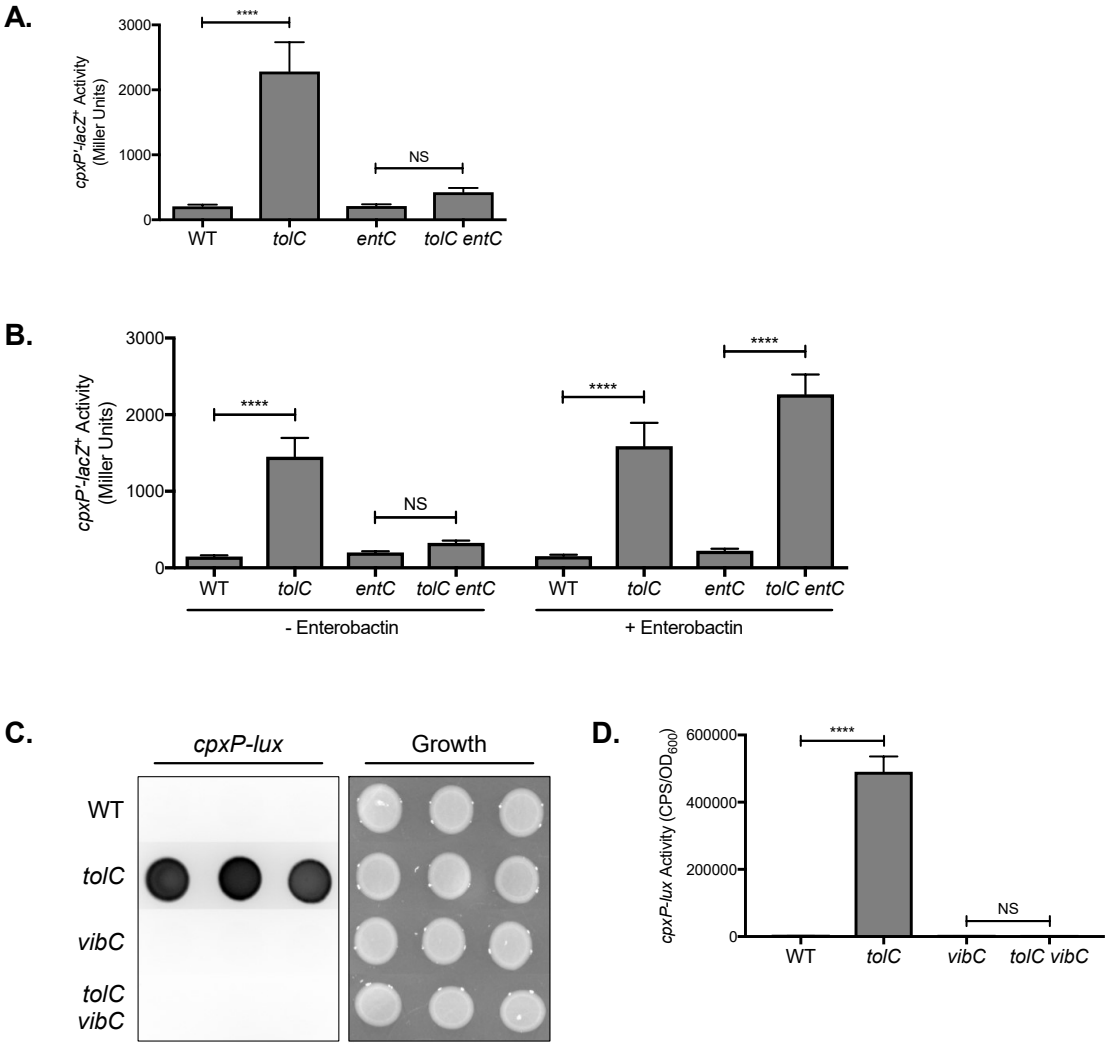


Figure 4-2 Siderophore accumulation in the *tolC* mutant activates the Cpx response

(A and B) Expression of the chromosomal *cpxP-lacZ* transcriptional reporter in wildtype *E. coli* strain MC4100, the *tolC* and *entC* single mutants, and the *tolC entC* double mutant. Strains were grown overnight in LB and then subcultured into (A) M9 minimal medium, or (B) M9 minimal medium with (+) or without (-) 10μM enterobactin. As enterobactin is dissolved in 42% DMSO, an equivalent volume of 42% DMSO was added to cultures without (-) enterobactin. Bacteria were grown for 20 hours at 37ºC. Cells were lysed with chloroform and SDS, and β-galactosidase levels were measured after addition of ONPG in a 96 well plate as described in section 4.2.3. Data represent the means and standard deviations of three replicate cultures. (C and D) Expression of the *cpxP-lux* transcriptional reporter in wildtype *V. cholerae* strain C6706, the *tolC* and

vibC single mutants, and the *tolC vibC* double mutant. Strains were grown in LB liquid broth overnight with shaking at 37°C. Bacteria were diluted to an OD_{600} of 1,10µL of each diluted culture was spotted onto LB agar, and bacteria were grown overnight at 37°C. (C) Luminescence from bacteria on agar plates was visualized using the ChemiDoc MP system (Bio-Rad). (D) Bacteria from (C) were scraped off the plate with plastic inoculating loops and resuspended in 1mL LB. 200µL of culture was transferred to a black-walled 96 well plate. Luminscence and OD_{600} were measured and *cpxP-lux* activity was calculated as described in section 4.4.5. Data show means and standard deviations of three biological replicates. Asterisks indicate a statistically significant difference from the indicated wildtype control (****, $P \leq 0.0001$ [one-way ANOVA with Sidak's post-hoc test]). NS indicates no statistically significant difference in *cpxP* reporter activity. WT, wildtype

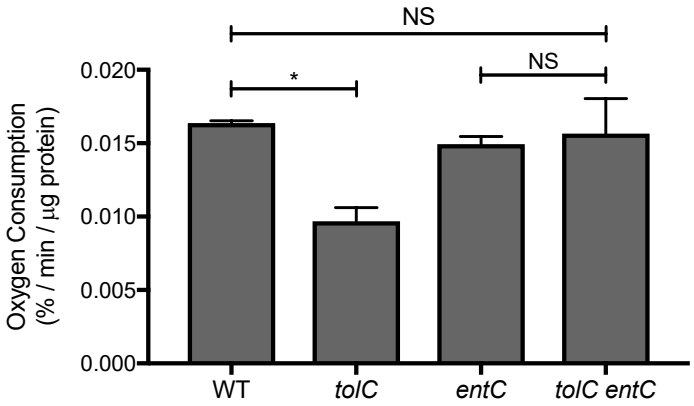


Figure 4-3 Enterobactin accumulation reduces NADH-dependent activity of the aerobic electron transport chain

Wildtype (WT) *E. coli* strain MC4100, the *tolC* and *entC* single mutants, and the *tolC entC* double mutant were subcultured into M9 minimal medium containing 0.4% glucose after overnight growth in LB medium and grown for 20 hours at 37ºC. Bacteria were collected by centrifugation and washed once in 50mM MES buffer, pH 6.0. Bacteria were then pelleted by centrifugation, weighed, and resuspended in 1mL of 50mM MES buffer, pH 6.0. 25µL of protease inhibitor was added for every 100mg of wet cell weight. Bacteria were then lysed by sonication. After centrifugation to remove intact cells, 100µL of cell lysate was diluted in 890µL 50mM MES buffer pre-warmed to 30ºC in a 1mL microrespiration chamber. Diluted lysate was covered in light mineral oil to prevent oxygen from dissolving into the system. Oxygen concentration was measured every 30 seconds for 10-15 minutes after the addition of 100µM β-NADH at 30ºC using oxygen MicroOptode sensor (Unisense). Oxygen concentration at each time point was standardized to the concentration present just prior to the addition of β-NADH. The rate of oxygen concentration per µg of total protein was calculated as described in section 4.2.4. Data represent the means and standard deviations of two biological replicates. Asterisks indicate a statistically significant difference from the indicated strain (*, $P \leq 0.05$ [one-way ANOVA with Sidak's post-hoc test]). NS indicates no statistically significant difference in the rate of oxygen consumption.

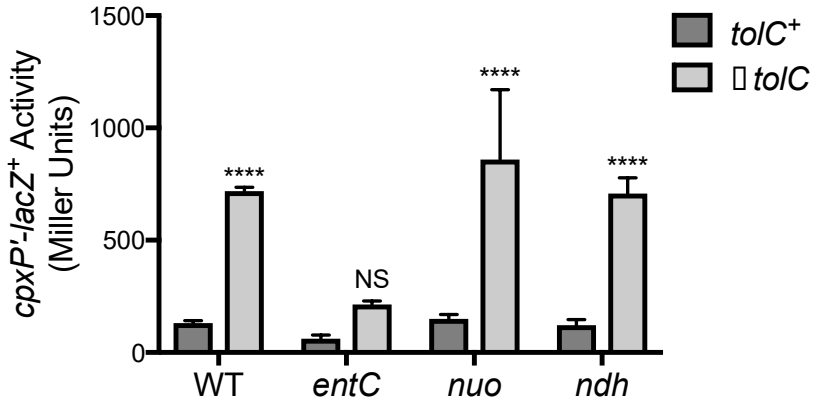


Figure 4-4 Activation of the Cpx response by deletion of *tolC* does not require NDH-I or NDH-II

After overnight growth in LB medium, bacteria containing the *cpxP-lacZ* transcriptional reporter were washed once, and resuspended in, phosphate-buffered saline. 10µL of culture was spotted onto M9 minimal medium agar containing 0.4% glucose and grown at 37°C for 24 hours. Bacteria were collected using plastic inoculating loops and resuspended in 1 x Z buffer. *cpxP-lacZ* activity was then measured as described in section 4.2.3. The strains shown are TR50 and the isogenic Δ*tolC, entC::kan, nuo::kan*, and *ndh::kan* single mutants, and the Δ*tolC entC::kan,* Δ*tolC nuo::kan,* Δ*tolC ndh::kan* double mutants. Data represent the means and standard deviations of three biological replicates. Asterisks indicate a statistically significant difference from the control strain containing a wildtype copy of *tolC* (*tolC*$^+$) (****, $P \leq 0.0001$ [two-way ANOVA with Sidak's post-hoc test]). NS indicates no statistically significant difference in *cpxP-lacZ* reporter activity.

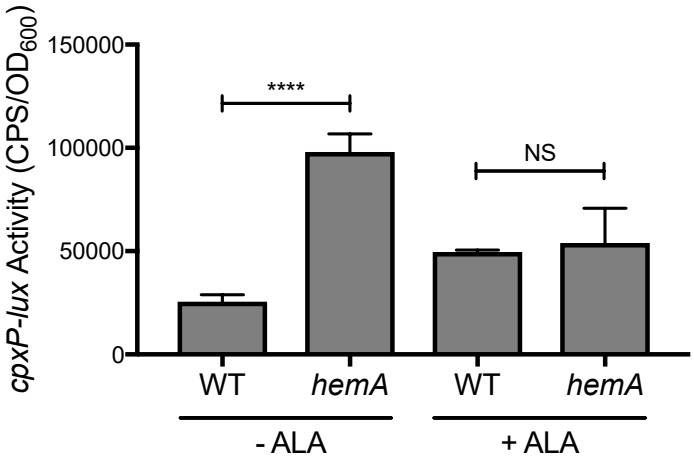
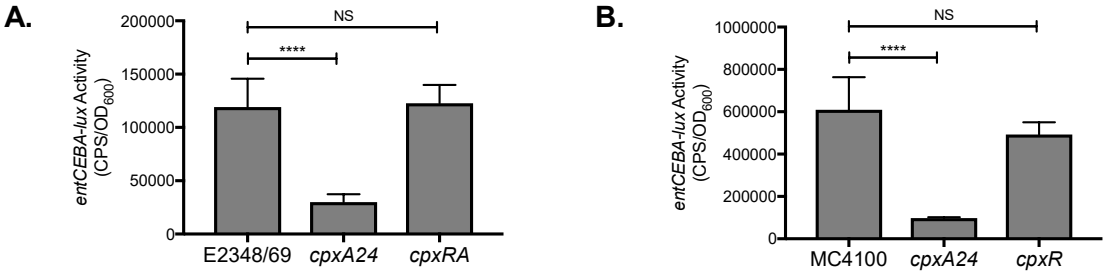


Figure 4-5 Deletion of *hemA* activates the Cpx response in EPEC

Wildtype (WT) or *hemA* mutant EPEC grown overnight in LB supplemented with 50µg mL^{-1} γ-aminolevulinic acid (ALA) were collected by centrifugation and washed twice in plain LB. Bacteria were then subcultured into 200µL LB containing 0.4% glucose with (+) or without (-) 50µg mL^{-1} ALA in a black-walled 96 well plate and grown for two hours. Luminescence (counts per second, CPS) and OD_{600} were measured, and *cpxP-lux* activity was calculated, as described in second 4.4.5. Data represent the means and standard deviations of three biological replicates. Asterisks indicate a statistically significant difference between the indicated strains (****, $P \leq 0.0001$ [one-way ANOVA with Sidak's post-hoc test]). NS indicates no statistically significant difference in *cpxP-lux* reporter activity.



C.

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  MC4100  CCTGAACTGCGGCTATTCCTGAAAGCAAAAGTCCTGTTAATAGAAGGGCG
E2348/69  CCTGCACTGCGGCTATTCCTGAAAGCAAAAGTCCTGTTAATAGAAGGGCG

  MC4100  TTGCGGTAGAGCGGGGCGAGTCTCACAAATCAGCTTCCTGTTATTAATAA
E2348/69  GTGCGGTAGAGCGGGGCGAGTCTCACAAATTAGCTTCCTGTTATTAATAG

  MC4100  GGTTAAGGGCGTAATGACAAATTCGACAAAGCGCACAATCCGTCCCCTCG
E2348/69  AGTTAA----------------------------------------------

  MC4100  CCCCTTTGGGGAGAGGGTTAGGGTGAGGGGAACAGCCAGCACTGGTGCGA
E2348/69  ------------------------------------------------

  MC4100  ACATTAACCCTCACCCCAGCCCTCACCCTGGAAGGGAGAGGGGGCAGAAC
E2348/69  ------------------------------------------------

  MC4100  GGCGCAGGACATCACATTGCGCTTATGCGAATCCATCAATAATGCTTCTC
E2348/69  ----------------------------------------TGCTTCTC

  MC4100  ATTTTCATTGTAACCACAACCAGATGCAACCCCGAGTTGCAGATTGCGTT
E2348/69  ATTTTCATTGTAACCACAAACAGATGCAACCCCGAGTTGCAGATTGCGTT

  MC4100  ACCTCAAGAGTTGACATAGTGCGCGTTTGCTTTTAGGTTAGCGACCGAAA
E2348/69  ACCTCAAGAGTTGACATAGTGCGCGTTTGCTTTTAGGTTAGCGACCGAAA

  MC4100  ATATAAATGATAATCATTATTAAAGCCTTTATCATTTTGTGGAGGATGAT
E2348/69  ATATAAATGATAATCATTATTAAAGCCTTTATCATTTTGTGGAGGATGAT

  MC4100  ATGGATACGTCACTGGCTGAGGAAGTA
E2348/69  ATGGATACGTCACTGGCTGAGGAAGTA
```

D.

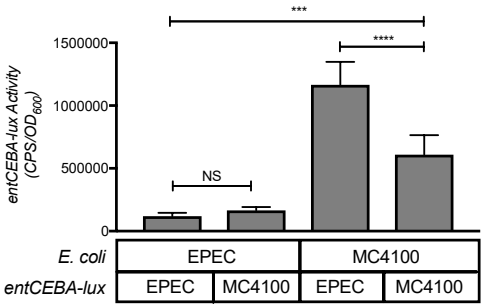

Figure 4-6 Expression of the *entCEBA* operon

Bacteria were grown overnight in LB broth at 30ºC with shaking. The following day, bacteria were subcultured into M9 minimal medium supplemented with 0.4% glucose,

5.34mM isoleucine, and 6.53mM valine and grown for 8 hours at 30°C with shaking. 200µL of culture was transferred to a black-wall 96 well plate and luminescence (expressed in counts per second [CPS]) and OD_{600} were read, and *entCEBA-lux* activity was calculated as described in section 4.4.5. (A) Activity of the EPEC *entCEBA-lux* reporter in wildtype EPEC and EPEC containing the *cpxA24* or *cpxRA* mutation. (B) Activity of the MC4100 *entCEBA-lux* reporter in wildtype MC4100 and MC4100 containing the *cpxA24* or *cpxR::spc* mutation. (C) Alignment of the *entCEBA* promoter region between MC4100 and E2348/69. DNA sequence of the *entCEBA* promoter DNA was determined by sequencing the insert of the pJW15-P*entCEBA*$_{K\text{-}12}$ and pJW15-P*entCEBA*$_{EPEC}$ plasmids. The DNA sequence of the insert for the pJW15-P*entCEBA*$_{K\text{-}12}$ and pJW15-P*entCEBA*$_{EPEC}$ was compared to the published genome of MG1655 and E2348/69, respectively, and was found to be 100% identical (data not shown). The DNA sequences were aligned using Multialin (http://multalin.toulouse.inra.fr/multalin). Bolded sequences represent the -35 box (TTGACA), the -10 box (TAGGTT), and the start codon (ATG), which were identified using the Ecocyc database (http://ecocyc.org) (281). Red sequences denote single base pair changes. -, absence of a base pair. (D) Activity of the EPEC *entCEBA-lux* reporter and the MC400 *entCEBA-lux* reporter in wildtype EPEC or wildtype MC4100. Data represent the means and standard deviations of five biological replicates. Asterisks indicate a statistically significant difference between the indicated strains (****, $P \leq 0.0001$; ***, $P \leq 0.001$ [one-way ANOVA with Sidak's post-hoc test]). NS indicates no statistically significant difference in *entCEBA-lux* reporter activity.

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CHAPTER 5

General Discussion

5.1 Overview

The work presented in this thesis aims to better understand the regulatory connections between the Cpx envelope stress response and respiration. We sought to determine how Cpx response regulates expression of the respiratory complexes NADH dehydrogenase I and cytochrome bo_3, and if regulation of these complexes facilitates adaptation to envelope stress. We also examined the mechanism by which deletion of *tolC* activates the Cpx response and the role of respiration in this process. This work furthers our understanding of how Gram-negative bacteria sense and adapt to stresses that disrupt envelope integrity.

5.2. Regulation of NADH dehydrogenase I and cytochrome bo3 expression by the Cpx response facilitates adaptation to envelope stress

5.2.1 Expression of NADH dehydrogenase I and cytochrome bo3 is repressed by the Cpx response.

NDH-I of the electron transport chain catalyzes the transfer of electrons from NADH to quinone, and uses the energy released during this process to translocate protons across the inner membrane. Microarray data indicate that expression of NDH-I is regulated during periods of envelope stress by the Cpx response (64). Here, we found that overexpression of the outer membrane lipoprotein NlpE decreased transcription of the *nuo* gene cluster in a CpxRA-dependent manner (Figure 2-1A). CpxR directly mediates this repression, as mutation of a putative CpxR binding site upstream of the *nuo* promoter nearly abolished regulation upon NlpE overexpression (Figure 2-1C). As the Cpx binding site is located upstream from the *nuo* promoter (Figure 2-1B), it is unclear how CpxR represses *nuo* transcription. Given that ArcA represses *nuo* transcription from a site near the CpxR binding site (Figure 2-1B), and that the Arc response is induced by envelope stress, it is possible that ArcA is involved in regulation of *nuo* transcription by CpxR (288). However, *nuo* transcription was still repressed, although to a slightly lesser extent, upon overexpression of *nlpE* in EPEC lacking *arcA* (Figure 2-1E). Accordingly, we conclude that ArcA plays a minor role in CpxR-mediated repression of *nuo* transcription. During our investigation, we identified a second potential CpxR binding site downstream of the *nuo* promoter (Figure 2-1B). We hypothesize that CpxR bound at the upstream and downstream binding sites interact to prevent *nuo* transcription through a looping mechanism that precludes RNA polymerase from

accessing the *nuo* promoter. To test this possibility, future experiments should be directed at mutating the putative CpxR binding side downstream from the *nuo* promoter.

We also found that activation of the Cpx response increases proteolysis of the NuoA protein subunit of the NDH-I complex (Figure 3-3). Several Cpx-regulated protein folding and degrading factors that are associated with the inner membrane protease FtsH alter NuoA protein levels when overexpressed, including DegP, YccA, HtpX, PpiD, and HflC (Figure 3-4 and Figure 3-7). Together, these results suggest that activation of the Cpx response may stimulate FtsH activity, which degrades NuoA. Indeed, overexpression of FtsH reduced NuoA protein levels (Figure 3-4A). In contrast to this hypothesis, however, activation of the Cpx response prevents FtsH-mediated degradation of the SecY subunit of the sec translocon (95). A major future direction for this project is to determine if FtsH is required for Cpx-mediated proteolysis of NuoA. This could be determined by deleting *ftsH* in a strain that carries the *sfhC21* allele, which suppresses the lethality of the *ftsH* null (338). If FtsH is responsible for NuoA proteolysis in the *cpxA24* mutant, then NuoA protein levels should remain unchanged in the *cpxA24 ftsH sfhC21* mutant in comparison to the *ftsH sfhC21* mutant. The data presented in this thesis suggest that activation of the Cpx response reduces expression of the NDH-I respiratory complex at both the transcriptional and post-translational level. A recent study has shown that mutational activation of the Cpx response increases the ratio of $NADH/NAD^+$ (339), supporting the hypothesis that NADH dehydrogenase activity is diminished upon Cpx pathway activation.

As with NDH-I, microarray data indicate that cytochrome bo_3 is a member of the Cpx regulon (64). Cytochrome bo_3 is one of three terminal oxidases of the electron transport chain in *E. coli* that reduce protons and molecular oxygen into water. Unlike the other terminal oxidases, cytochrome bo_3 contributes to the proton motive force by transporting protons across the inner membrane (176). In this work, we found that overproduction of NlpE reduced transcription of a *cyoABCDE* luminescent transcriptional reporter in a CpxRA-dependent manner. The mechanism by which activation of the Cpx response reduces transcription of the *cyo* gene cluster is unknown, however there are several possibilities. It is possible that CpxR could bind to the *cyo* promoter to directly repress *cyo* transcription. In support of this hypothesis, bioinformatic analysis identified a potential CpxR binding site that overlaps with the -35 box in the *cyo* promoter. As such, we are unable to mutate the putative CpxR binding site without potentially interfering with RNA polymerase binding. This possibility could instead be addressed by performing

electrophoretic mobility shift assays to determine whether CpxR binds to the *cyo* promoter *in vitro*. In the likely event that CpxR does bind to the *cyo* promoter, the electrophoretic mobility shift assay could be repeated using DNA in which the putative CpxR binding site in the *cyo* promoter has been mutated. Alternatively, the Cpx response may indirectly inhibit *cyo* transcription or may require additional regulators to mediate repression. In this work, we identified a minor role for ArcA in the Cpx-mediated repression of *cyo* transcription, however, CpxR is still able to repress *cyo* transcription in the absence of ArcA. Future studies could examine the role of other known *cyo* regulators in the Cpx-mediated repression of *cyo* transcription, including Fnr (340), Fur (341, 342), Crp (343), and PdhR (344).

Activation of the Cpx response also regulates expression of cytochrome bo_3 at the post-transcriptional level, since mutational activation of the Cpx response reduced levels of a CyoA protein that is transcribed from an inducible promoter (Figure 3-2). While we did not investigate the mechanism by which the Cpx response post-transcriptionally regulates CyoA, it is possible that CyoA is a substrate for Cpx-regulated protein folding and degrading factors. As we have proposed a role for FtsH in degradation of NuoA upon activation of the Cpx response, future experiments should investigate whether Cpx-mediated proteolysis of CyoA requires FtsH. Alternatively, the Cpx response may reduce translation of the *cyoA* mRNA transcript. In this regard, expression of several small regulatory RNAs is regulated by the Cpx response (96, 106, 118). At least one of these has been shown to protect against stresses that dissipate the proton motive force (96, 118), while another has been shown to reduce activity of the Cpx response (106), much like deletion of the *cyo* gene cluster (Figure 2-4A). Such post-transcriptional effects of the Cpx pathway on *cyoA* could be investigated by determining CyoA protein levels in *E. coli* lacking the RNA chaperone Hfq, which is required for regulation mediated by many small RNAs (345).

In agreement with the finding that activation of the Cpx response reduces expression of the cytochrome bo_3 terminal oxidase, mutational activation of the Cpx response reduces oxygen consumption (Figure 2-2A). Surprisingly, inhibition of the Cpx response by deletion of *cpxRA* also reduces oxygen consumption despite increased transcription of the *cyo* gene cluster (Figure 2-2 and Figure 2-1D). The rate of oxygen consumption in the *cpxRA* mutant is similar to the *cyo* mutant (Figure 2-2B), suggesting that the respiratory defect of the *cpxRA* mutant is may be due to defects in cytochrome bo_3 activity. As expression of several Cpx-regulated protein folding and degrading

factors is decreased in the absence of *cpxR*, we hypothesized that stability of the cytochrome bo_3 proteins may be affected by loss of *cpxRA*. However, protein levels of at least CyoA are unchanged in the *cpxRA* mutant compared to wildtype (Figure 3-2). Instead, it is possible that loss of the Cpx response affects stability of another subunit(s) of cytochrome bo_3. Alternatively, inhibition of the Cpx response may prevent assembly of the cytochrome bo_3 subunits into a functional multiprotein complex. Assembly of the cytochrome bo_3 complex in wildtype and *cpxRA* mutant *E. coli* could be determined by blue-native polyacrylamide gel electrophoresis.

Several envelope localized multiprotein complexes are regulated by the Cpx response at both the transcriptional and post-transcriptional level. Activation of the Cpx response inhibits the transcription of both the type three secretion system (109) and the bundle forming pilus (110). Unlike the NDH-I and cytochrome bo_3, transcription of these complexes is likely indirectly regulated by the Cpx response (109, 110). Overexpression of the Cpx-regulated chaperone/protease *degP* decreased activity of the type three secretion system without affecting transcription (101). Furthermore, activity of the type three secretion system is unaffected by loss of *cpxR* (101). The post-transcriptional effect of the Cpx response on the bundle forming pilus appears to be different than that of the type three secretion system or respiratory complexes. Expression of the bundle-forming pilus proteins is decreased in a *cpxR* mutant, but not when the Cpx response is activated (110). Elaboration of the bundle-forming pilus is increased under conditions in which it is not normally formed if the Cpx response is activated (56), suggesting that Cpx regulated chaperones assist in the biogenesis of the bundle-forming pilus. While these results clearly indicate a role for the Cpx response in the regulation of envelope localized multisubunit complexes, the rationale for this regulation remains enigmatic. It has been proposed that downregulation of these proteins may relieve the burden on the envelope protein folding machinery and/or help to conserve finite cellular resources during times of stress (25, 84, 100).

Overall, the work presented in this thesis has established a novel role for the Cpx response in regulating expression of at least two respiratory complexes: NDH-I and cytochrome bo_3. Activation of proteases that degrade existing complexes coupled with direct repression of *nuo* and *cyo* transcription ensures that expression of NDH-I and cytochrome bo_3 is reduced during periods of envelope stress. As discussed in the next section, regulation of these complexes is vital in generating an adaptive response to Cpx-dependent stress.

5.2.2 Regulation of respiratory complexes facilitates adaptation to Cpx-dependent envelope stress.

Activation of the Cpx response occurs upon overexpression of *nlpE* (58), exposure to aminoglycoside antibiotics (89), and growth at alkaline pH (54). The Cpx pathway is required to generate an effective adaptive response to these stressors, as the *cpxR* mutant grows poorly under these conditions (Figure 2-3). However, molecular details of the adaptive response established by the Cpx pathway remain poorly understood. Given that the Cpx response regulates expression of NDH-I and cytochrome bo_3 at multiple levels, expression of these complexes during stress may be toxic. In support of this hypothesis, we found that deletion of the *nuo* and *cyo* operons rescues the sensitivity of the *cpxR* mutant to *nlpE* overexpression, the aminoglycoside antibiotic amikacin, and alkaline pH (Figure 2-3). How does regulation of respiratory complexes facilitate adaptation to Cpx-dependent envelope stress? It is possible that under these conditions, chaperones and proteases that normally assist in the biogenesis of the NDH-I and cytochrome bo_3 complexes are titrated away, resulting in misassembled respiratory complexes that may compound the ensuing inner membrane stress. Activation of the Cpx response under these conditions could prevent *de novo* synthesis of NDH-I and cytochrome bo_3 while also increasing expression of protein folding and degrading factors that clear the membrane of improperly assembled complexes. In the absence of *cpxR*, expression of NDH-I and cytochrome bo_3 is not repressed during stress (Figure 2-1), and as such, newly synthesized complex subunits may be inserted into the membrane. Misassembled respiratory complexes in the absence of Cpx-regulated chaperones and proteases may dramatically increase stress on the inner membrane.

Alternatively, or in addition, decreased activity of the respiratory complexes may facilitate adaptation to Cpx-dependent envelope stress. As NDH-I and cytochrome bo_3 contribute to the PMF, it is possible that decreased expression of these complexes prevents stresses from reaching their site of toxicity. In this regard, NlpE is secreted through the PMF dependent Sec translocon, and aminoglycosides require the PMF for uptake. Regulation of proton-pumping complexes is also known to occur at alkaline pH to maintain the cytoplasmic pH. In contrast to this hypothesis, however, activation of the Cpx response is not known to affect the PMF (41, 45, 46), and a previous study has shown that deletion of both *nuo* and *cyo* in *E. coli* does not alter the PMF (292) .

Furthermore, overexpression of NlpE in the absence of both NDH-I and cytochrome bo_3 induces the Cpx response to the same extent as when both complexes are present (Figure 3-6B), suggesting that NlpE is secreted and can still induce envelope stress in this mutant. As such, it is unlikely that deletion of *nuo* and *cyo* promotes adaptation to envelope stress by preventing stressors from reaching their target. Instead, we favor the hypothesis that multileveled regulation of envelope-localized protein complexes reduces stress by preventing the formation of misassembled subunits that are a burden on the protein folding machinery and disrupt envelope integrity. We hypothesize that deletion of other multisubunit protein complexes, such as the bundle forming pilus or the type three secretion system, would rescue the sensitivity of the *cpxR* mutant to Cpx-dependent envelope stressors as well.

Several other phenotypes associated with the Cpx response can be explained by regulation of respiration. One of the first phenotypes associated with mutations at the *cpxRA* locus was the inability to grow using non-fermentable carbon sources (38, 41). In light of the work presented in this thesis, it is likely that decreased expression of respiratory complexes when the Cpx response is active prevents the generation of energy required to support growth under these conditions. Furthermore, several studies have shown that mutational activation of the Cpx response provides resistance to oxidative stress (64, 339, 346, 347). As the respiratory chain is known to generate damaging reactive oxygen species (293), activation of the Cpx response, and the resulting reduction in electron transport activity, could promote survival under these conditions. It has been observed that respiration is decreased upon surface adhesion (348). Given that the Cpx response is activated upon surface adhesion, the decrease in respiration under these conditions is likely mediated by the Cpx response. Finally, several systems that regulate respiration are required for colonization of the mammalian host (349-351), suggesting that the colonization defects displayed by *cpx* mutants may be related to inappropriate regulation of respiratory complexes (349, 352, 353). Accordingly, regulation of respiration may unify several seemingly unrelated phenotypes associated with the Cpx response.

5.3 Effects of NADH dehydrogenase I, cytochrome bo_3, and TolC on Cpx pathway activity.

Although the molecular nature of the Cpx inducing cue has not yet been determined, several conditions known or predicted to generate misfolded or mislocalized envelope proteins activate the Cpx response, including overexpression of pilin subunits in the absence of their cognate chaperone (55), overexpression of *nlpE* (58), aminoglycoside antibiotics (89), and expression of a misfolded variant of the periplasmic protein MalE (57). Accordingly, the Cpx response is believed to sense protein folding status within the envelope. In an effort to better understand the stress that is sensed by the Cpx response, in this work we a) determined the role of respiratory complexes in the activation of the Cpx response, and b) investigated the mechanism by which inhibition of the outer membrane channel TolC activates the Cpx response.

5.3.1 NADH dehydrogenase I and cytochrome bo_3 affect Cpx pathway activity

In this work, we found that expression of NDH-I and cytochrome bo_3 contributes to basal Cpx pathway activity and promotes pathway activation during stationary phase (Figure 2-4A). The mechanism by which these complexes activate the Cpx response is currently unknown, however there are several possibilities. One possibility is that the Cpx response detects some aspect related to the respiratory activity associated with these complexes. In support of this hypothesis, Cpx pathway activity in *Salmonella* is stimulated by the proton ionophore CCCP (118). However, we were unable to replicate this finding in *E. coli* K-12 strain MC4100 (Figure 2-5A) or EPEC strain E2348/69 (Figure 2-5B). Assuming that the Cpx responses in *E. coli* and Salmonella detect a similar stress, it is possible that CCCP does not generate this stress in *E. coli*. Alternatively, it is possible that CCCP activates the Cpx response in *E. coli*, however this cannot be detected using the method by which we quantified Cpx pathway activity. Chao and Vogel (118) measured Cpx pathway activity by determining the level of *cpxP* mRNA transcript after exposure to CCCP. In this work, we determined Cpx pathway activity using a *cpxP-lacZ* transcriptional reporter and by determining CpxA and CpxR protein levels, both of which rely on translation. It is possible that energy depletion in the presence of CCCP prevents translation, thus we were not able to accurately measure Cpx pathway activity. As such, future experiments should assess the effect of CCCP on Cpx pathway activity in *E. coli* by quantitative real-time PCR or via northern blot to directly measure *cpxP* transcript levels.

It is also possible that deletion of the *nuo* and *cyo* operons decreases Cpx pathway activity by reducing the production of reactive oxygen species that are

generated by the respiratory chain (293). In support of this hypothesis, paraquat, which generates superoxide radicals (354), was recently shown to activate the Cpx response in *V. cholerae*. As a redox-cycling agent, however, paraquat affects many redox active molecules within the cell (355) and thus it cannot be concluded with certainty that superoxide generation in the presence of paraquat activates the Cpx response. *E. coli* encode multiple superoxide dismutase enzymes that maintain low levels of superoxide and the level of superoxide is thought to rise in their absence (356). Assessing Cpx pathway activity in *E. coli* lacking superoxide dismutase could more directly test the hypothesis that the Cpx response is activated by superoxide. This strategy has been employed to determine the effect of superoxide on activity of the SoxRS oxidative stress response (355, 357). Conversely, however, deletion of NDH-I does not decrease production of superoxide or peroxide (358). As such, the effect of at least the *nuo* mutant on the Cpx response is unlikely to be related to reactive oxygen species.

Previous studies have shown that the central metabolite acetyl-CoA inhibits transcription of *cpxP* by acetylating lysine residues within the α subunit of RNA polymerase (69, 70). It is possible that acetyl-CoA accumulates in the *nuo* and/or *cyo* mutants as a result of decreased flux through the tricarboxylic acid (TCA) cycle. Accordingly, decreased *cpxP* expression in these mutants may arise though accumulation of acetyl-CoA.

We favor the possibility that the Cpx response senses some aspect related to the assembly of the NDH-I and cytochrome bo_3 complexes within the inner membrane. Overexpression of individual subunits of the cytochrome bo_3 complex activates the Cpx response (Figure 2-4C) and this activation is still observed in *E. coli* lacking the entire cytochrome bo_3 complex (J Wang and TL Raivio, unpublished observation). Furthermore, while deletion of the entire *nuo* operon decreases Cpx pathway activity, deletion of only the *nuoF* subunit activates the Cpx response (JL Wong and TL Raivio, unpublished observation). As NADH oxidase activity is decreased in both strains (160), this result argues against the Cpx response sensing a stress related to the activity of NDH-I. Instead, we believe that during normal biogenesis of NDH-I and cytochrome bo_3 some subunits may not assemble correctly, and these subunits engage in nonproductive interactions that generate the stress that is sensed by the Cpx response (chapter 2). Activation of the Cpx response would alleviate this stress by reducing *nuo* and *cyo* transcription as well as by increasing proteolysis of existing NDH-I and cytochrome bo_3 subunits (chapter 2 and 3; Figure 5-1). This hypothesis predicts that conditions that

affect biogenesis of the NDH-I or cytochrome *bo*$_3$ complexes would activate the Cpx response. Given that the protease HtpX affects NuoA protein expression (Figure 3-4), and that deletion of *htpX* activates the Cpx response (24), we hypothesized that improper biogenesis of NDH-I or cytochrome *bo*$_3$ in the *htpX* mutant may activate the Cpx response. However, deletion of the entire *nuo* and/or *cyo* gene cluster(s) did not reduce Cpx pathway activation in the *htpX* mutant (Figure 3-6A). Furthermore, deletion of *nuo* and/or *cyo* did not prevent Cpx pathway activation by *nlpE* overexpression (Figure 3-6B). We believe that the cumulative stress of improper biogenesis of multiple envelope localized complexes activates the Cpx response under these conditions, and that deletion of the NDH-I and cytochrome *bo*$_3$ complexes is not sufficient to reduce Cpx pathway activity. It is also possible that CpxA directly senses misfolded NlpE subunits (73), and as such, deletion of *nuo*, *cyo* or any other gene would not prevent pathway activation under these conditions. The possibility that the Cpx response senses the assembly, rather than the activity, of NDH-I and cytochrome *bo*$_3$ could be further tested by analyzing Cpx pathway activity in mutants with inactive variants of these enzymes. For NDH-I, it has previously been shown that introducing the $D_{79}N/E_{81}Q$ mutations in NuoA decreases NDH-I activity by 98% while leaving the complex intact (359). If activation of the Cpx response were related to the biogenesis of the NDH-I complex, we would not expect a decrease in Cpx pathway activity in this mutant. However, if the Cpx response were influenced by the activity of the enzyme, we would expect that Cpx pathway activity would decrease in the NuoA$D_{79}N/E_{81}Q$ mutant.

5.3.2. Activation of the Cpx envelope stress response by deletion of the outer membrane channel *tolC*

Another major finding of this work is that activation of the Cpx response that occurs in the *tolC* mutant is the result of improper secretion of the siderophore enterobactin (chapter 4). At this point, we are unable to rule out the possibility that enterobactin directly stimulates CpxA. However, as enterobactin accumulation causes several toxic phenotypes that are unlikely to be the result of Cpx pathway activation (229), we believe that this activation is indirect. Several observations support a role for respiration in the activation of the Cpx response in the *tolC* mutant. First, NADH oxidase activity is decreased in *tolC* mutants grown in minimal medium, but not rich medium (264). In this work, we provide evidence to suggest that this is due to enterobactin accumulation (Figure 4-3). It is possible that enterobactin directly damages the NADH

dehydrogenase enzymes, thus inhibiting their activity. As activation of the Cpx response by deletion of *tolC* is unaffected in *E. coli* lacking *nuo* or *ndh* (Figure 4-4), it is unlikely that damage to NADH dehydrogenase enzymes is responsible for activation of the Cpx response under these conditions. It is also possible that enterobactin accumulation indirectly decreases NADH-dependent activity of the electron transport chain through activation of regulatory pathways that decrease expression of the NDH enzymes, such as the Cpx response (Figure 2-1). Second, vibriobactin-mediated activation of the Cpx response in the *V. cholerae tolC* mutant can be suppressed if *sdhA* of succinate dehydrogenase is mutated (294). Together, these results suggest that activation of the Cpx response upon accumulation of siderophores occurs through succinate dehydrogenase, but not NADH dehydrogenase. Like NDH-I and cytochrome bo_3, succinate dehydrogenase is a multisubunit complex located in the inner membrane. We believe that enterobactin accumulation prevents the proper assembly of succinate dehydrogenase by affecting biosynthesis of the heme cofactors (chapter 4). In support of this hypothesis, deletion of *hemA* activates the Cpx response (Figure 4-5). Mutating the heme-binding site in succinate dehydrogenase could test the possibility that improper heme incorporation into this enzyme activates the Cpx response. Furthermore, deletion of the *cyo* genes should also reduce Cpx pathway activity in the *tolC* mutant as insertion of heme into cytochrome bo_3 is essential for proper biogenesis of the complex (190). Overall, this work provides support for the hypothesis that improper biogenesis of multisubunit respiratory complexes activates the Cpx envelope stress response. It is important to note that heme cofactors are also found in catalase enzymes involved degradation of reactive oxygen species (360, 361). As such, it is possible that disrupting heme biosynthesis increases reactive oxygen species that may generate a Cpx-inducing signal. To test this possibility, Cpx pathway activity could be monitored in *E. coli* lacking heme-containing catalases.

We also provide evidence to suggest that the Cpx response rids the cell of stressors that may interfere with proper biogenesis of respiratory complexes. We found that the Cpx response represses genes involved in enterobactin biosynthesis, as well as increases expression of the enterobactin secretion machinery. Accordingly, the Cpx envelope stress response mounts an effective adaptive response to stresses that disrupt biogenesis of respiratory complexes by clearing the envelope of the stressor and removing the target of the stress (Figure 5-2).

5.4 Role of the Cpx response in the assembly of inner membrane protein complexes

Overall, the data presented in this thesis suggest an intimate association between the Cpx response and multisubunit complexes with components in the inner membrane. We provide evidence to suggest that improper biogenesis of several respiratory complexes activates the Cpx response. Activation of the Cpx response then represses *de novo* synthesis of at least NDH-I and cytochrome bo_3 and activates the expression of chaperones and/or proteases to clear the cell of existing complexes. Given that the Cpx response regulates and/or is regulated by additional multisubunit complexes in a similar fashion, including various pili (55, 56, 103, 110, 315), secretion systems (101, 109, 111), and the flagella (63, 100), we propose that the Cpx response plays a general role in monitoring and maintaining the biogenesis of macromolecular protein complexes within the inner membrane and that improper assembly of these complexes disrupts inner membrane integrity.

Several observations provide support for this model. The Cpx response regulates several proteins that modulate the activity of the zinc metalloprotease FtsH (chapter 3). Inner membrane proteins that fail to associate into multisubunit complexes, such as SecY of the sec translocon (362) and subunit a of the F_1F_o ATP synthase (320), are subject to FtsH-mediated degradation . Overproduction of protein variants that inhibit FtsH activity is toxic in *E. coli* lacking the Cpx response (24). Furthermore, overexpression of *secY* and *atpB* in the absence of FtsH activates the Cpx response (24). Together, these results suggest that the Cpx response functions to facilitate assembly of the sec translocon and F_1F_o ATP synthase and/or to prevent the toxicity of misassembled subunits. Another piece of evidence that supports a role for the Cpx response in the biogenesis of inner membrane multisubunit complexes comes from studies on the insertase/assembly factor YidC. YidC is required for inserting several proteins into the inner membrane, including subunits of NDH-I and cytochrome bo_3 (173, 191). Depletion of YidC activates the Cpx response, suggesting that the Cpx pathway is attuned to conditions that prevent the proper insertion or assembly of complexes within the inner membrane (273). Overall, these results support the conclusion that the Cpx response serves to monitor and maintain the biogenesis of inner membrane proteins.

5.5 Concluding remarks

The gram-negative envelope establishes a protective barrier that separates bacteria from their environment. As the integrity of the envelope is fundamental to this function, several regulatory systems are tasked with modifying and repairing the envelope in response to environmental assaults. The purpose of this thesis was to better understand the mechanism by which the Cpx envelope stress response detects and responds to conditions that threaten protein folding within the envelope. More specifically, we investigated the association between the Cpx response and the respiratory chain. In this thesis, we have demonstrated that the Cpx response is attuned to the biogenesis of several respiratory complexes. We have shown that NDH-I and cytochrome bo_3 are members of the Cpx regulon and that regulation of these complexes facilitates adaptation to Cpx-specific stresses. These results suggest a completely new role for the Cpx response in controlling respiration, and provide an explanation for observations that link the Cpx response to antibiotic resistance, oxidative stress resistance, and host colonization. In conclusion, we propose that the Cpx response serves as the sentry for inner membrane protein biogenesis.

5.6 Figures

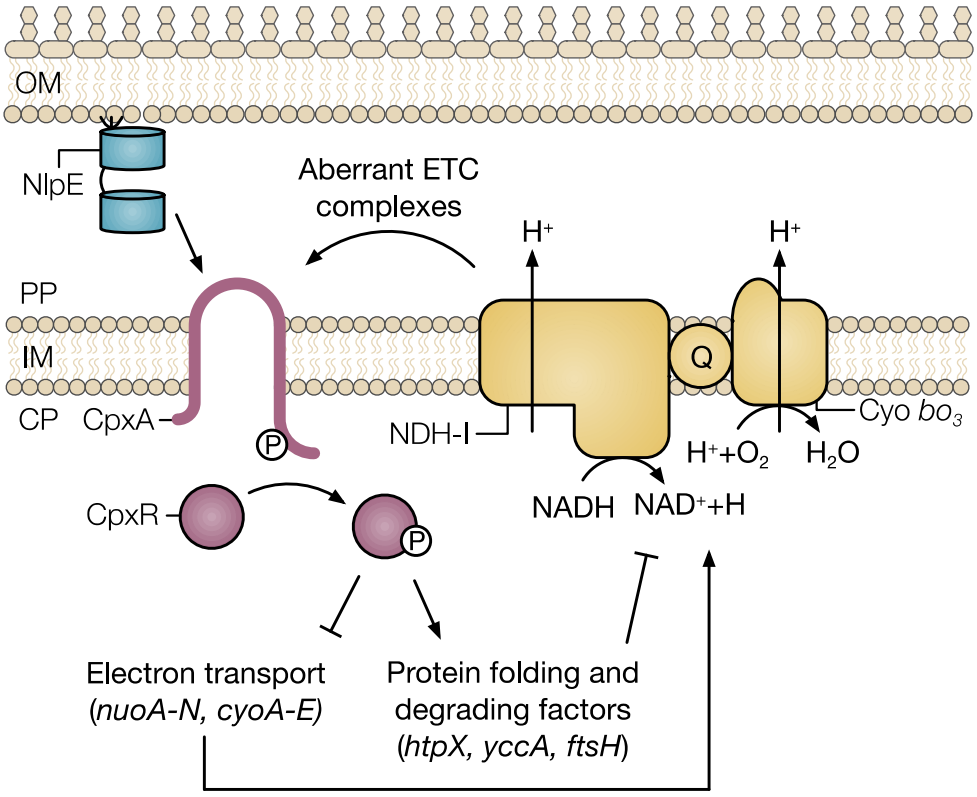

Figure 5-1 Proposed association between the Cpx envelope stress response and the electron transport chain

In the presence of envelope stress, such as that generated by NlpE, the Cpx response directly represses transcription of the *nuo* and *cyo* operons that encode NADH dehydrogenase I (NDH-I) and cytochrome bo_3 (Cyo bo_3), respectively. The Cpx response increases activity of protein folding and degrading factors, such as HtpX, YccA, and FtsH, that increase proteolysis of NADH dehydrogenase I and cytochrome bo_3. NDH-I and cytochrome bo_3 contribute to Cpx pathway activity. OM, outer membrane; PP, periplasm; IM, inner membrane; CP, cytoplasm. Figure adapted from (304)

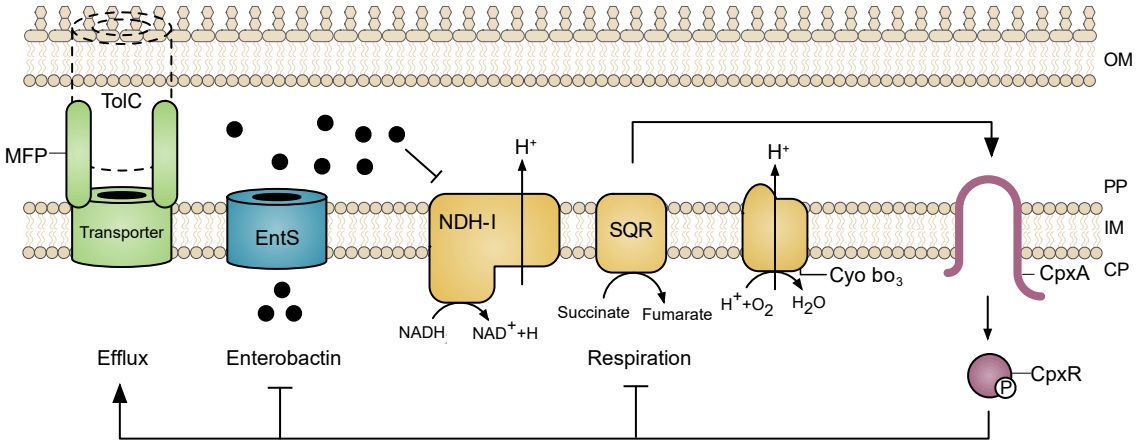

Figure 5-2 Proposed association between the Cpx envelope stress response and TolC

Under conditions in which efflux through TolC is compromised, the siderophore enterobactin accumulates within the periplasm of *E. coli*. Enterobactin may disrupt heme-containing components of the electron transport chain, which may generate a Cpx inducing signal. Cpx regulation of multidrug efflux pumps, respiratory complexes, and enterobactin biosynthesis may provide an adaptive response to accumulation of enterobactin. NDH-I, NADH dehydrogenase I; SQR, succinate dehydrogenase; Cyo *bo*$_3$, cytochrome *bo*$_3$; OM, outer membrane; PP, periplasm; IM, inner membrane; CP, cytoplasm; MFP, membrane fusion protein. Figure adapted from (336).

5.7 References

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