Molecular Mechanisms Used by Auxiliary Regulator NlpE to Signal the Cpx Envelope Stress Response in *Escherichia coli*

by

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Abstract

The Cpx two-component system senses and mediates adaptation to envelope stresses in *Escherichia coli*. NlpE is an outer membrane lipoprotein that senses surface adhesion and signals the Cpx pathway. This work focused on investigating the molecular mechanisms used by NlpE to signal the Cpx pathway when overexpressed and upon surface adhesion in *E. coli*. The previously solved crystal structure of NlpE revealed two distinctly formed β-barrel domains connected by an unstructured flexible linker. Mutagenesis studies revealed that both the amino- and carboxyl-terminal β-barrel domains, as well as the flexible linker contribute to signaling the Cpx pathway. The results suggest a signaling model that involves collaboration between all regions of NlpE. The amino-terminus and the flexible linker also stabilize the lipoprotein for its proper function in *E. coli. In vitro* pull-down, *in vivo* co-immunoprecipitation and cross-linking assays were carried out to identify proteins that may interact with NlpE in *E. coli.* The outer membrane adhesin OmpA was identified. OmpA is also required for NlpE to sense surface adhesion and convey this signal to the Cpx response. In summary, the findings of this thesis suggest a model in which outer membrane lipoprotein NlpE senses surface adhesion through a direct interaction with the adhesin OmpA and signals the Cpx pathway in a manner that involves all three structural domains of NlpE. The results provide molecular details of the manner in which auxiliary regulator NlpE communicates a signal generated at the outer membrane to a sensory kinase in the inner membrane and may be useful in the future for the design of tools to manipulate bacterial behavior.

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List of Abbreviations

Amp: ampicillin

Cam: chloramphenicol

Co-IP: co-immunoprecipitation

cps: counts per second

Cpx: conjugative plasmid expression

DNA: deoxyribonucleic acid

IM: inner membrane

IPTG: isopropyl β- D-thiogalactoside

Kan: kanamycin

LB: Luria-Bertani

NlpE: new lipoprotein E

OD_{600}: optical density at 600 nm

OM: outer membrane

OMP: outer membrane protein

PCR: polymerase chain reaction

PBS: Phosphate-buffered saline

RNA: ribonucleic acid

RNase: ribonuclease

rpm: rotations per minute

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TCS: two-component system

Tris: tris(hydroxymethyl)aminomethane

VC: vector control

WT: wild-type

1. General Introduction

1.1 Gram-negative Bacterial Cell Envelope

The bacterial cell envelope is a sophisticated structure that separates living cells from the environment. It maintains cell integrity while allowing selective passage of nutrients and wastes. It also contains proteins, protein complexes and even cellular appendages involved in a variety of activities such as chemotaxis, motility, surface attachment, secretion, pathogenesis and signal transduction (Glauert and Thornley, 1969; Duong *et al.*, 1997; Costerton *et al.*, 1974; Binet *et al.*, 1997; Silhavy *et al.*, 2010). To better understand envelope stress responses, the composition, organization, and biogenesis of the Gram-negative bacterial cell envelope will be reviewed.

1.1.1 The Inner Membrane

For Gram-negative bacteria, the envelope consists of an outer membrane, inner membrane and the periplasm in between (Glauert and Thornley, 1969) (Fig.1-1). The inner membrane (cytoplasmic membrane) is a phospholipid bilayer that surrounds the cytoplasm. Phospholipid content may vary in different species. There are integral inner membrane proteins, peripherally associated proteins and lipoproteins embedded in the inner membrane, some of which diffuse freely. Inner membrane proteins or protein complexes are involved in respiration, energy production, cell division, signal transduction and transport processes, to name a few. Integral inner membrane proteins contain trans-membrane domains that are mainly α-helices. Integral inner membrane proteins are synthesized in the cytoplasm and assembled through the signal recognition particle (SRP)-dependent co-translational targeting pathway. During co-translational targeting, SRP

recognizes a signal anchor sequence in proteins emerging from the ribosome and delivers ribosome nascent protein chains to the SecYEG translocon (Kudva *et al.*, 2013; Luirink *et al.*, 2012). The SecYEG translocon is a dual functional channel that allows translocation of secreted protein across the inner membrane and the integration of α-helical inner membrane proteins. The YidC insertase can cooperate with the SecYEG translocon during this process (Kudva *et al.*, 2013; Dalbey *et al.*, 2011; Luirink *et al.*, 2005; Luirink *et al.*, 2012).

1.1.2 The Outer Membrane

The outer membrane is a selective barrier that protects cells from detrimental environments. Unlike the inner membrane, the outer membrane is asymmetrical with lipopolysaccharide (LPS) on its outer leaflet and phospholipids in the inner leaflet (Nikaido, 1988). There are outer membrane proteins and lipoproteins embedded in the outer membrane (Fig.1-1). In addition, there are many cell appendages embedded in the outer membrane such as flagella, pili, and secretion systems. All the components found in the outer membrane including LPS, phospholipids and proteins are synthesized in the cytoplasm. The biogenesis and transport of all the components of the outer membrane are fundamental topics in biology.

1.1.2.1 Lipopolysaccharide (LPS)

LPS is a glycolipid species consisting of three main parts: lipid A (endotoxin) that anchors LPS to the OM, a core oligosaccharide and an O-antigen polysaccharide of various lengths. The O-antigen domain is missing in laboratory *E. coli* K12 strains.

LPS induces a strong innate immune response in animals (Sperandeo *et al.*, 2009; Zhang *et al.*, 2013; Miyake, 2004). The strong interaction between LPS molecules through divalent cations, fatty-acid chains and the sugar components makes LPS a highly compacted gel-like lipid with very low fluidity. Therefore LPS plays a major role in maintaining the barrier function by preventing detrimental molecules from diffusing into the cell (Zhang *et al.*, 2013). LPS is essential for most gram-negative bacteria with the rare exceptions such as *Neisseria meningitides*, *Moraxella catarrhalis* and *Acinetoobacter baumannii* (Steeghs *et al.*, 1998; Peng *et al.*, 2005; Moffatt *et al.*, 2010). The biogenesis of LPS is extensively studied, but aspects of this process remain elusive. The core-lipid A synthesized on the cytoplasmic side of the inner membrane is flipped across the inner membrane to the periplasmic side by the ATP-binding cassette (ABC) transporter MsbA, where mature LPS forms (Doerrler *et al.*, 2004; Doerrler and Raetz, 2002). The transport and insertion of LPS into the OM is facilitated by the lipopolysaccharide transport (Lpt) machinery (Sperandeo *et al.*, 2009). The Lpt complex consists of an inner membrane ABC transporter LptBFG, a periplasmic chaperone LptA and an outer membrane translocon LptDE (Sperandeo *et al.*, 2009) (Fig.1-1). LPS biogenesis is monitored by the σ^E stress response system (Lima *et al.*, 2013) (Fig.1-3).

1.1.2.2 Outer Membrane Protein (OMPs)

Unlike inner membrane proteins, the trans-membrane domains of outer membrane proteins adopt a β-barrel structure, instead of α-helices. Outer membrane proteins function as channels, porins, transport machineries, adhesins, and enzymes (Ruiz *et al.*, 2006).

Newly synthesized outer membrane proteins contain an amino-terminal signal sequence that is recognized by the SecYEG translocon for subsequent translocation across the inner membrane. Misfolding of β-barrel OM protein precursors in the periplasm is prevented by molecular chaperones, such as SurA and Skp, which are believed to assist OMP transport across the periplasm and delivery to the β-barrel assembly machinery – the Bam Complex. The Bam complex is composed of an OMP, BamA, and four lipoproteins, BamB, BamC, BamD and BamE (Knowles *et al.*, 2009). The four lipoproteins form a ring-like structure and interact with BamA at the periplasmic side creating a substrate-exit pore located at the outer membrane-periplasm interface. The chaperone-bound substrates entry the complex via the ring-like apparatus and insert into the outer membrane via the substrate-exit pore (Han *et al.*, 2016; Gu *et al.*, 2016).

1.1.2.3 Lipoprotein

Bacterial lipoproteins are proteins with an amino-terminal lipid modification at a conserved cysteine residue. The lipid moiety anchors the lipoprotein to the membrane. Recently, many lipoproteins have been revealed to play important roles in a variety of bacterial physiological processes including virulence, surface adhesion, cellular signaling, envelope biogenesis, and regulation of cell wall biogenesis (Polissi and Sperandeo, 2014; Paradis-Bleau *et al.*, 2010; Castanié-Cornet *et al.*, 2006a; Hirano *et al.*, 2007; Tsukahara *et al.*, 2009; Kim *et al.*, 2012). The most well studied lipoprotein, Lpp (Braun's lipoprotein), is one of the most abundant proteins in *E. coli* (Braun and Wolff, 1970). Lpp physically staples the outer

membrane to the peptidoglycan through its carboxyl-terminus lysine residue that reacts with *meso*-diaminopimelic acid of peptidoglycan (Braun and Wolff, 1970).

Lipoprotein biogenesis is well studied in *E. coli*. Lipoproteins are synthesized in the cytoplasm as a precursor called preprolipoprotein, with an amino-terminal signal peptide sequence, which has a characteristic consensus sequence [LVI][ASTVI][GAS] C+1 often referred to as the lipobox (C+1 is the conserved C that is lipid modified and three amino acids ont the amino-terminal of C+1) (Tokuda and Matsuyama, 2004). The cysteine is the target of lipid modification and is referred to as +1 in the mature lipoprotein. Similar to OMPs, the signal peptide of lipoproteins is required for their transport by the Sec translocon system across the inner membrane to its outer leaflet, where lipid modification takes place. The lipid modification is catalyzed by three sequentially acting enzymes: preprolipoprotein diacylglycerol transferase (Lgt) catalyzing formation of the thioether linkage between diacylglycerol and cysteine, prolipoprotein signal peptidase (LspA) that cleaves the signal peptide, and apolipoprotein N-acyltransferase (Lnt), which catalyzes amino-acylation of the N-terminal cys residue (Sankaran and Wu, 1994). The mature lipoprotein with proper lipid modification may be retained in the inner membrane or be transported to the outer membrane by the localization of lipoproteins (Lol) system (Tokuda and Matsuyama, 2004). In *E. coli*, most lipoproteins are destined to the outer membrane (Konovalova and Silhavy, 2015). Extensive molecular studies revealed that inner membrane lipoproteins have a Lol avoidance signal with Asp at position +2 and either Gln, Glu, or Asp at position +3 (Yamaguchi *et al.*, 1988; Gennity and Inouye, 1991; Terada *et al.*, 2001). In contrast, the ABC transporter

LolCDE selectively recognizes outer membrane lipoproteins (Yakushi *et al.*, 2000). The LolCDE complex releases lipoproteins from the cytoplasmic membrane by hydrolyzing ATP, and delivers them to the periplasmic chaperone protein LolA, which accommodates the hydrophobic lipid moiety of the lipoprotein (Matsuyama *et al.*, 1995). The LolA-lipoprotein complex is hydrophilic and travels across the periplasm, probably by diffusion, to the outer membrane lipoprotein LolB. The LolA-lipoprotein complex interacts with lipoprotein LolB, which assembles lipoproteins properly at the outer membrane through an unknown mechanism (Tsukahara *et al.*, 2009; Matsuyama *et al.*, 1997).

Historically, lipoproteins were thought to be largely soluble, with the majority of the protein residing in the periplasm (Tokuda and Matsuyama, 2004). However, recent studies revealed several lipoproteins that exist in more complicated forms (Konovalova and Silhavy, 2015). The outer membrane LPS translocon consists of an outer membrane protein, LptD, and a lipoprotein LptE. A crystal structure of the complex revealed an unusual two protein 'barrel and plug' architecture in which LptD forms a 26- stranded β-barrel with a quarter of LptE embedded inside its barrel (Konovalova and Silhavy, 2015). The rest of LptE resides in the periplasm. CsgG is an outer membrane lipoprotein that is required for secretion of the subunits that make up the cell surface associated curli adhesin - CsgA and CsgB (Robinson *et al.*, 2006). Nine CsgG subunits with proper lipid modification each donate four β-strands to form a 36-stranded β-barrel that functions as a transmembrane channel (Goyal *et al.*, 2014). In contrast, soluble CsgG without lipid modification forms an octamer. This suggests that lipid modification is essential for the proper assembly of

CsgG oligomers at the outer membrane. The auxiliary regulator RcsF is an outer membrane lipoprotein that is required for signaling by the envelope stress sensitive Rcs phosphorelay (Castanié-Cornet *et al.*, 2006a; Farris *et al.*, 2010; Majdalani *et al.*, 2005). Unusually, RcsF is tethered to the outer leaflet of the outer membrane through its lipid moiety, leaving the unstructured region at its amino-terminus surface exposed. The rest of the protein is threaded through OmpC or OmpF to reside in the periplasm (Konovalova *et al.*, 2014; Cho *et al.*, 2014). Its unique morphology is thought to sequester it from signaling the inner membrane Rcs phosphorelay in the absence of inducing cues, and therefore be critical for its signaling. These more complicated forms of lipoproteins necessitate experimental verification of the location and topology of each specific lipoprotein.

1.1.3 Periplasm

In Gram-negative bacteria, the periplasm refers to the viscous space between the inner membrane and outer membrane (Silhavy *et al.*, 2010). This compartment is packed with proteins including periplasmic chaperones involved in envelope biogenesis, binding proteins facilitating small molecule transport, and enzymes that play roles in hydrolytic reactions, nucleic acid and protein degradation, as well as detoxification (Beacham, 1979; Silhavy *et al.*, 2010). The periplasm is devoid of ATP. In addition, the structurally important bacterial cell wall is localized in the periplasm.

1.1.3.1 Cell Wall

In Gram-negative bacteria, the cell wall is a mesh like layer that resides in the periplasm and surrounds the plasma membrane. Peptidoglycan (also known as murein) is made of polymers of the disaccharide N-acetyl glucosamine-N-acetylmuramic acid that are cross-linked by short peptides (Vollmer *et al.*, 2008). The rigidity of peptidoglycan defines the cell shape and provides the cell with more tension to withstand osmotic pressure. Due to its significance, cell wall synthesis and remodeling must be tightly regulated to coordinate with outer membrane biogenesis and the cell division cycle (Typas *et al.*, 2012). This regulation is just beginning to be understood. For example, maintenance of the rod shape of *E. coli* requires a bacterial homologue of actin, MreB, to direct peptidoglycan synthesis specifically at curved regions of the cell (Ursell *et al.*, 2014). Similarly, the outer membrane proteins LpoA and LpoB act as regulators to sense outer membrane inputs, and modify the activities of the main peptidoglycan synthases PBP1A and PBP1B (Paradis-Bleau *et al.*, 2010).

1.1.4 Trans-envelope Protein Complexes

Many cellular functions, such as transport, require large protein complexes formed by multiple components located in all compartments of the envelope (inner membrane, periplasm, outer membrane). These molecular machines include trans-envelope complexes such as flagella, type III secretion systems, and efflux pumps (Koronakis *et al.*, 2000). Such protein assemblies are particularly interesting because of three aspects: the synthesis of these complexes involves envelope quality control, their activities must be tightly regulated, and their function impacts

envelope activity. Two examples of trans-envelope complexes include the Tol-Pal and TonB-ExbBD protein assemblies, reviewed below.

1.1.4.1 Tol-Pal Complex

The Tol-Pal complex consists of an inner membrane complex of TolA, TolQ and TolR and an outer membrane complex of Pal and TolA (Cascales *et al.*, 2000) (Fig.1-2). It also involves a periplasmic protein, TolB, that is required for the interaction of TolA and Pal (Walburger *et al.*, 2002). The TolQ and TolR complex interacts with TolA through its trans-membrane helices (Cascales *et al.*, 2001). The TolQR complex acts as a proton translocator to harvest the PMF and energize TolA, resulting in conformational changes that facilitate interaction with Pal (Germon *et al.*, 2001). Pal and TolA interaction connect the outer membrane to the inner membrane by consuming proton motive force (Cascales *et al.*, 2000). The Tol-Pal complex is a conserved complex in Gram-negative bacteria (Cascales *et al.*, 2000). However, the function of the Tol-Pal complex is not clear. It has been shown that the Tol-Pal complex contributes to maintaining outer membrane integrity by mediating physical interaction between the OM and the peptidoglycan. Pal is one of the most abundant proteins in the outer membrane and interacts with the cell wall non-covalently (Koebnik, 1995; Mizuno, 1979). Mutation in *pal* or *tolA* results in increased sensitivity to SDS and elevated formation of outer membrane vesicles (Cascales *et al.*, 2002; Lazzaroni *et al.*, 1999; Lazzaroni and Portalier, 1992; Santos *et al.*, 2015; Yeh *et al.*, 2010). The Tol-Pal complex is part of the cell division machinery as a *tol-pal* complex mutant showed abnormal OM invagination and OM blebs at constriction sites and cell poles (Gerding *et al.*, 2007). In *pal* and *tolA* mutants,

chemoreceptors lost their polar localization leading to loss of cell motility and chemotaxis perturbation (Santos *et al.*, 2014). EHEC Δ*tolA* mutants showed complete loss of motility, attenuated survival in a *Galleria mellonella* waxmoth larvae model and reduced adherence to HT-29 colonic epithelial cells. These results indicate that the Tol-pal is involved in EHEC pathogenesis (Morgan *et al.*, 2014). Cumulatively, the above studies implicate the Tol-Pal complex in structural integrity, cell division, chemotaxis, motility, and pathogenesis.

1.1.4.2 TonB-ExbBD Complex

The TonB-ExbBD complex energizes the transport of macromolecular substrates such as siderophores and vitamin B12 across the outer membrane (Skare and Postle, 1991; Shultis *et al.*, 2006) (Fig.1-2). TonB has an amino-terminal trans-membrane domain that serves as an anchor to the inner membrane and a large periplasmic region that spans the periplasm and binds to receptors in the outer membrane. In the inner membrane, TonB also interacts with an inner membrane complex consisting of ExbB and ExbD. ExbB and ExbD are homologs of TolQ and TolR respectively (Cascales *et al.*, 2001; Germon *et al.*, 2001). ExbB and ExbD are proposed to harvest the proton motive force to energize TonB dependent uptake of substrates. The energized mode of TonB scans the outer membrane until it interacts with a ligand loaded TonB-dependent receptor. The energy derived from the PMF is used to transfer the loaded ligand across the outer membrane into the periplasm (Sverzhinsky *et al.*, 2014). Once the ligand is in the periplasm, it is bound by other receptors that mediate its translocation through the inner membrane via an ABC transporter (Braun, 2003). FecA is one such outer membrane TonB-dependent

receptor that binds diferric dicitrate in *E. coli*. In addition to transport, FecA is involved in a signaling cascade that up-regulates the genes encoding citrate-dependent iron (III) transport upon ligand binding. This signal transduction involves the TonB-ExbBD transport system and is dependent upon protein-protein interactions involving sigma and anti-sigma factors (Braun *et al.*, 2003; Koebnik, 2005). Accordingly, precedent exists for the connection between the activity of trans-envelope protein complexes and associated signal transduction processes.

1.2 Envelope Stress Responses

The bacterial envelope directly encounters the environment and therefore is challenged constantly. Bacteria have evolved multiple mechanisms to respond to stresses in the envelope. In the model organism *E. coli*, the envelope stress responses are well studied.

1.2.1 The σ^E Stress Response

The σ^E stress response pathway monitors and maintains the integrity of the outer membrane (Ades, 2008)(Fig.1-3). σ^E was first identified as a regulator of the heat shock response sigma factor RpoH/σ^H and the periplasmic serine protease DegP (Erickson *et al.*, 1987). Since then, it has been extensively studied with regard to its molecular mechanism of action and physiological relevance. The σ factor is a transcriptional initiation factor, which is required for enabling binding of RNA polymerase to its target promoters. σ^E is an alternative sigma factor for responding to extracytoplasmic stresses. To inhibit its activity when not necessary, σ^E is sequestered by its anti-sigma factor RseA through direct binding, as revealed by co-

immunoprecipitation studies (De Las Peñas *et al.*, 1997; Missiakas *et al.*, 1997; Campbell *et al.*, 2003). This interaction directly sequesters σ^E to the inner membrane and blocks it from binding RNA polymerase and consequently transcription initiation. RseA is an integral inner membrane protein with periplasmic and trans-membrane domains. RseA is degraded by a DegS initiated degradation cascade upon encountering stresses that affect the outer membrane (Ades *et al.*, 1999; Alba *et al.*, 2002). Thus, the signal in the periplasm is transmitted to the cytoplasmic sigma factor, which consequently regulates gene transcription in the cytoplasm. Another negative regulator of σ^E activity is the periplasmic protein RseB that increases RseA affinity for σ^E by 2-3 fold through direct binding to the RseA C-terminus (Collinet *et al.*, 2000).

Regulation of the signaling process is achieved at different points. First, degradation of RseA by the DegS-initiated degradation cascade relieves sequestration of σ^E (Chaba *et al.*, 2007; Walsh *et al.*, 2003). Along with a transmembrane domain and a protease domain, protease DegS also has a PDZ (PSD-95, Discs-large, ZO-1) domain that recognizes and binds to the carboxyl terminal sequences of OMPs containing a conserved YXF (Tyr, any amino acid, phenylalanine) motif (Walsh *et al.*, 2003). The PDZ domain is an abundant protein module characteristic of 5 or 6 β-stranded and 2 or 3 α-helical structures that mediate protein interaction by recognizing the carboxyl-terminus of its taget proteins (Walsh *et al.*, 2003). The binding of the PDZ domain to the OMP sequence activates DegS cleavage of RseA, which alleviates inhibition of σ^E. Thus, DegS senses envelope stresses that lead to misfolded OMPs through its PDZ domain (Walsh *et al.*, 2003). In addition, inactivation of RseB also

induces σ^E activity (Chaba *et al.*, 2011)(Lima et al. 2013). LPS and various LPS intermediates antagonize RseA-RseB binding and subsequently relieve inhibition of DegS-dependent degradation of RseA (Lima et al. 2013). This suggests that RseB serves as a sensor for off-pathway LPS intermediates. Also, further investigation revealed that activation of σ^E in the presence of misfolded OMPs involves both activation of DegS, as previously discovered, and inactivation of RseB (Chaba *et al.*, 2011).

σ^E is activated upon heat shock, ethanol treatment, and in the presence of misfolded outer membrane proteins such as overexpression of OmpC and OmpF (Mecsas *et al.*, 1993). The σ^E regulon has been intensively studied and the genes fall into several functional categories including periplasmic chaperones and proteases such as DsbA, Skp, SurA and DegP; phospholipid and lipopolysaccharide biosynthesis; outer membrane synthesis; and transporters and genes of unknown function (Erickson *et al.*, 1987; Dartigalongue *et al.*, 2001; Rezuchova *et al.*, 2003; Rhodius *et al.*, 2006).

As a sigma factor, σ^E functions as a positive regulator of its downstream targets by directly initiating transcription. Additionally, σ^E employs sRNAs to repress gene expression. sRNAs are non-coding RNA species that mediate gene regulation instead of encoding protein products. Trans-acting sRNAs regulate gene expression at the post-transcriptional level by modulating target mRNA stability and/or accessibility to ribosomes through direct base pairing (Storz *et al.*, 2011). The sRNA chaperone Hfq protects sRNA species from degradation and facilitates sRNA and target mRNA interaction by interacting with sRNAs (Vogel and Luisi, 2011). Three σ^E-regulated sRNAs have been identified so far including MicA (Udekwu and Wagner, 2007),

RybB (Johansen *et al.*, 2006) and MicL (Guo et al., 2014). Both RybB and MicA are positively regulated by activation of σ^E (Thompson *et al.*, 2007). Previous studies of RybB and MicA in both *E. coli* and *Salmonella* highlight their roles as global repressors of several abundant porins such as OmpC, OmpF, OmpA, and OmpW (Johansen *et al.*, 2006; Gogol *et al.*, 2011). MicL (mRNA-interfering complementary RNA regulator of Lpp) is a newly discovered σ^E-dependent sRNA that represses synthesis of the major outer membrane lipoprotein Lpp, as identified in an RNAseq analysis in the presence of over-expressed MicL (Guo *et al.*, 2014). MicL decreases *lpp* mRNA levels by 20-fold. Reversely, σ^E activity responds to Lpp levels in the cell. In *E. coli*, an *lpp* mutant has decreased σ^E activity (Mecsas *et al.*, 1993), while mild over-expression of Lpp significantly induces σ^E activity and leads to growth arrest. σ^E activity is reduced when MicL is overexpressed. Lpp is the most abundant (~1million copies/cell) protein in *E. coli* (Cowles *et al.*, 2011); its translation engages 5% of all active ribosomes (Li *et al.*, 2014). Keeping its biogenesis under control is critical to maintain outer membrane homeostasis (Guo *et al.*, 2014). In summary, during envelope stress, σ^E-regulated sRNAs repress all the abundant proteins destined to the outer membrane.

1.2.2 Phage Shock Protein (Psp) Stress Response

The Phage Shock Protein stress response monitors inner membrane homeostasis (Brissette *et al.*, 1990; Darwin, 2005) (Fig.1-3). Its inducing cues include filamentous phage infection (Brissette *et al.*, 1990), ethanol, osmotic shock, heat shock, and aberrant insertion of secretin proteins in the inner membrane (Flores-Kim and Darwin, 2015). The Psp stress response is well studied in *E. coli* and the intestinal

pathogen *Yersinia enterocolitica*. PspA, PspB, PspC, PspD and PspF are conserved in both organisms, therefore are considered as the core components of the Psp stress response. PspF is a cytoplasmic transcriptional regulator of σ^{54}-dependent promoters (Jovanovic *et al.*, 1996). Under normal conditions, inner membrane protein PspA inhibits PspF activity through direct interaction (Dworkin *et al.*, 2000). In response to stresses, a complex of the inner membrane proteins PspB and PspC sequesters PspA from interacting with PspF and relieves inhibition of PspF activity in the cytoplasm (Yamaguchi *et al.*, 2013). Signal transmission involves a partner-switching interaction in which the PspC carboxyl-terminus interacts with PspA during stress, instead of PspB (Flores-Kim and Darwin, 2015). The manner in which the Psp response mediates adaptation to inner membrane perturbation is not fully understood. It likely involves Psp proteins that act directly in controlling PMF at the inner membrane (Joly et al., 2010). In *E. coli*, the Psp response contributes to survival in stationary phase, maintenance of the proton motive force across the inner membrane, and transport of proteins by the SecYEG translocon (Darwin, 2005). It is critical for bacterial virulence in *Y. enterocolitica, Salmonella enterica* and *Typhimurium* (Darwin, 2013; Karlinsey et al., 2010; Maxson and Darwin, 2004).

1.2.3 Two-component Signal Transduction Systems (TCS)

Two-component signal transduction systems constitute a predominant mechanism that bacteria employ to sense and respond to the environment. Two-component systems are found in organisms from all domains including Eubacteria, Archaea, and Eukarya except animals, which makes makes them strong targets for drug development (Gotoh *et al.*, 2010). A classical two-component system consists of a

histidine sensor kinase (HK) and a response regulator (RR). HKs generally have a variable periplasmic sensor domain, two transmembrane helices and a carboxyl-terminus cytoplasmic portion, which contains a histidine-containing phosphotransfer (DHp) domain and a catalytic domain. The histidine sensor kinase senses environmental stimuli, auto-phosphorylates itself at a conserved histidine residue located in the DHp domain, followed by phosphoryl transfer to a conserved Asp residue on the response regulator. Phosphorylation of the response regulator triggers conformational changes that modulate its effector domain affinity for its targets (Casino *et al.*, 2009; Gao and Stock, 2009; Casino *et al.*, 2010; Stock *et al.*, 2000). The variability of sensor domains is thought to enable sensor kinases to sense a wide variety of stimuli. Sensor kinases normally function as homodimers. Perception of stimuli in the periplasm is believed to be transmitted through the inner membrane by alteration of transmembrane domain interactions between protomers (Gao and Stock, 2009; Fung *et al.*, 2016). Complexity in two-component system signaling may arise from cross talk between different two-component systems and auxiliary regulators (Buelow and Raivio, 2010). More complicated phosphorelays such as the Rcs phosphorelay, that involves extra components, function to introduce more regulatory checkpoints (Gao and Stock, 2009; Majdalani and Gottesman, 2005).

1.2.4 The Rcs Phosphorelay

The Rcs phosphorelay senses diverse envelope-stresses that lead to outer membrane and peptidoglycan damage (Laubacher and Ades, 2008; Majdalani and Gottesman, 2005)(Fig.1-3). Its activation impacts processes such as flagellar

synthesis, motility, biofilm maturation, antibiotic resistance, cell wall synthesis, and pathogenesis (Wang *et al.*, 2009; Erickson and Detweiler, 2006; Laubacher and Ades, 2008).

The Rcs phosphorelay is more complicated than the classical two-component system since it involves a separate phosphotransfer protein, RcsD, and an auxiliary response regulator, RcsA, in addition to sensor kinase RcsC and response regulator RcsB (Majdalani and Gottesman, 2005). In consequence, its phosphor transfer reactions begin with the autophosphorylation of the Dhp domain of hybrid sensor kinase RcsC upon signal reception. The phosphate is then transferred to an RcsC receiver domain with a conserved aspartate residue before being transferred to the phosphotransfer protein RcsD, that phosphorylates the primary response regulator RcsB (Takeda *et al.*, 2001). Phosphorylated RcsB is active and functions in two modes. An RcsB homodimer binds directly upstream of the -35 region of its target genes, such as *rprA* and *osmC* (Majdalani et al., 2002; Davalos-Garcia et al., 2001; Sturny et al., 2003). Also, phosphorylated RcsB binds to its auxiliary response regulator RcsA. An RcsAB heterodimer binds far upstream of target gene start codons (-100bp) differently from the RcsB homodimer and is responsible for transcriptional regulation of *cps*-capsule synthesis genes and *rcsA* itself (Fig. 1-3).

The Rcs Phosphorelay is subject to regulation by the auxiliary regulators IgaA and RcsF. IgaA is an integral inner membrane protein that inhibits the activity of the Rcs phosphorelay (Cano *et al.*, 2002). IgaA is essential unless the Rcs phosphorelay is not active, however its function remains unclear (Cano *et al.*, 2002). Depletion of cellular IgaA levels induces the Rcs phosphorelay (Cho *et al.*, 2014). RcsF is an outer

membrane lipoprotein that is required for induction of the Rcs phosphorelay upon antimicrobial peptide exposure and overexpression of RcsF itself (Farris *et al.*, 2010; Majdalani *et al.*, 2005; Castanié-Cornet *et al.*, 2006b). RcsF activates the Rcs phosphorelay by alleviating inhibition of IgaA through direct binding, as IgaA is co-purified with RcsF (Cho *et al.*, 2014).

1.2.5 The Cpx Two-component System

The Conjugative pilus expression locus (Cpx pathway) was first identified through mutations that resulted in a reduction of expression of the F-plasmid conjugative pilus in *E. coli* (McEwen and Silverman, 1980). Later studies revealed that the Cpx pathway is made up of a two-component signaling system consisting of inner membrane sensor kinase CpxA and cytoplasmic response regulator CpxR (Nixon *et al.*, 1986; Weber and Silverman, 1988; Dong *et al.*, 1993)(Fig.1-3, 1-4). CpxA acts as both a phosphatase and a kinase of response regulator CpxR. Under normal conditions, CpxA acts as a phosphatase and dephosphorylates CpxR to keep CpxR-P at low levels when not necessary. In the presence of inducing cues, CpxA autophosphorylates itself at the conserved site of autophosphorylation, H248, and subsequently phosphorylates CpxR. Phosphorylated CpxR is active and regulates transcription of its downstream targets (Raivio and Silhavy, 1997). CpxA is required for sensing envelope stresses, as a CpxA mutant with a deletion in the soluble periplasmic domain is blind to inducing signals (Raivio and Silhavy, 1997). However, CpxA is not required for sensing cell growth, which requires only CpxR (Raivio and Silhavy, 1997). Cpx is the third highest expressed two-component system in *E. coli* with a synthesis rate of about 2.5K molecules/generation (Li *et al.*, 2014).

1.2.5.1 Inducing Cues

In *E. coli*, the Cpx pathway senses signals in the cytoplasm, inner membrane, periplasm and also the outer membrane (Fig.1-4). These include cell growth (De Wulf *et al.*, 1999), cellular metabolites (Lima *et al.*, 2016), alkaline pH, inner membrane lipid composition changes (Mileykovskaya and Dowhan, 1997; Danese *et al.*, 1998), NlpE over-expression, surface adhesion through NlpE, and misfolded pilus subunits PapE and PapG (Lee *et al.*, 2004; Hunke *et al.*, 2012; Danese and Silhavy, 1998).

In addition, cell wall perturbations also increase Cpx pathway activity in *E. coli*. Simultaneous absence of four penicillin-binding proteins, PBP4, PBP5, PBP7 and AmpH, activates the Cpx pathway (Evans *et al.*, 2013). Cell wall anomalisms caused by inactivation of elongasome Pbp2 or divisome Pbp3 or upon depolymerization of MreB also activate the Cpx pathway through sensor kinase CpxA (Delhaye *et al.*, 2015). Deletion of the genes *ldtD* and *ygaU*, encoding CpxR-regulated cell wall modification proteins, results in mild activation of the Cpx pathway (Bernal-Cabas *et al.*, 2014). In *V. cholerae*, ion chelation induces the Cpx pathway (Acosta *et al.*, 2015). Cumulatively, these studies demonstrate that a wide variety of signals converge at the Cpx response, and the mechanisms by which these are sensed are poorly understood.

1.2.5.2 Auxiliary Regulators of the Cpx Pathway

Signaling pathways are often regulated by other components called auxiliary regulators in addition to their core members (Buelow and Raivio, 2010). The Cpx

two-component system is subjected to regulation by at least two auxiliary regulators, CpxP and NlpE (Fig.1-4).

Overexpression of CpxP strongly decreases activity of the Cpx pathway. Also, when *cpxP* was mutated, there was an elevation of Cpx pathway activity (Raivio *et al.*, 1999), suggesting that CpxP functions to feedback inhibit the Cpx response. CpxP inhibits the activity of the Cpx pathway under normal conditions through direct interaction with the periplasmic domain of CpxA (Tschauner *et al.*, 2014). CpxP is one of the most strongly up-regulated genes when CpxR is active and also contributes to combating envelope stresses (Danese and Silhavy, 1998). CpxP shares homology with the novel periplasmic chaperone Spy, and has been demonstrated to possess weak chaperone activity (Quan *et al.*, 2011; Zhou *et al.*, 2011). Genetic evidence suggests that CpxP is necessary for the degradation of misfolded P pilus subunits, which are strong activators of the Cpx response. Together, these data posit a model in which CpxP functions as a chaperone to target misfolded proteins for degradation by the protease DegP. It is proposed that titration of CpxP in the presence of misfolded proteins leads to induction of the Cpx response, and subsequent elevated CpxP expression. Upon relief of the misfolded protein stress, CpxP would accumulate and shut off the pathway (Isaac *et al.*, 2005).

Another important auxiliary regulator of the Cpx pathway is the outer membrane lipoprotein NlpE (new lipoprotein E). NlpE was first identified in a screen for factors contributing to the suppression of toxicity of a periplasmic LamB-LacZ-PhoA fusion protein (Snyder *et al.*, 1995). It was shown that over-expression of NlpE increased transcription of the gene encoding the periplasmic protease DegP by activating the

Cpx pathway. Upon adhesion to an abiotic hydrophobic surface (coated glass beads), the Cpx pathway is activated about two-fold and NlpE is essential to this process since its mutation abolishes activation of the Cpx pathway and also decreases surface adhesion (Otto and Silhavy, 2002). Further investigation revealed that NlpE is dispensable for Cpx activation by most inducing cues of the Cpx pathway such as alkaline pH, misfolded PapE or entry into stationary phase growth (DiGiuseppe and Silhavy, 2003; Danese *et al.*, 1995; Danese and Silhavy, 1997). Thus, NlpE appears to function as an auxiliary regulator of the Cpx pathway that senses surface adhesion specifically. However, the molecular mechanism remains unclear.

Structural and cellular localization studies indicate that NlpE contains two domains and must be localized to a membrane to exert Cpx signaling effects. Crystallization studies revealed that soluble NlpE consists of two well-formed β-barrel domains – an amino-terminal domain resembling lipocalin and a carboxyl-terminal oligonucleotide/oligosaccharide-binding (OB) fold (Hirano *et al.*, 2007). Mis-locating NlpE to the inner membrane induced the Cpx pathway to a greater level at lower over-expression levels as compared to the wild-type form. Mislocating NlpE to the periplasm failed to induce the Cpx pathway, suggesting that the lipid modification is critical to the signaling of the Cpx pathway upon NlpE over-expression (Hirano *et al.*, 2007; Miyadai *et al.*, 2004).

NlpE was also identified as CutF, and was thought to mediate copper transport since its mutation conferred a copper sensitive phenotype in *E. coli*. Additional studies, however, indicated that the copper sensitive phenotype observed was a result of mutation of an additional gene, *cutC* (Gupta *et al.*, 1995). Later, the copper sensitive

phenotype of the *cutC nlpE* double mutant was found to be a result of misregulation of Lpp in the *cutC* mutant (Guo *et al.*, 2014). An sRNA MicL, which is transcribed from a promoter located in the middle of the *cutC* gene, was identified in a tiled microarray expression profiling study after σ^E was overexpressed. MicL is dedicated to inhibition of expression of the most abundant outer membrane lipoprotein, Lpp. Thus, inactivation of *cutC* diminishes MicL levels in the cell and consequently leads to misregulation of Lpp expression, likely destabilizing the envelope and leading to copper sensitivity. Indeed, in an *E. coli Δlpp* mutant, the same copper sensitive phenotype observed in the *cutC, nlpE* double mutant was observed (Guo *et al.*, 2014). These results might suggest that NlpE is not directly involved in copper homeostasis in *E. coli*.

1.2.5.3 Physiological Significance of the Cpx Regulon

In *E. coli*, early studies highlighted the role of the Cpx pathway in suppressing the toxicity of a LamB-LacZ-PhoA fusion protein in the envelope through up-regulated production of the periplasmic protease DegP (Cosma *et al.*, 1995). In addition, Cpx activation was shown to lead to increased levels of periplasmic chaperones including PpiA, Dsb, Skp and SurA (Pogliano *et al.*, 1997). These observations suggested that the role of the Cpx pathway is to sense and relieve stresses caused by misfolded envelope proteins (Pogliano *et al.*, 1997).

The Cpx regulon was further investigated by microarray expression profiling study in *E. coli* (Raivio *et al.*, 2013). In addition to previously described protein folding and degrading factors, genes that were Cpx-regulated in both *E. coli* K-12 and enteropathogenic *E. coli* (EPEC) strains were enriched for those encoding proteins

and functions that are inner membrane-associated. This indicates that the Cpx two-component system plays an important role in inner membrane homeostasis. Several small 'Y genes' predicted to be located in the periplasm and inner membrane and with unknown function were identified. Among these Y genes, some contribute to inner membrane integrity (Price and Raivio, 2009; Raivio *et al.*, 2013). Genes encoding protein complexes involved in energization were shown to be down regulated, such as succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase and the EfeUOB ferrous iron transporter (Raivio *et al.*, 2013).

The Cpx pathway also regulates target gene expression at the post-transcriptional level through noncoding small RNAs (sRNAs). When NlpE was over-expressed, about 35 intergenic regions-including six known sRNAs (CyaR, MicF, OmrA, OmrB, RprA and RybB)-were shown to be either up or down regulated by at least 2-fold (Raivio *et al.*, 2013). Further research revealed that CpxR regulates expression of *cyaR* and *rprA* through direct binding to their promoter regions. CyaR is a negative regulator of CpxR regulated inner membrane protein YqaE. In response to Cpx inducing conditions, CpxR increases *yqaE* transcription directly at the transcriptional level and also indirectly through down-regulation of its negative regulator CyaR (Vogt *et al.*, 2014). Recently, an unusual Cpx pathway employed sRNA CpxQ was identified (Grabowicz *et al.*, 2016; Chao and Vogel, 2016). CpxQ is a 3'- untranslated region (UTR) processing product of the *cpxP* mRNA that is produced through RNase E action. CpxQ does not appear to be involved in combating periplasmic misfolded pilus subunit stress like CpxP. Rather, its main target is the periplasmic chaperone Skp that is required for OMP biogenesis

(Grabowicz *et al.*, 2016). It is thought that in the presence of certain envelope stresses, Skp causes toxicity by mistargeting OMPs to the inner membrane. By employing CpxQ to down regulate *skp* at the translational level, the Cpx pathway combats this stress at the inner membrane. These results all suggest an additional sRNA-dependent arm of the Cpx response that awaits further investigation.

The Cpx envelope stress response also directly regulates genes involved in cell wall remodeling including LdtD, a putative L, D-transpeptidase, YgaU, a putative peptidoglycan binding protein of unknown function, and Slt, a lytic transglcosylase (Bernal-Cabas *et al.*, 2014). Activation of the Cpx pathway resulted in increases in the abundance of diaminopimelic acid (DAP)-DAP cross-links in an LdtD- and YgaU-dependent manner (Bernal-Cabas *et al.*, 2014). These data indicate that the Cpx response stabilizes the bacterial envelope by modifying the existing cell wall in the presence of envelope stresses.

In pathogenic strains of *E. coli,* including EPEC and enterohemmorhagic *E. coli* (EHEC), the Cpx two-component system is involved in pathogenesis. In EPEC, the Cpx response is both required for efficient assembly of the type IV bundle forming pilus (BFP), and when activated, the inhibition of both BFP production as well as the type III secretion system (Vogt *et al.*, 2010). Clues about how this might occur come from studies in EHEC, where CpxR has been shown to repress transcription of *grlA* and *ler,* two main positive virulence regulators of the Locus of enterocyte effacement (LEE). In agreement with this, both EHEC and EPEC Cpx mutants altered killing in an insect larva model of virulence (Miguel *et al.*, 2015; Leuko and Raivio, 2012).

The Cpx response contributes to resistance to the antimicrobials hydroxyurea (HU) and aminoglycoside antibiotics (AGAs), most likely through down-regulation of succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase and the EfeUOB ferrous iron transporter (Mahoney and Silhavy, 2013). Also, the Cpx two-component system was shown to up-regulate tripartite multidrug efflux systems through activation of the multiple antibiotic resistance (Mar) regulator and this is thought to contribute to resistance to cationic antimicrobial peptides and antibiotics (Weatherspoon-Griffin *et al.*, 2014).

The role of the Cpx two-component system in other organisms has also been studied. *Haemophilus ducreyi* is a gram-negative bacterium that causes the sexually transmitted disease chancroid (Gangaiah *et al.*, 2013; Labandeira-Rey *et al.*, 2010). In *H. ducreyi*, the Cpx two-component system is critical for the regulation of virulence determinants. An *H. ducreyi cpxA* mutant in which the Cpx response is activated, was avirulent. RNA-Seq studies of both *cpxA* and *cpxR* mutants, showed that the Cpx pathway repressed 70% of its targets, including seven well studied determinants of serum resistance, phagosome escape, heme and iron uptake, adherence and microcolony formation (Gangaiah *et al.*, 2013). Functional classification of genes or operons regulated by the Cpx pathway revealed that they fell into divergent categories including amino acid biosynthesis, cell surface structures and associated proteins, cell wall biosynthesis and remodeling, cofactor biosynthesis, generation of precursor metabolites and energy, membrane transport and uptake and hypothetical proteins (Raivio *et al.*, 2013; Gangaiah *et al.*, 2013). In addition, only one protein peptidyl-prolyl cis-trans isomerase, FkpA, was identified

in the protein folding and degradation category, suggesting that the facilitation of protein folding and degradation is not the main function of the Cpx pathway as proposed in *E. coli* (Raivio *et al.*, 2013; Raivio, 2014).

In the *Vibrio cholerae* El Tor C6706 strain, the Cpx regulon was investigated upon CpxR overexpression by microarray expression profiling study (Acosta *et al.*, 2015). The transcriptional profile featured iron-related transport genes, membrane-localized proteins, outer membrane pore TolC and RND efflux systems. It was further demonstrated that iron chelation and deletion of RND efflux leads to activation of Cpx, which was suppressed by iron. These observations indicate that the *V. cholerae* Cpx pathway mediates adaptation to iron depletion and toxic compounds (Acosta *et al.*, 2015).

Finally, in *Yersinia pseudotuberculosis,* the Cpx two-component system directly regulates the transcription of the global transcriptional regulator RovA (Liu *et al.*, 2011). RovA regulates multiple pathways such as secretion systems and also virulence genes. Altogether, the literature indicates that the Cpx response serves important roles in envelope protein quality control and inner membrane function that affect multiple cellular attributes including virulence and antibiotic resistance.

1.3 Bacterial Surface Adhesion and Biofilm Formation

In nature, bacteria exist in two forms, planktonic and adherent cells, more predominantly as cells adhered to either biotic or abiotic surfaces. Bacteria adhered to surfaces tend to develop more complicated, structured communities called biofilms. Adherent bacteria or biofilm–associated microorganisms exhibit different behaviors from planktonic cells including altered growth characteristics and

resistance to antimicrobial treatments. Biofilm development within indwelling medical devices and in industry causes huge problems.

1.3.1 Bacterial surface adhesion

Since the twentieth century, it has been known that bacteria have a tendency to associate with surfaces, since surface adhesion is nutritionally favorable (Dunne, 2002). In marine environments surface-associated microbes outnumber planktonic organisms (Dunne, 2002). Bacteria that interact with surfaces adapt different behaviors from planktonic cells in terms of gene expression profiles, metabolism, respiration, and pathogenesis (Costerton *et al.*, 1987; Zhang and Normark, 1996; Costerton *et al.*, 1999; Moorthy *et al.*, 2016; Geng *et al.*, 2014; Otto *et al.*, 2001). However, in the laboratory, bacteria are often studied in well-controlled conditions using planktonic cultures derived from single colonies of pure isolates.

Cells that approach close approximation with surfaces first establish initial attachment. The initial attachment is affected by multiple factors including cell surface adhesive structures, the characteristics of the surface, and the hydrodynamics of the interface. To resist repulsive forces, cellular organelles such as pili and flagella could act as propellers or grappling hooks to contribute greatly to attachment during this stage (Costerton *et al.*, 1987; Beloin *et al.*, 2008). The initial attachment is reversible and cells could easily flow away. Thus, once the initial interaction is established, bacteria usually employ a combination of multiple adhesins to promote irreversible attachment. For adhesion to abiotic surfaces, non-specific adhesins are involved, while specific adhesins are needed for adhesion to biotic surfaces (Pizarro-Cerdá and Cossart, 2006; Berne *et al.*, 2015). Bacterial

contact with abiotic surfaces induces adaptive responses that involve multiple pathways and interplay amongst regulatory mechanisms.

1.3.2 Biofilms

Bacteria adhered to surfaces often form a biofilm, which is a structured bacterial community embedded in a self-produced matrix of extracellular polymeric substances featuring protein, nucleic acids, nutrients, and cell wall material (Dunne, 2002). A biofilm protects its inhabitants from biocides, antibiotics, disinfectants and dynamic environments (Anwar *et al.*, 1990; Costerton *et al.*, 1999; Dunne, 2002). The protection of the biofilm is attributed to low antibiotic penetration through its extracellular matrix, an altered microenvironment that slows down growth, adaptive resistance of its inhabitant, and persisters (Hathroubi *et al.*, 2016; Stewart, 2002). Microbial biofilms can be harmful for human activities, as causes of contamination in the food industry, hospital-related infections, and chronic diseases (Costerton *et al.*, 1987; von Rosenvinge *et al.*, 2013; Zottola and Sasahara, 1994). The properties and prevalence of biofilms challenge our studies of antibiotic treatments using planktonic cells and necessitate more research on mechanisms of microbial adhesion, biofilm formation and properties.

1.3.3 Molecular Mechanisms of Bacterial Adhesins

Bacteria usually employ multiple adhesins in different combinations at different stages during adhesion (Berne *et al.*, 2015; Pizarro-Cerdá and Cossart, 2006). An adhesin is any molecules or protein complex involved in promoting cell-surface adhesion (Berne *et al.*, 2015; Pizarro-Cerdá and Cossart, 2006). The molecular

nature of adhesins varies dramatically and includes protein complexes, extracellular organelles, and single proteins (Maier and Wong, 2015).

1.3.3.1 Surface Appendages

In addition to cell mobility, flagella were demonstrated to mediate EPEC adhesion to epithelial cells and *E. coli* K-12 adhesion to hydrophobic surfaces (Girón *et al.*, 2002; Haiko and Westerlund-Wikström, 2013; Friedlander *et al.*, 2015). Flagella mediate bacterial adhesion not only through motility that drives bacteria towards a surface but also through direct binding to cells (Friedlander *et al.*, 2015; Haiko and Westerlund-Wikström, 2013). Pili or fimbriae are proteinaceous, hair-like cell appendages that extrude from the cell surface. There are several examples of pili involved in adhesion to biotic or abiotic surfaces such as P pili, type IV pili and curli (Cordeiro *et al.*, 2016; Maier and Wong, 2015; Zhang and Normark, 1996; Sauer *et al.*, 2000). One of the first characterized examples is the pyelonephritis-associated (P or pap) pilus that mediates colonization of the urinary tract by uropathogenic *E. coli* (UPEC) and subsequently infection of the kidney (Sauer *et al.*, 2000). The P pili are encoded by the *pap* gene cluster and are representative members of a class of pili that are assembled through a so-called "chaperone-usher" pathway. The periplasmic chaperone PapD binds to pilus subunits such as PapE and PapG as they emerge into the periplasm and guides them to the outer membrane usher PapC that translocates the subunits to the cell surface where they are assembled. The binding specificity to host tissues is determined by the pilus tip-adhesin PapG.

Different from P pili, the type IV pili are formed at the cytoplasmic membrane and the whole organelle is extruded from that location, through the periplasm and

across the outer membrane (Wolfgang *et al.*, 2000). Type IV pili subunits accumulate in the inner membrane and are assembled by a multi-protein complex at this location into a fiber of repeating subunits that is pushed through a secretin in the outer membrane. In EPEC, bundle-forming pili (BFP) mediate initial attachment to epithelial cells, a precursor for the later intimate adhesion that leads to the characteristic attaching and effacing lesions (Hicks *et al.*, 1998).

Curli biosynthesis and assembly requires seven genes encoded by two operons, *csgDEFG* and *csgBAC*. CsgD is the major transcriptional regulator of curli biogenesis and is required for transcription of curli subunits CsgA and CsgB. CsgG is an outer membrane lipoprotein that forms a pore-like structure that mediates secretion of the major subunit CsgA and the minor subunit CsgB across the outer membrane where nucleation of CsgA into an amyloid fiber occurs (Barnhart and Chapman, 2006).

1.3.3.2 Autotransporters

Autotransporters are adhesive proteins that facilitate their own transport to the outer membrane with a limited number of accessory secretion factors (Dautin and Bernstein, 2007). The most extensively studied example is Antigen 43 (Ag43) in *E. coli*. Ag43 consists of a passenger domain, Ag43α, and a translocation domain, Ag43β. It is encoded by the *flu* gene and expressed as a precursor protein containing a signal sequence that directs translocation across the inner membrane into the periplasm. It is processed into Ag43α and Ag43β, probably through autocatalytic cleavage. The translocation domain Ag43β is an integral membrane protein that still interacts with Ag43α at the outer membrane and presents Ag43α at the cell surface

for its function. Ag43β is a 14-stranded β-barrel domain (Konieczny MPJ *et al.*, 2001; Maurer *et al.*, 1997), based on its similarity to the adhesin involved in diffuse adherence (AIDA) autotransporter. Ag43β is heat-modifiable in a similar fashion to OmpA (see below), which suggests that it forms an extremely stable β-structure. Ag43α is an L-shaped β–helix domain protein and is surface exposed. In addition, proper transport and conformation of Ag43 requires O-glycosylation of its Ag43α subunit that is catalyzed by the specific heptosyltransferase Aah (Reidl *et al.*, 2009; Charbonneau and Mourez, 2008; Benz and Schmidt, 2001). Ag43 recognizes itself and facilitates self-association and cell aggregation as well as adherence to abiotic surfaces (Klemm *et al.*, 2004; Diderichsen, 1980) and pathogenesis in uropathogenic *E. coli* (Ulett *et al.*, 2007). Expression of Ag43 was significantly higher in biofilms compared to planktonic cells, indicating its contribution to biofilm formation (Danese *et al.*, 2000). It has been shown that Ag43 mediated cell-cell interactions contribute to *E. coli* biofilm formation in minimal media but not in rich media (Schembri and Klemm, 2001; Danese *et al.*, 2000). Cumulatively, studies of Ag43 implicate it as an important factor in the formation of adherent biofilm communities.

1.3.3.3 OmpA

OmpA is the major component of the outer membrane of *E. coli* with 100,000 copies per cell (Koebnik *et al.*, 2000). It plays diverse roles in maintaining outer membrane integrity as well as in adhesion and biofilm formation (Gaddy *et al.*, 2009). It is also a target of the mammalian immune response, colicins, and bacteriophage (Achouak *et al.*, 2001). There are two models for OmpA structure. In one, OmpA is proposed to be a large porin with 14-transmembrane domains, while a second model proposes

that it exists as a smaller eight-stranded outer membrane porin (Sugawara and Nikaido, 1994; Nikaido, 2003) with a globular C-terminus that resides in the periplasm and interacts with peptidoglycan (Ishida *et al.*, 2014; Pautsch and Schulz, 1998; Arora *et al.*, 2001). Both conformations of OmpA can be purified from the outer membrane with about 2-3% of OmpA forming the larger pore structure (Ishida *et al.*, 2014). The structural transition from the small pore to the larger pore was observed when the temperature reached 24-39°C (Zakharian and Reusch, 2003).

The role of OmpA as an adhesin/invasin has been well established in several models. In *E. coli* K1, OmpA is required for invasion of brain micro-vascular endothelial cells, subsequently causing meningitis (Prasadarao *et al.*, 1996). In EHEC, a mutation leading to elevated expression of OmpA resulted in a hyperadherent phenotype to Caco-2 and HeLa cells (Torres and Kaper, 2003). Also, OmpA mediates *E. coli* adhesion to abiotic surfaces through direct binding, as revealed by atomic force microscopy (Lower *et al.*, 2005). OmpA homologs also function as adhesins/invasins in other organisms such as *Rickettsia conorii* (Hillman *et al.*, 2013), *Acinetobacter baumannii* (Gaddy *et al.*, 2009), *Acinetobacter nosocomialis* (Gaddy *et al.*, 2009), *Coxiella burnetii* (Martinez *et al.*, 2014), *Cronobacter sakazakii* (Kim *et al.*, 2010) and *Yersinia pestis* (Bartra *et al.*, 2012).

1.3.4 Envelope Stress Responses and Biofilm Formation

Envelope stress responses sense envelope stresses and regulate envelope structures and activities. They play important regulatory roles in biofilm formation. Microarray transcriptomic study of mature biofilm formation in *E. coli* K-12 revealed elevated

expression of components of the Psp, σ^E, and Cpx envelope stress responses (Beloin *et al.*, 2004). Further investigation revealed that mutation of *cpxR* or *cpxP* resulted in a decreased ability to initiate surface adhesion, indicated by an increased prevalence of single cells, smaller surface-associated colonies, and altered biofilm morphology. These results indicate that the Cpx pathway may impact initial adhesion to surfaces during biofilm formation (Beloin *et al.*, 2004). The Cpx pathway is known to impact biofilm formation by regulating curli biogenesis. The Cpx pathway negatively regulates transcription of *csgA*, which encodes the major subunit of curli (Dorel *et al.*, 1999). Moreover, OmpA inhibits cellulose production by altering Cpx-dependent repression of CsgD-the activator of AdrA that activates cellulose production. The resulting reduction in cellulose increases biofilm formation on hydrophobic surfaces (Ma and Wood, 2009). In addition, other members of the Cpx regulon were shown to impact biofilm formation. Three genes *yccA, ybeE, and ycfS*, when mutated, resulted in decreased biofilm formation, while *ybaJ* overexpression resulted in increased biofilm formation (Price and Raivio, 2009). YccA is a modulator of the ATP-dependent zinc metalloprotease FtsH. Genes *ybaJ ybeE and ycfS* encode proteins of unknown function. Further study is necessary to reveal the molecular mechanisms by which these genes influence biofilm formation.

In *E. coli*, the Rcs response is involved in biofilm formation due to its regulation of the production of extracellular polysaccharide (EPS), which is critical for biofilm maturation (Ferrières and Clarke, 2003).

Adherent-invasive *E. coli* (AIEC) is able to adhere to and invade intestinal epithelial cells of patients with Crohn's disease. Inhibition of the σ^E response in AIEC led to

decreased ability to adhere to and invade intestinal epithelial cells and therefore affected colonization and biofilm formation. Comparative *in silico* analysis led to the identification of the *waaWVL* operon as being essential for AIEC to colonize the intestinal mucosa and promote biofilm formation as overexpression of the *waaWVL* operon restored biofilm formation in the σ^E impaired AIEC mutant. WaaWVL depletion was shown to alter LPS structure and composition (Chassaing and Darfeuille-Michaud, 2013; Chassaing *et al.*, 2015). Together these studies indicate the involvement of envelope stress responses in regulating genes, including those encoding surface-associated structures, that impact adherence and biofilm formation.

1.4 Objectives

The Cpx two-component system senses a variety of envelope stresses in *E. coli*. The auxiliary regulator NlpE signals Cpx activation upon surface adhesion and when overexpressed. The molecular nature and mechanism behind any of the Cpx inducing cues identified thus far remain elusive. The main goal of this thesis was to elucidate the signaling process utilized by NlpE to induce the Cpx two-component system. The specific objectives were:

1) To investigate how NlpE signals the Cpx pathway when overexpressed and upon surface adhesion by mutagenesis study of NlpE
2) To determine if NlpE interacts with any other proteins in *E. coli* and, if so, to assess their involvement in Cpx signaling

1.5 Tables and Figures

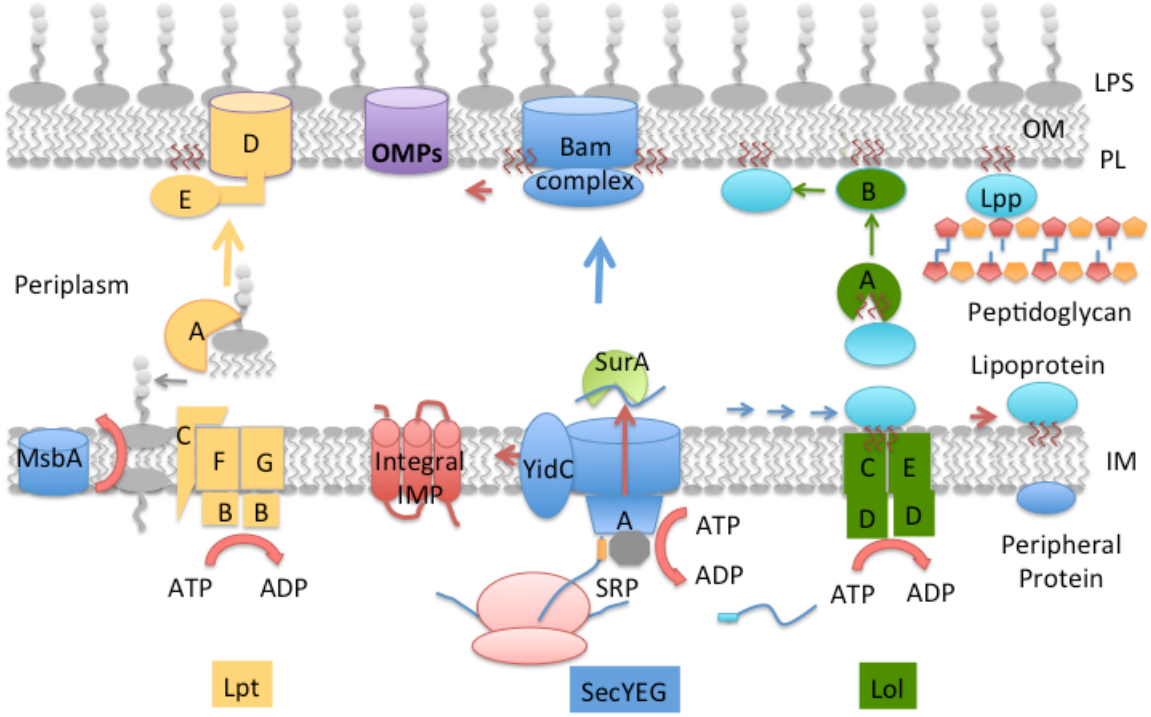

Figure 1-1 General structure of gram-negative bacterial envelope

The Gram-negative bacterial envelope consists of the inner membrane (phospholipid bilayer), the outer membrane (asymmetric bilayer with the inner leaflet of phospholipids and the outer leaflet of lipopolysaccharide) and the periplasm in between. The core-lipid A synthesized in the cytoplasm side of the inner membrane is flipped across the inner membrane to the periplasmic side where mature LPS forms through an ATP-binding cassette (ABC) MsbA. The transport and insertion of LPS to the OM is facilitated by lipopolysaccharide transport (Lpt) machinery. The peptidoglycan resides in the periplasmic space. There are integral inner membrane protein, peripheral protein and lipoprotein. The integral inner membrane protein is transported across the inner membrane by SRP-dependent secretion through SecYEG. Unfolded outer membrane proteins are recognized by molecular chaperones, such as SurA and Skp, which is believed to assist OMPs transport across the periplasm and deliver them to the β-barrel assembly machinery-Bam Complex. Lipoprotein precursors are synthesized in the cytoplasm and transported across the inner membrane where lipid modification occurs. Lipoprotein destined to the outer membrane is assembled by the Lol system. Lpp is a well-studied outer membrane lipoprotein that connects outer membrane and cell through interaction with peptidoglycan.

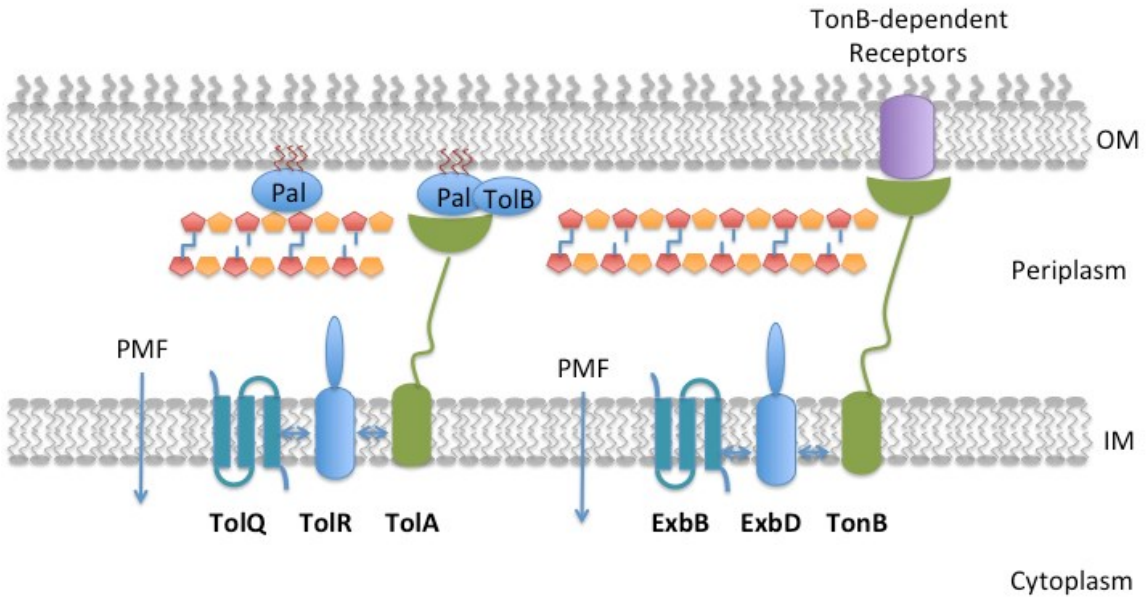

Figure 1-2 Current model of Tol-Pal complex and TonB-ExbBD complex in *E.coli*

The Tol-Pal complex consists of inner membrane complex of TolA, TolQ and TolR and outer membrane complex of Pal-TolB. Interaction of TolA and Pal requires PMF collected through TolQR. The TonB-ExbBD complex consists of the inner membrane complex of TonB ExbB and ExbD. The TonB is energized by PMF and scans the outer membrane for ligand-loaded TonB-dependent receptors.

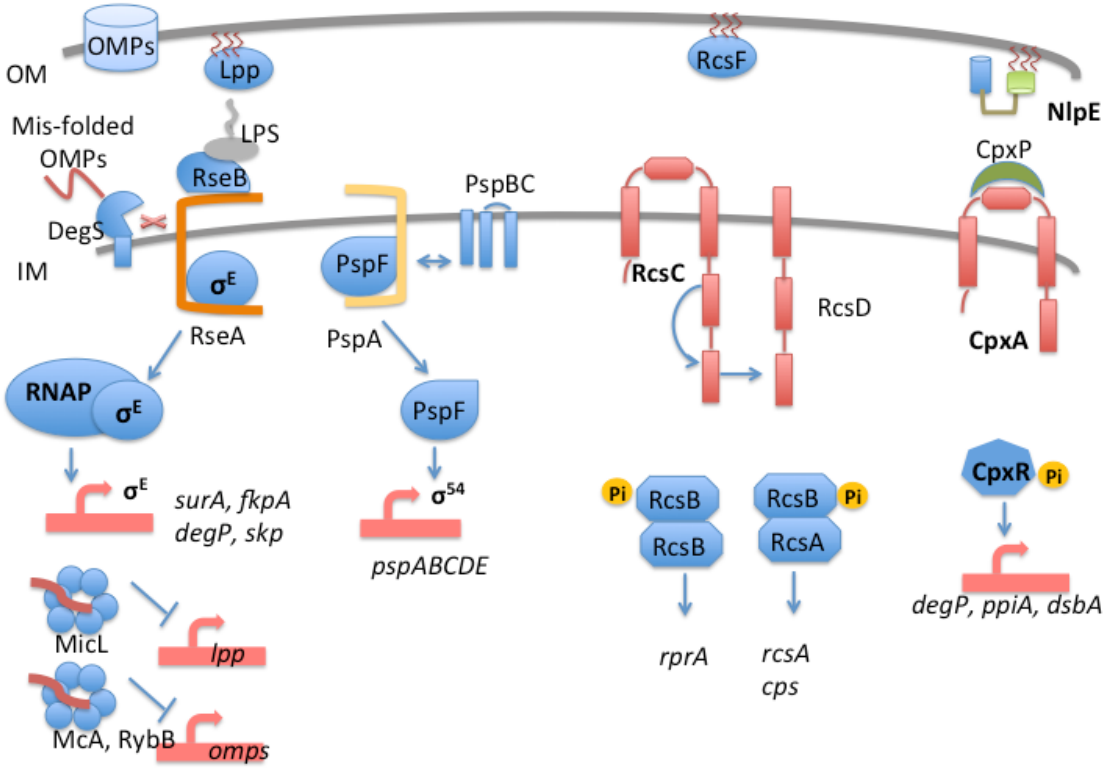

Figure 1-3 Envelope stress responses in *E.coli*

The σ^E is sequestered by the anti-sigma factor RseA to the inner membrane. It senses mis-folded OMPs and off-pathway LPS. The Psp stress response is maintained off by inner membrane protein PspA, which is sequestered by complex PspBC in presence of stresses. The Rcs phosphorelay involves a separate phosphotransfer protein, RcsD, and an auxiliary response regulator, RcsA, in addition to sensor kinase RcsC and response regulator RcsB. The Cpx two-component system consists of the sensor kinase CpxA and the response regulator CpxR. Its activity is subjected to regulation of auxiliary regulator CpxP and NlpE.

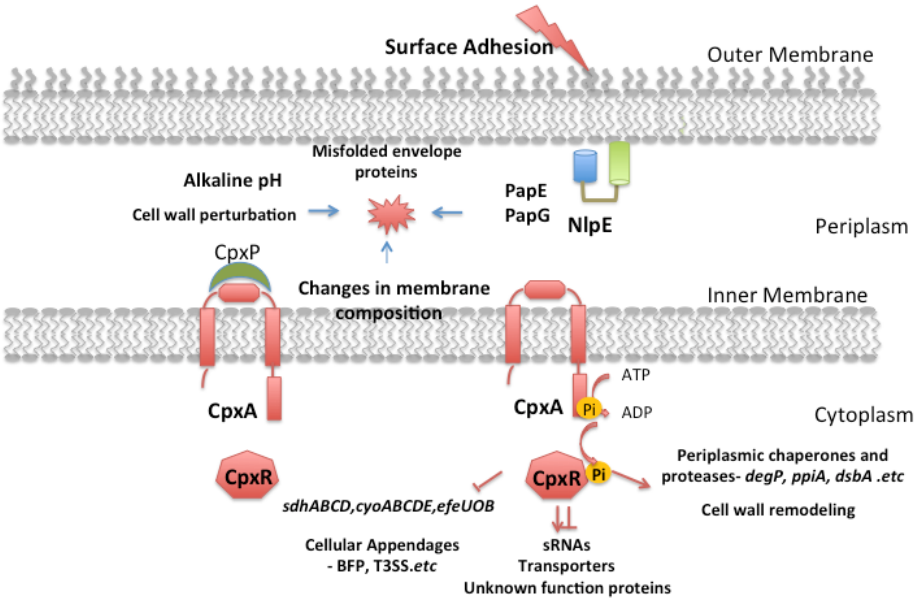

Figure 1-4 Current model of the Cpx pathway in *E.coli*

The Cpx pathway consists of the inner membrane sensor kinase CpxA and the cytoplasmic response regulator CpxR. The periplasmic chaperone CpxP inhibits the Cpx pathway through direct binding. Under inducing conditions such as changes in membrane composition, alkaline pH, mis-folded pilus subunits PapE, PapG, surface adhesion through NlpE, CpxA phosphorylates CpxR. Phosphorylated CpxR becomes active and mediate cellular response.

1.6 Literature Cited

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2 Materials and Methods

2.1 Bacterial Strains and Growth Conditions

All bacterial strains used in this study are listed in Table 2-1. All bacterial strains were routinely cultured in Luria-Bertani (LB) broth containing the appropriate antibiotics at 37°C with aeration at 225 rpm. *E. coli* Δ*cpxA* and Δ*cpxR* mutants were cultured at 30°C with aeration at 225 rpm. Ampicillin was added to LB broth to a final concentration of 100 μg/mL; chloramphenicol was added to a final concentration of 25 μg/mL; kanamycin was added to a final concentration of 50 μg/mL. To induce gene expression from isopropyl-β-D-thiogalactopyranoside (IPTG) -inducible promoters, IPTG (Invitrogen) was added to a final concentration of 0.1 mM.

2.2 Strain Construction

Transformation and P1 transduction were carried out following standard molecular techniques (Sambrook and Russell, 2001; Silhavy *et al.*, 1984). All plasmids used in this study are listed in Table 2-2. All oligonucleotide primers used in this study are listed in Table 2-3.

MC4100 *ΔnlpE* was constructed by λ-Red recombination as previously described (Datsenko & Wanner, 2000). Briefly, in *E. coli* MC4100, λ-Red recombinase was expressed from the pKD46 plasmid using 0.2% arabinose at 30°C (Datsenko and Wanner, 2000). A PCR fragment of the Kan-resistant cassette flanked with sequences homologous to *nlpE* was amplified and transformed into the MC4100 strain expressing the λ-Red recombinase by electroporation. The resulting transformants were selected on kanamycin agar plates overnight at 37°C. Single

colonies were patched onto a fresh kanamycin agar plate. Kanamycin resistant mutants were subsequently verified by sequencing PCR products derived from single colonies. The kanamycin resistance cassette was removed from the resulting MC4100 *nlpE::kan* mutant by FRT-dependent recombination catalyzed by the Flp recombinase expressed from pFLP2 plasmid (Hoang *et al.*, 1998).

Single gene mutants MC4100 *ΔompA,* MC4100 *ΔcpxA* and MC4100 *ΔcpxR* were constructed by P1 Transduction using the corresponding mutants from the Keio library as previously described (Baba *et al.*, 2006; Silhavy *et al.*, 1984). Kanamycin resistance was the selective marker used. The kanamycin cassette was removed by FRT-mediated recombination using the pFlp2 plasmid as previously described (Hoang *et al.*, 1998).

2.3 Construction of Chromosomal *nlpE* Mutants

All chromosomal *nlpE* mutants were constructed by allelic exchange as previously described (Edwards *et al.*, 1998). Briefly, PCR product containing the desired *nlpE* mutation, flanked by 500-1000 bp of homologous sequence up- and downstream of *nlpE* was amplified. The resulting PCR products were purified and first cloned into pUC19 using the In-Fusion HD Cloning kit (Takara company) following the manufacturer's instructions. The pUC19:*nlpE* plasmids were digested with *Sac*I and *Kpn*I and the *nlpE* mutant genes were ligated into *Sac*I and *Kpn*I digested suicide vector pRE-112 (Edwards *et al.*, 1998). GT115 competent cells (Invitrogen) were used for suicide vector cloning and propagation. Plasmid pRE112 with insertion was transformed into conjugal donor strain SM10 and then into MC4100 by conjugation. Donor and recipient cells were mixed in a ratio of 2:1. This mix was spotted onto a

plain LB plate and incubated at 37°C overnight. The next day, the resulting lawn of bacteria was scraped off and re-suspended in 1 mL fresh LB media and plated onto an LB plate supplemented with chloramphenicol. Single colonies were picked and inoculated into 2 mL fresh LB media, and cultured at 30°C for 6 hours with shaking. Serial dilutions were made and plated onto regular LB plates and those containing 5% sucrose to screen for cells in which the gene replacement plasmid had recombined out of the chromosome. These recombinants were patched on LB plates and LB plates containing chloramphenicol to confirm that the plasmid was no longer present in the chromosome. Single colonies were screened for the presence of the correct *nlpE* mutation by sequence analysis of PCR products derived from single colonies.

2.4 Cloning

Cloning was carried out using standard molecular procedures as previously described (Sambrook and Russell, 2001). To construct pET-NlpE, pET-NlpE Δ100-216, pET-NlpE Δ126-216, and pET-cytoNlpE, DNA fragments were synthesized by PCR using the primers listed in Table 2-3. The resulting PCR products were purified using a QIAquick PCR purification Kit (Qiagen) following the manufacturer's instructions, digested with restriction enzymes *Nde*I and *Xho*I, then ligated into *Nde*I and *Xho*I digested pET-22b plasmid. The sequences of the resulting plasmids were verified by DNA sequencing (by Molecular Biology Service Unit, University of Alberta).

2.5 Random Mutagenesis

Random mutagenesis of NlpE was performed using XL-1 Red Competent Cells (Agilent Technologies) following the manufacturer's instructions. The pET-22b based NlpE overexpression plasmid pET-NlpE was transformed into XL-1 Red competent cells made from an *E. coli mutS mutD mutT* triple mutant strain. The resulting transformants were inoculated into 5mL LB and cultured overnight at 37°C. Pools of randomly mutagenized plasmids were extracted using a GenElute™ Plasmid Miniprep Kit (Sigma) following the manufacturer's instructions. SL195, an MC4100 derivative carrying a chromosomal, Cpx-dependent, *yjfN-lacZ* reporter gene was transformed with the randomly mutagenized plasmids. To screen for NlpE mutants with a decreased ability to induce the Cpx pathway, the resulting transformants were patched onto MacConkey agar plates supplemented with 0.1 mM IPTG and 100mg/mL ampicillin. Less red colonies (either pink or white), as compared to SL195 transformed with the wild-type NlpE construct, indicated the presence of NlpE mutants with a decreased ability to activate the Cpx two-component system. Such single colonies were patched onto fresh MacConkey agar plates along with the SL195(pET-22b) and SL195(pET-NlpE) strains. Mutants that retained their phenotypes were analyzed by DNA sequencing (by Molecular Biology Service Unit, University of Alberta).

2.6 Site-directed Mutagenesis

Site-directed mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs Inc) following the manufacturer's instructions. Briefly,

primers used for site-directed mutagenesis (Table.2-2) were designed with the desired mutation in the DNA sequence. pET-NlpE was amplified using the designed primers to introduce the desired mutation into the DNA sequence. The desired mutation was verified by DNA sequencing (by Molecular Biology Service Unit, University of Alberta). All primers used were synthesized by Sigma-Aldrich Company.

2.7 β-galactosidase Assays

β-galactosidase assays were performed as previously described (Silhavy *et al.*, 1984; Slauch and Silhavy, 1991). Briefly, single colonies of each strain were inoculated in 2 mL of LB broth containing appropriate antibiotics and cultured overnight at 37 °C with shaking at 225 rpm. The following day the overnight culture was sub-cultured into fresh LB medium and cultured at 37°C with aeration. To induce expression of NlpE, IPTG was added to a final concentration of 1 mM 1 h after subculture. Cells were collected when the OD_{600} reached ~0.6-0.8 by centrifugation at 4500 g for 10 min. Cells were re-suspended in buffer Z (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$; containing 270 µL β-mercaptoethanol), then 250 µL of the cell mixtures were transferred to a 96-well polystyrene plate. The OD_{600} was read using a Perkin Elmer Wallac Victor^2 1420 plate reader. Remaining cells were lysed using two drops of chloroform and one drop of 0.1% SDS for 10 min and the cellular debris were removed by centrifugation. The lysed cell mixture was added to 1X Z-buffer in 96-well plates (50 µL lysed cell mixture, 150 µL 1X Z-buffer for *yjfN-lacZ* reporter), and 50 µL 10 mg/mL ONPG (o-

nitrophenyl β–D-galactopyranoside)(Sigma) was added to initiate the reaction. The A_{420} was read 20 times over approximately 30 min in the plate reader and Miller Units were calculated (Zhang and Bremer, 1995). For each experiment, all samples were analyzed in triplicate, and each experiment was repeated three times.

2.8 NlpE Protein Purification

The soluble carboxyl-terminal His-tagged cytoplasmic NlpE that has no lipid modification signal was purified by affinity purification using Cobalt resin (Pierce). BL21 (DE3) harboring plasmid pET-cytoNlpE were cultured overnight and sub-cultured 1:100 into 500 mL fresh LB media supplemented with ampicillin and chloramphenicol. The subculture was grown at 37°C with shaking at 225 rpm for an hour. To induce expression of soluble NlpE, IPTG was added to a final concentration of 0.1 mM. The subcuture was grown at 30°C with shaking at 225 rpm for 24 h. Cells were collected by centrifugation at 3214 g for 30 min. The bacterial pellet was re-suspended in 50 mL PBS and lysed by French Press. Protein inhibitor complex (Sigma) was added to the supernatant immediately. The cell lysate was centrifuged at 12857 g for 15 min to pellet unbroken cells and cell debris. The resulting lysate was used for protein purification. To equilibrate the cobalt resin, 1 mL resin (0.5 mL bed volume) was washed with two bed volumes of wash buffer (50mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 7.4) three times. The cobalt resin was handled gently and centrifuged at 700 g at 4°C for all steps. The cell lysate was mixed with pre-equilibrated cobalt resin (Pierce) overnight at 4°C with agitation. The following day, the cell lysate and resin mixture was loaded on a 10 mL column and allowed to settle while the flow-through was collected. The column was

washed using 20 bed volumes of wash buffer. Flow-through was collected in 1 mL fractions in separate tubes. About 20 bed volumes of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole; pH 7.4) were added to the column to elute His-tagged NlpE. Flow-through was collected in 1 mL fractions in separate tubes. All collected samples were analyzed by SDS-PAGE followed by coomassie staining. The eluted protein was buffer exchanged in PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) using Ultra-15 Centrifugal Filter Units (Amicon). Protein concentration was determined using the PierceTM BCA protein assay kit (Pierce) following the manufacturer's instructions.

2.9 NlpE Antibody Purification

Purified NlpE protein was used as antigen for antibody production in rabbit (Rockland Immunochemicals). The crude antiserum was first centrifuged to remove debris. Then total antibodies were precipitated by ammonium sulfate saturated solution. Briefly, 10 mL antiserum was transferred to a beaker and left stirring at 4°C. An equal volume of freshly prepared, saturated ammonium sulfate solution was slowly added to the antiserum. The sample was left stirring at 4°C overnight. Precipitant was collected by centrifugation at 3000 g for 30 min and resuspended in 10 mL of binding buffer (50 mM Tris, 5 mM EDTA; pH 8.5) for further affinity purification.

To immobilize NlpE, purified NlpE (1 mg) was solublized in 1 mL coupling buffer (50 mM Tris, 5 mM EDTA-Na; pH 8.5) through buffer exchange and applied to 1 mL (bed volume) of equilibrated SulfoLink Coupling Resin (Thermo Scientific). Then the mix was incubated for 15 min at room temperature with agitation and an additional

30 min without mixing to allow coupling to occur. The mix was applied to a 10 mL column and allowed to settle. Nonspecific binding sites on the resin were blocked by a solution of 50 mM L-cysteine•HCl in coupling buffer. Coupling efficiency was examined by monitoring the protein concentration of purified NlpE sample prior to and after coupling using the Pierce BCA Protein Assay Kit. Resin with immobilized NlpE was washed with six resin bed volumes of wash solution (1 M NaCl) and stored in PBS buffer for further affinity purification of the anti-NlpE sera.

For affinity purification of the anti-NlpE sera, 1 mL ammonium sulfate precipitated sera sample was added to the packed NlpE immobilized Sulfolink resin column and allowed to flow through the column. This was followed by washing with six bed volumes of PBS. Antibody was eluted using ten bed volumes of elution buffer (0.2 M glycine•HCl, pH 2.5) and collected in separate tubes. The eluted fraction was neutralized by adding an equal volume of 1 M Tris-HCl (pH 9). The concentration of the affinity-purified antibody was determined using a Pierce BCA Protein Assay Kit and buffer-exchanged in PBS buffer to a final concentration of 1mg/mL using Ultra-15 Centrifugal Filter Units (Amicon).

2.10 Western Blotting

Electrophoresis and western blotting were performed as previously described (Raivio *et al.*, 1999b). Blots were incubated with primary anti-NlpE (1mg/mL), anti-OmpA (Antibody Research Corporation), anti-PoxB (Abcam) and anti-PhoA (Abcam) in 1: 10,000 dilutions in 2% (wt/vol) skimmed milk for 1 h with agitation at room temperature. Blots were washed with wash buffer (1 M Tris, 0.9 M NaCl with 0.2% vol/vol tween 20) for 30 min three times. The secondary antibody anti-rabbit

immunoglobulin G-alkaline phosphatase conjugate (Sigma) was applied to blots at a concentration of 1:25,000 in 2% (wt/vol) skimmed milk and incubated for 1 h with agitation at room temperature. Blots were washed with wash buffer (1 M Tris, 0.9 M NaCl with 0.2% vol/vol tween 20) for 30 min three times. Blots were developed with the chemiluminescent Immun-Star AP Substrate Pack (Bio-Rad) following the manufacturer's directions. Blots were imaged using a Chemi-Doc Imager (Bio-Rad).

2.11 Membrane Preparation

Crude membrane extracts were prepared as previously described (Lobos and Mora, 1991). Bacterial strains were subcultured into 5 mL fresh LB at 1:100 dilution and grown at 37°C with aeration at 225 rpm. To induce protein overexpression from IPTG-inducible promoters, IPTG was added to the culture to a final concentration of 0.1 mM 1 h after subculture. When cell grown to OD_{600} of 0.6-0.8, equal numbers of cells were collected by adjusting cell volumes to a standard OD_{600} and re-suspended in PBS before being lysed by sonication. The resulting cell lysate was centrifuged at 9391 g at 4°C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet the membranes. The membrane pellet was solubilized in 50 mM Tris-HCl (pH 8.0, with 2% Triton X-100). In order to account for the low level of NlpE produced from the chromosomal locus, 10 mL of LB culture were used for membrane preparations in the absence of NlpE overexpression.

Bacterial membrane extracts used for pull-down and co-immunoprecipitation assays were collected as previously described with modifications (Lobos and Mora, 1991). Overnight cultures were sub-cultured into 500 mL fresh LB at 1:100 dilution

and cultured at 37°C with aeration at 225 rpm for 15 h. Cells were collected by centrifugation at 4500 g for 30 min. The pellet was resuspended in 50 mL PBS buffer and lysed by French press. Protein protease inhibitor complex solution (Sigma) was added following the manufacturer's instructions immediately after cell lysis. The resulting cell lysate was centrifuged at 3214 g for 30 min to remove cell debris and unbroken cells. Supernatant was transferred to clean tubes and ultracentrifuged for 45 min at 130,000 g to pellet membranes. The resulting pellet was solubilized in 10 mL sodium phosphate based buffer with Triton X-100 overnight for pull-down assays. For co-immunoprecipitation assays, the membrane pellet was solubilized in Tris-based buffer with 1% Triton X-100 overnight. Buffer exchange of membrane extracts was achieved using PD-10 Desalting Columns (GE healthcare) following the manufacturer's instructions. Total protein concentration was determined using the PierceTM BCA protein assay kit (Pierce) following the manufacturer's instructions.

2.12 Analysis of Gene Expression in Adhered Cells

An assay to measure gene expression in adhered cells was developed, based on a previously described protocol (Otto *et al.*, 1999; Otto and Silhavy, 2002). Acid washed glass bead (212-300 µm; cat No. G1277. Sigma) served as an abiotic surface for cells to adhere to. Briefly, to make the glass beads hydrophilic, acid-washed glass beads were treated in flask with a mixture of H_2O:H_2O_2:NH_4OH (Ratio 6:1:1, V:V) at 80°C for 10 min followed by thorough rinsing with distilled water. Then, glass beads were treated with a mixture of H_2O:H_2O_2:HCl (Ratio 5:1:1, V:V) at 80°C for 10 min followed by thorough rinsing with distilled water. Hydrophilic glass beads were immersed in distilled water for storage at room temperature overnight. To prepare

hydrophobic glass beads, hydrophilic beads were first thoroughly washed with two-volumes of 99% ethanol and trichloroethylene (TCE) and treated with 10% dimethyldichlorosilane (DDS) dissolved in trichloroethylene for 10 min. Excess DDS was thoroughly washed away by sequential rinses with ethanol, TCE and ethanol. Hydrophobic glass beads were immersed in ethanol for storage at room temperature overnight.

To initiate the adherence assay, 1 mL of cells cultured overnight for 14-16 h was added to 3 g glass beads in a 10 mL flask and left at 37°C in an incubator for 6 h, allowing adherence to occur. Planktonic cells were aspirated by removing LB culture from the glass beads with a pipette. Glass beads were washed three times with 1 mL of fresh LB medium. 1 mL of fresh LB was added to the glass beads after the washes. Adherent cells were collected by vortexing the glass beads for 15 sec and then transferring the supernatant to a new eppendorf tube. The activity of the Cpx response was determined by measuring the light produced from a Cpx-dependent luminescent reporter gene in both planktonic and adherent cells. The luminescence assay was performed as previously described (MacRitchie *et al.*, 2008). Assays were performed at least three times in triplicate. For each sample, 250 µL was aliquoted to a black, clear-bottom 96-well plate (Corning). Optical dencity at 600nm and luminescence (counts per second, CPS) were measured using a Perkin Elmer Wallac Victor^2 1420 plate reader. The final bioluminescence (CPS/OD_{600}) values were calculated by dividing the corrected CPS (CPS sample-CPS blank control) by the corrected OD_{600} (OD_{600} sample-OD_{600} blank control).

2.13 Pull-down Assay

Cobalt resin (Pierce) was used to immobilize NlpE for pull down assays. For all steps, cobalt resin was centrifuged at 700 g at 4°C for 5 min. Supernatant was removed by syringe. First, 100µL of cobalt resin was centrifuged and equilibrated with 6 bed volumes of equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 1% Triton X-100; pH 7.4) three times. Then, 100 µg purified NlpE diluted with equilibration buffer was added to freshly equilibrated cobalt resin in a microfuge tube. The resulting mix was agitated at 4°C for 1 h. Resin was collected by centrifugation. Supernatant was collected to monitor binding affinity by measuring protein concentration using the PierceTM BCA protein assay kit (Pierce) following the manufacturer's instructions. Resin was washed three times with 20 bed volumes of wash solution (50 mM sodium phosphate, 300 mM sodium chloride, 1% Triton X-100; pH 7.4). Prepared membrane extract (1 mL of a 1 mg/mL preparation) was added to resin containing immobilized NlpE to initiate the pull down assay. Prepared membrane extract was added to plain cobalt resin as a control for nonspecific binding. The resulting mix was agitated at 4°C overnight. Then resin was collected and washed three times with 20 bed volumes of wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 1% Triton X-100; pH 7.4). Protein complexes retained were eluted using 50 µL elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 1% Triton X-100, 150 mM imidazole; pH 7.4). Elution was collected after 2 min incubation at room temperature. Elution samples were analyzed by SDS-PAGE followed by silver staining.

2.14 Silver Staining

Silver staining was performed as previously described (Rabilloud *et al.*, 1988). Following SDS-PAGE, polyacrylamide gels were fixed with 50% methanol and 12% acetic acid solution for 1 h. The gel was washed with a 50% ethanol solution for 20 min, three times with agitation and then sensitized in 0.2 g/mL sodium thiosulfate for 1 min in the dark followed by three washes with distilled water for 20 sec each. Gels were then incubated in 2 g/L silver nitrate solution (with 0.75 mL/L formaldehyde) for 20 min in the dark and washed three times for 20 sec each using distilled water. Gels were developed with 60 g/L sodium carbonate solution (with thiosulfate 0.04 g/mL, 0.5 mL/L formaldehyde) and the reaction was stopped immediately when bands appeared by removing the sodium carbonate solution. Gels were washed with a 50% methanol and 12% acetic acid solution for 10 min. Silver stained gels were stored in a 50% methanol solution and imaged using a Chemi-Doc Imager (Bio-Rad).

2.15 In Gel Protein Identification

To identify proteins visualized in silver stained gels, bands were excised using a sterile blade and stored in sterile microfuge tubes before being sent for mass-spectrometry analysis performed by the Alberta Proteomics and Mass Spectrometry Facility.

2.16 Co-immunoprecipitation Assay

The co-immunoprecipitation assay was performed using protein A agarose and Pierce spin columns (Pierce) following the manufacturer's instructions. The protein

A agarose was handled gently and centrifuged at 700 g at 4°C for 5 min for all steps. First, 100 µL protein A agarose was transferred to a Pierce spin column and washed with three bed volumes of PBS three times. 0.1 mg of purified anti-NlpE antibody was diluted in 0.5 mL PBS and mixed with equilibrated protein A resin with agitation at 4°C for 1 h. This mix was centrifuged at 700 g for 5 min to remove buffer. The resin was washed gently with 20 bed volumes of PBS buffer three times. To avoid co-elution of antibody with any retained protein complexes, NlpE antibody was cross-linked to the protein A agarose resin using the cross linker dimethyl pimelimidate (DMP) (Abcam) following the manufacturer's instructions. Freshly prepared 13 mg/mL cross-linker DMP solution (dissolved in PBS) was added to the resin at a 1:1 ratio. Resin was left agitating at room temperature for 30 min. Resin was washed with PBS buffer containing 0.2 M triethanolamine and treated with the cross linker DMP two more times. Quench buffer (PBS with 50 mM ethanolamine) was added to the resin twice to quench the crosslinking reaction and the resin was then treated with 100 µL 1 M glycine (pH 3.0) solution to remove excess unlinked antibody. Finally, the resin was washed with PBS and stored in the last wash at 4°C for a maximum of one week.

To initiate the co-IP experiment, 1mg of membrane extract was added to antibody cross-linked protein-A agarose and mixed with agitation at 4°C overnight. The following day, the agarose was washed three times using 20 bed volumes of co-IP buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100) to remove non-specific binding. To elute protein complexes that were retained, 50 µL of 1 M glycine

(pH 3.0) was added to the resin. Eluates were analyzed by SDS-PAGE followed by western blotting.

2.17 *In vivo* Formaldehyde Cross-linking

Formaldehyde cross-linking experiments were performed as previously described (Postle, 2007). Briefly, wildtype MC4100(pCA-NlpE), MC4100 *ΔompA*(pCA-NlpE), and MC4100 *ΔnlpE* were cultured overnight and sub-cultured into 5 mL fresh LB media at a 1:100 dilution for 1h. IPTG was added to induce overexpression of NlpE and the culture was grown to mid-log phase (OD_{600}~ 0.5-0.6). Equal numbers of cells were collected by adjusting cell volumes to a standard OD_{600}, pelleted, and re-suspended in 938 µL PBS buffer. Samples were treated with 62 µL 16% monomeric p-formaldehyde (to a final concentration of 1%) (Electron Microscopy Services) for 15 min. Treated cells and non-treated control were harvested by centrifugation, re-suspended in 1mL fresh PBS buffer, and lysed by sonication. The resulting cell lysate was centrifuged at 9391 g at 4°C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet membranes. The resulting pellet was solubilized in 100 mM Tris-HCl (pH 8.0, with 2% Triton X-100) and immediately analyzed by SDS-PAGE followed by western blotting.

2.18 Tables

Table 2-1 Bacterial strains used and constructed in this study

Strain	Description	Source or Reference
MC4100	F- *araD139 Δ(argF-lac)* U169 *rpsL150* (Str^R) *relA1 flhD5301 deoC1 ptsF25 rbsR*	(Casadaban, 1976)
SL195	MC4100 *λRS88* [*yjfN-lacZ*]	(Raivio *et al.*, 2013)
TR50	MC4100 *λRS88* [*cpxP-lacZ*]	(Raivio and Silhavy, 1997)
BL21 (DE3)	B F– *ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)*	(Studier and Moffatt, 1986)
RM53	TR50 *ΔcpxA*	Raivio Lab
JSW1	TR50 *ΔcpxR*	This study
JSW153	MC4100 *ΔnlpE*	This study
	MC4100 *ΔcpxA*	This study
JSW157	MC4100 *ΔcpxR*	This study
SV195	MC4100 *ΔompA*	(Vogt and Raivio, 2014)
JSW215	MC4100 *ΔnlpE ΔompA*	This study
JSW118	BL21 (DE3)/pET-CytoNlpE	This study
Nlp15	MC4100/pNlp15	(Price and Raivio, 2009)
JSW158	MC4100 *ΔcpxA*/pNlp15	This study
JSW159	MC4100 *ΔcpxR*/pNlp15	This study
JSW160	MC4100 *ΔnlpE*/pNlp15	This study
JSW131	MC4100 *ΔompA*/pNlp15	This study
JSW223	MC4100 *ΔnlpE ΔompA*/pNlp15	This study
JSW204	TR50 *ΔnlpE*	This study

JSW237	TR50 *ΔnlpE* /pCA-24N	This study
JSW238	TR50 *ΔnlpE* /pCA-NlpE	This study
JSW239	TR50 *ΔnlpE* /pCA-OmpA	This study
JSW245	MC4100/pCA-NlpE	This study
JSW248	MC4100 *ΔompA*/pCA-NlpE	This study
SY327	*Δ*(*lac pro*) *argE*(Am) *rif nalA recA56*	(Edwards *et al.*, 1998)
SM10	*thi thr leu tonA lac Y supE recA*::RP4-2-Tc: :Mu Km	(Edwards *et al.*, 1998)
JSW264	SL195/pET-22B	This study
JSW100	SL195/pET-NlpE	This study
JSW99	SL195/pET-NlpE Δ126-216	This study
JSW235	SL195/pET-NlpE Δ100-216	This study
JSW265	SL195/pET-NlpE Δ100-108	This study
JSW266	SL195/pET-NlpE Δ108-112	This study
JSW267	SL195/pET-NlpE Δ108-115	This study
JSW268	SL195/pET-NlpE L43G	This study
JSW269	SL195/pET-NlpE Y90A	This study
JSW231	SL195/pET-NlpE E103A	This study
JSW233	SL195/pET-NlpE M127A	This study
JSW193	MC4100 *nlpE* Δ126-216	This study
JSW191	MC4100 *nlpE* Δ100-216	This study
JSW198	MC4100 *nlpE* L43G	This study
JSW199	MC4100 *nlpE* Y90A	This study

JSW197	MC4100 *nlpE* E103A	This study
JSW240	MC4100 *nlpE* M127A	This study
JSW194	MC4100 *nlpE* Δ100-108	This study
JSW270	MC4100 *nlpE* Δ108-112	This study
JSW195	MC4100 *nlpE* Δ108-115	This study
JSW244	MC4100 *nlpE* Δ126-216/pNlp15	This study
JSW225	MC4100 *nlpE* Δ100-216/pNlp15	This study
JSW219	MC4100 *nlpE* L43G/pNlp15	This study
JSW243	MC4100 *nlpE* Y90A/pNlp15	This study
JSW242	MC4100 *nlpE* E103A/pNlp15	This study
JSW241	MC4100 *nlpE* M127A/pNlp15	This study
JSW227	MC4100 *nlpE* Δ100-108/pNlp15	This study
JSW271	MC4100 *nlpE* Δ108-112/pNlp15	This study
JSW229	MC4100 *nlpE* Δ108-115/pNlp15	This study

Table 2-2 Plasmids used and constructed in this study

Plasmid	Description	Source or Reference
pNlp15	Low copy number plasmid with a *cpxP-lux* reporter	(Price and Raivio, 2009)
pKD46	Red recombinase expression plasmid	(Datsenko and Wanner, 2000)
pFLP2	Flp recombinase expression plasmid	(Hoang *et al.*, 1998)
pCA-24N	Empty high copy number cloning plasmid	(Kitagawa *et al.*, 2005)
pCA-NlpE	NlpE overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-OmpA	OmpA overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pET-22B	Cloning vector with sequence encoding carboxyl-terminus His-tag	Novagene
pET-NlpE	NlpE overexpression plasmid	This study
pET-Δ126-216	*nlpE*Δ126-216 cloned into pET-22B	This study
pET-Δ100-216	*nlpE*Δ100-216 cloned into pET-22B	This study
pET-cytoNlpE	*nlpE*ΔPRE1-20 cloned into pET-22B	This study
pET-NlpE Δ100-108	*nlpE*Δ100-108 cloned into pET-22B	This study
pET-NlpE Δ108-112	*nlpE*Δ108-112 cloned into pET-22B	This study
pET-NlpE Δ108-115	*nlpE*Δ108-115 cloned into pET-22B	This study
pET-NlpE L43G	*nlpE*L43G cloned into pET-22B	This study
pET-NlpE Y90A	*nlpE*Y90A cloned into pET-22B	This study
pET-NlpE E103A	*nlpE*E103A cloned into pET-22B	This study
pET-NlpE M127A	*nlpE*M127A cloned into pET-22B	This study
pUC-19	High-copy number cloning vector, Amp^R	Invitrogen

pUC-Δ126-216	*nlpE*Δ126-216 cloned into pUC-19	This study
pUC-Δ100-216	*nlpE*Δ100-216 cloned into pUC-19	This study
pUC-NlpE Δ100-108	*nlpE*Δ100-108 cloned into pUC-19	This study
pUC-NlpE Δ108-112	*nlpE*Δ108-112 cloned into pUC-19	This study
pUC-NlpE Δ108-115	*nlpE*Δ108-115 cloned into pUC-19	This study
pUC-NlpE L43G	*nlpE*L43G cloned into pUC-19	This study
pUC-NlpE Y90A	*nlpE*Y90A cloned into pUC-19	This study
pUC-NlpE E103A	*nlpE*E103A cloned into pUC-19	This study
pUC-NlpE M127A	*nlpE*M127A cloned into pUC-19	This study
pRE112	Suicide vector used for allelic exchange	(Edwards *et al.*, 1998)
pRE112-Δ126-216	*nlpE*Δ126-216 cloned into pRE112	This study
pRE112-Δ100-216	*nlpE*Δ100-216 cloned into pRE112	This study
pRE112-NlpEΔ100-108	*nlpE*Δ100-108 cloned into pRE112	This study
pRE112-NlpEΔ108-112	*nlpE*Δ108-112 cloned into pRE112	This study
pRE112-NlpEΔ108-115	*nlpE*Δ108-115 cloned into pRE112	This study
pRE112-NlpE L43G	*nlpE*L43G cloned into pRE112	This study
pRE112-NlpE Y90A	*nlpE*Y90A cloned into pRE112	This study
pRE112-NlpE E103A	*nlpE*E103A cloned into pRE112	This study
pRE112-NlpE M127A	*nlpE*M127A cloned into pRE112	This study

Table 2-3 Oligonucleotide primers used in this study

Primer Name	Sequence (5'-3') restriction enzyme sequence is underlined
NlpE-F	GGGAATTCCATATGGTGAAAAAAGCGATAGTGAC
NlpE-R	GCCCTCGAGCTGCCCCAAACTACTGCAATC
Kan-NlpE	AAATATATCCTTCTGGCCTGTTTTGCGTTTGTTTCTGTCTCAAGACG GGTTAATGGGAATTAGCCATGGTCC
NlpE-Kan	GGCGATGCGCGGCAAAGTGCGCAGCGGTCGGGAATAAAAAGAAGGAA TGGGTGTAGGCTGGAGCTGCTTC
Δ126-216-R	GCCCTCGAGCGGCGTCATAGGTAAACTGG
Δ100-216-R	GTGGTGGTGCTCGAGCATCTCCAGCGC
CytNlpE-F	GGGAATTCCATATGAATAATCGGGCCGAAGTCGAT
L43G-1	AATCGAAACCTCTCTGTTCGGCGAAAAAGACGGAACATGG
L43G-2	CCATGTTCCGTCTTTTTCGCCGAACAGAGAGGTTTCGATT
Y110A-1	CGACAGCAAAGGTGAAAAGTCATATGCTCGGGCGAAAGGC
Y110A-2	GCCTTTCGCCCGAGCATATGACTTTTCACCTTTGCTGTCG
E103A-1	GAGATGCTCGATCGTGCAGGCAATCCGATTGAATCG
E103A-2	CGATTCAATCGGATTGCCTGCACGATCGAGCATCTC
M127A-1	ACCTATGACGCCGGCGACCCTGCGGGGC
M127A-2	GCCCCGCAGGGTCGCCGGCGTCATAGGT
slNlpE-1	GGCGATGCGCTGGAGATGGAATCGCAGTTCAACTAT
slNlpE-2	ATAGTTGAACTGCGATTCCATCTCCAGCGCATCGCC
slNlpE-3	AAGGCAATCCGATTGAA GCGGCACAATCCAGTTTA

slNlpE-4	TAAACTGGATTGTGCCGCTTCAATCGGATTGCCTT
T7-F	TAATACGACTCACTATAGGG
T7-R	GCTAGTTATTGCTCAGCGG
UpNlpE	ACGGCCAGTGAATTCGAGCTCCCATCCTTCAGAAACCCAGG
DownNlpE	TCTAGAGGATCCCCGGGTACCCTTTTCGTCTTACGAGTCCC
InTNlpE-R	GTGGTGGTGCTCGAGCATCTCCAGCGC
Δ100-216-1	GGCGATGCGCTGGAGATGTAACCCGTCTTGAGACAGA
Δ100-216-2	TCTGTCTCAAGACGGGTTACATCTCCAGCGCATCGCC
Δ126-216-1	AGTTTACCTATGACGCCGtaaCCCGTCTTGAGACAG
Δ126-216-2	CTGTCTCAAGACGGGttaCGGCGTCATAGGTAAACT
M13-FP	TGTAAAACGACGGCCAGT
M13-RP	CAGGAAACAGCTATGACC

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3 Mutagenesis Study of Surface Sensing Regulator NlpE in *Escherichia coli*

3.1 Introduction

Sensing environmental stresses and mediating an appropriate cellular response is crucial for bacterial survival. Two-component systems are the most prevalent mechanism that bacteria employ to do so (Stock *et al.*, 2000). The Cpx two-component system is one of the envelope stress responses in *E. coli*. It consists of the inner membrane sensor kinase CpxA and the cytoplasmic response regulator CpxR (Nixon *et al.*, 1986; Weber and Silverman, 1988; Dong *et al.*, 1993). Under normal conditions, CpxA is inhibited by a periplasmic chaperone CpxP through direct binding and functions primarily as a phosphatase that dephosphorylates CpxR (Raivio *et al.*, 1999b; Thede *et al.*, 2011; Tschauner *et al.*, 2014). However, in the presence of inducing cues, CpxA functions as a kinase that phosphorylates and activates CpxR. Subsequently, phosphorylated CpxR impacts transcription of its regulon to effect a cellular response (Price and Raivio, 2009; Raivio *et al.*, 2013).

The activity of the Cpx envelope stress response is subjected to regulation by additional auxiliary regulators (Raivio *et al.*, 1999a; Snyder *et al.*, 1995; Otto and Silhavy, 2002; Buelow and Raivio, 2010). One of the auxiliary regulators of the Cpx pathway is the outer membrane lipoprotein NlpE (new lipoprotein E). NlpE was first identified in a screen designed to identify over-expressed genes that suppressed the toxicity of a secreted LamB-LacZ-PhoA tripartite fusion protein in *E. coli* (Snyder *et al.*, 1995). Overexpression of NlpE resulted in increased levels of a CpxR-regulated periplasmic serine protease, DegP, through activation of the Cpx pathway. Further characterization revealed that NlpE has a lipoprotein signal peptide and is anchored

to the outer membrane through its amino-terminal lipid modification. Overexpression of NlpE is commonly used as an inducing cue of the Cpx pathway.

The involvement of NlpE in sensing other inducing cues of the Cpx pathway was investigated. It is not required to sense other activating signals, such as alkaline pH, misfolded pilus subunits PapG or PapE, or cell growth, since an *nlpE* mutant still responded to these inducing cues by activating the Cpx response (DiGiuseppe and Silhavy, 2003). The only known signal that NlpE senses is adhesion to hydrophobic surfaces, as revealed by the Silhavy lab (Otto and Silhavy, 2002). In enterohaemorrhagic *Escherichia coli* (EHEC), NlpE mediated induction of the Cpx pathway was observed in EHEC adhering to hydrophobic beads and also to human intestinal Caco-2 cells and contributes to EHEC virulence regulation (Shimizu *et al.*, 2015). NlpE mediated signaling might be important for the differences that are exhibited by adherent cells as compared to planktonic cells in gene expression profile, metabolism, respiration and pathogenesis (Costerton *et al.*, 1987; Zhang and Normark, 1996; Costerton *et al.*, 1999; Moorthy *et al.*, 2016; Geng *et al.*, 2014; Otto *et al.*, 2001). The mechanism by which NlpE controls the Cpx response is largely unknown.

The structure of NlpE was revealed in a crystallization study (Hirano *et al.*, 2007). It consists of two distinct, well-formed β-barrel domains linked by an unstructured region. The amino-terminus of NlpE is an eight-stranded antiparallel β-barrel domain that resembles lipocalin Blc, but with one α-helix missing in NlpE. Lipocalins belong to a multifunctional protein family that is proposed to bind hydrophobic small molecules (Flower, 1996). The first bacterial lipocalin identified was *E. coli* Blc

(Campanacci et al., 2004). Blc binds to lysophospholipids (Campanacci *et al.*, 2004). Lipocalin Blc functions as an asymmetric dimer that binds to hydrophobic small molecules (Schiefner *et al.*, 2010). The carboxyl-terminus of NlpE is a five-stranded β-barrel domain with an α-helix connecting β3-β4 (Fig.3-1A). Structurally, it is similar to the oligonucleotide/oligo-saccharide-binding (OB) fold that often mediates protein-protein interactions and substrate binding (Murzin, 1993). The unstructured region of 29 amino acids connecting the amino- and carboxyl-terminus is often referred to as the flexible linker.

Despite knowledge of a variety of inducing cues, the specific molecular signal that the Cpx pathway senses remains elusive. Understanding the mechanism of how NlpE signals the Cpx pathway upon its overexpression and during surface adhesion will provide us with more information about Cpx signaling at the molecular level. In this study, NlpE was subjected to truncating mutations, random, and site-directed mutagenesis and its role in signaling the Cpx pathway when overexpressed and upon surface adhesion was assayed. The results revealed that the carboxyl-terminus is important for signaling both upon NlpE overexpression and surface adhesion. Mutations that shorten or even alter single amino acids of the flexible linker negate signaling and affect protein stability indicating that this domain may control important interactions between the N- and C-termini and/or other molecules. Finally, our results show that in addition to the role the amino-terminal lipocalin-resembling domain plays in lipid modification, it may also bind a ligand, as suggested by the phenotype of mutations affecting residues that are oriented towards the inside of the β-barrel structure. In summary, our mutagenesis study

highlights the complexity of NlpE signaling and suggests that it involves collaboration of all regions of the protein, and potentially ligand binding.

3.2 Results

3.2.1 Truncated NlpE with the C-terminus Deleted Fails to Induce the Cpx Pathway upon Overexpression

The NlpE homolog from *Vibrio cholerae* contains the amino-terminal lipocalin resembling domain and the unstructured region close to the flexible linker in *E. coli* NlpE, but is missing the carboxyl-terminus β-domain. To investigate the role of the carboxyl-terminus in signaling, truncated NlpE mutants (NlpEΔ126-216 and NlpE Δ100-216) were constructed (molecular details of truncated NlpE are shown in Fig.3-1.B). NlpE Δ126-216 contains the amino-terminal lipocalin resembling domain and the flexible linker, but lacks the carboxyl-terminus. The molecular weight of NlpE Δ126-216 is predicted to be 17kDa (ExPASy Compute pI/Mw tool, http://web.expasy.org/compute_pi/). NlpE Δ100-216 contains only the amino-terminus and is missing both the flexible linker and the carboxyl-terminal domain. The molecular weight of NlpE Δ100-216 is 13kDa, as predicted from its amino acid sequence using the ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The expression levels of the truncated NlpE constructs were investigated by western blotting. NlpE Δ126-216 was expressed to a similar level as wild-type NlpE (Fig. 3-4). However, further deleting the flexible linker leaving only the amino-terminus renders the protein unstable, as NlpE Δ100-216 was not detected in membrane fractions by western blotting (Fig. 3-4).

To examine the activity of the Cpx pathway upon overexpression of the truncated NlpE mutants, plasmids expressing whole length NlpE, NlpE Δ100-216 and NlpE Δ126-216 were transformed into SL195, which contains a chromosomal CpxR-regulated *yjfN-lacZ* reporter gene. The abilities of the NlpE constructs to induce the Cpx pathway upon overexpression were assessed by monitoring β-galactosidase activity from a Cpx-regulated *yjfN-lacZ* reporter gene (Raivio *et al.*, 2013). Overexpression of NlpE showed about 50-fold induction of the Cpx pathway compared to that of vector control (Fig. 3-1). On the contrary, overexpression of NlpE Δ126-216 induced the Cpx pathway about 4-fold despite being highly expressed (Fig. 3-1), demonstrating that deleting the carboxyl-terminal domain severely reduces the ability of NlpE to signal the Cpx pathway. The NlpE Δ100-216 construct also failed to induce the *yjfN-lacZ* reporter gene, but since this protein was unstable, we were unable to make any conclusions regarding the impact of the flexible linker on Cpx signaling (Fig. 3-1, 3-4).

3.2.2 Random Mutagenesis Reveals the Involvement of the NlpE Flexible Linker in Signaling the Cpx Pathway

As an alternative approach to identification of amino acids and domains in NlpE that are important for signaling, we subjected an NlpE overexpression plasmid to random mutagenesis and screened the resulting mutant plasmid pools for those that altered the ability of NlpE overexpression to induce the Cpx pathway. Pools of mutated pET-NlpE plasmids were transferred into the SL195 strain carrying the *yjfN-lacZ* reporter gene. The resulting transformants were plated on MacConkey plates supplemented with 0.1 mM IPTG to induce overexpression of NlpE. On

MacConkey plates, SL195(pET-22B) shows white colonies due to the low basal expression of the *yjfN-lacZ* reporter gene. Upon NlpE overexpression, SL195(pET-NlpE) produced red colonies, indicating increased β-galactosidase expression and activation of the Cpx response. Colonies with decreased β-galactosidase activity (lighter red or white on the MacConkey plates) were patched onto fresh MacConkey plates to verify the phenotypes and then the *nlpE* genes of these plasmids were sequenced. Out of 2400 colonies screened, eleven white colonies were identified. Among these, four were verified to be frame shifting nonsense mutations. Two mutants were mapped to T9 (all mutations are listed relative to C1 at the start of the mature, modified protein), and resulted in T9A and T9S mutations. Mutating T9 to alanine and serine affected the stability of NlpE as no protein was detected by western blotting and therefore these mutants were not further investigated (data not shown). Two mutants were mapped to M127 and resulted in M127I mutations. Three mutants converted E103 to isoleucine. To confirm the decreased abilities of the M127 and E103 mutants to signal the Cpx pathway, M127A and E103A substitutions were constructed by site-direct mutagenesis. The stabilities of NlpE M127A and E103A were examined by western blotting. Both NlpE E103A and M127A were stable on overexpression as shown in Fig. 3-4, where it is evident that the mutant proteins are present at levels similar to those of the wild-type NlpE. The abilities of NlpE E103A and M127A to activate the Cpx pathway upon overexpression were examined by measuring the β-galactosidase activity of the Cpx-regulated *yjfN-lacZ* reporter gene. Overexpression of NlpE E103A and M127A resulted in less than 2-fold induction of the Cpx pathway (Fig 3-2B), as compared to

approximately 50-fold induction upon overexpression of the wild-type NlpE. In the NlpE structure, both E103 and M127 are located near the flexible linker region (as shown in Fig 3-2.A). E103 is at the amino-terminal end of the flexible linker, while M127 is located at the carboxyl-terminus. The diminished ability of these mutant NlpE proteins to activate the Cpx response, despite that fact that they did not appear to affect stability, led us to focus on the flexible linker region of NlpE.

To investigate the involvement of the flexible linker in signaling the Cpx pathway, three NlpE mutants with varying portions of the flexible linker deleted were constructed: NlpE Δ100-108, NlpE Δ108-115 and NlpE Δ108-112 (Fig. 3-2A). NlpE Δ100-108, NlpE Δ108-115 and NlpE Δ108-112 were expressed and stable as revealed by western blotting (Fig. 3-4). In fact, NlpE Δ108-115 and NlpE Δ108-112 appear to be more stable on overexpression than the wildtype NlpE protein (Fig. 3-4), confirming that mutations to the flexible linker affect stability. The activity of the Cpx pathway was examined upon overexpression of NlpE Δ100-108, NlpE Δ108-115 and NlpE Δ108-112. As shown in Fig. 3-2B, while overexpression of NlpE showed about 50-fold induction of the Cpx pathway, overexpression of NlpE Δ100-108, NlpE Δ108-115 and NlpE Δ108-112 failed to activate the Cpx pathway at all. Together, these experiments show that the unstructured flexible linker region that connects the two well-structured β-barrel domains of NlpE is critical for signaling the Cpx pathway when NlpE is overexpressed.

3.2.3 Specific NlpE N-terminal Amino Acids are Required for Signaling the Cpx Pathway

In order to investigate the role of the amino-terminal domain of NlpE, derivatives were constructed that contained the lipid modified or periplasmic localized carboxyl-terminus alone or the carboxyl-terminus with the flexible linker. However, none of the constructs were stably expressed in *E. coli* as no protein was detected by western blotting (data not shown). We therefore took another approach and analyzed NlpE homologues to identify conserved amino acids in the N-terminal lipocalin domain. Directed mutagenesis was carried out to mutate the conserved single amino acids L43 and Y90 found in the N-terminus of both the *E. coli* and *V. cholerae* NlpE proteins. As shown in Fig. 3-3, an NlpE L43G substitution was not capable of inducing the Cpx pathway when overexpressed, while NlpE Y90A overexpression resulted in an 8.6-fold induction of the Cpx pathway compared to the vector control. NlpE L43G and Y90A substitutions did not compromise protein stability since both mutants were expressed to a similar level as wild-type NlpE (Fig. 3-4). These data indicate that conserved amino acid L43 and Y90 located in the NlpE amino-terminus are required for signaling the Cpx pathway when NlpE is overexpressed.

3.2.4 Abilities of NlpE Mutants to Sense Surface Adhesion

We hypothesized that NlpE signals the Cpx pathway upon overexpression and surface adhesion using similar molecular mechanisms. Thus NlpE amino acids or regions that are required for signaling the Cpx pathway upon overexpression should also be involved in signaling the Cpx pathway upon surface adhesion. To test this

hypothesis, chromosomal *nlpE* mutants were constructed and these strains were used to measure the ability of the various NlpE mutants that were stable on overexpression to induce the Cpx response upon adhesion. Decreased activation of the Cpx pathway upon surface adhesion could result from a reduced NlpE expression level. Therefore, the expression level of all chromosomal NlpE mutants was examined by western blotting. As shown in Fig. 3-5, wild-type NlpE, NlpE L43G, NlpE Y90A, NlpE E103A and NlpE M127A were detected at the correct size of 27 kDa, while NlpE Δ100-108, NlpE Δ108-115, NlpE Δ108-112, and NlpE Δ126-216 were detected at smaller molecular weights corresponding with the deletions contained within these mutants. Although wild-type NlpE is expressed at low levels, all of these mutant proteins were expressed at similar levels, suggesting that they have stability comparable to that of the wild-type protein (Fig. 3-5). However, the NlpE Δ100-216 mutant was not detected when it was expressed from the chromosomal locus, suggesting that it is unstable (Fig. 3-5).

Adhesion assays were performed to assess the abilities of the chromosomally located NlpE mutants to sense surface adhesion and signal the Cpx pathway appropriately. Strains carrying various NlpE mutants were transformed with plasmid pJW15 carrying a luminescent *cpxP–luxCDABE* reporter. The resulting strains were inoculated and cultured for 14-16h. The following day, cultures were added to hydrophobic glass beads and incubated to allow adhesion. Planktonic cells and adherent cells were collected 6h later. The activity of the Cpx pathway in planktonic and adherent cells was assayed by measuring luminescence. As shown in Fig.3-6A, in wildtype MC4100, the Cpx pathway showed 2.3-fold induction in

adhered cells compared to planktonic cells (two sample t-test, P =0.0035). Also, as previously observed, when *cpxA* was mutated, the activation of the Cpx pathway in adherent cells was abolished (Fig.3-6A). Further, when *nlpE* was absent, the activity of the Cpx pathway in adherent and planktonic cells was not statistically significant (two sample t-test, p= 0.1924). Thus, NlpE and CpxA are critical for signaling the Cpx pathway upon surface adhesion in our modified assay, as previously shown (Otto and Silhavy, 2002). For NlpE L43G, NlpE Y90A, NlpE Δ126-216, NlpE Δ100-108, NlpE Δ108-115, NlpE Δ108-112, NlpE E103A and NlpE M127A, the activation of the Cpx pathway upon surface adhesion was abolished (Fig.3-6). Therefore, regions or single amino acids that are critical for signaling the Cpx pathway when NlpE is overexpressed are also critical for signaling the Cpx pathway upon surface adhesion.

3.3 Discussion

NlpE is an important auxiliary regulator of the Cpx pathway. It was first identified as a multi-copy activator of the Cpx pathway (Snyder *et al.*, 1995). Since then, NlpE overexpression is often used as an inducing cue of the Cpx pathway. Auxiliary regulators of two-component systems often integrate signals, and NlpE has been shown to sense cellular adhesion to hydrophobic surfaces in MC4100 and EHEC strains and subsequently signal the Cpx pathway (Otto and Silhavy, 2002; Shimizu *et al.*, 2015). However, the mechanism of how NlpE overexpression induces the Cpx pathway is not clear. In this study, we sought to investigate the molecular details of NlpE signaling to the Cpx pathway; both upon overexpression and surface adhesion.

Each domain in a multiple-domain protein tends to fold and function independently (Vogel *et al.*, 2004). NlpE has two well-structured β-barrel domains linked by an

unstructured region. In order to investigate the role of the carboxyl-terminus of NlpE, truncated NlpE mutant NlpE Δ126-216 was constructed (Fig.3-1B). Deleting the carboxyl-terminus alone did not compromise protein stability as NlpE Δ126-216 overexpressed from the pET-22b based plasmid and from the chromosomal locus was detected by western blotting at levels similar to that of the wild-type protein (Fig.3-4 and Fig.3-5). NlpE Δ126-216 showed a significantly decreased ability to induce the Cpx two-component system when overexpressed (Fig.3-2). Upon surface adhesion, NlpE Δ126-216 also failed to induce the Cpx pathway as full length NlpE did (Fig.3-6). Thus, the carboxyl-terminus of the NlpE protein is required for NlpE signaling when overexpressed and upon surface adhesion.

The unstructured region of NlpE connecting the amino- and carboxyl-termini is referred to as the flexible linker. Structurally, flexible linkers are disordered regions that are short, containing 2-31 amino acids. Flexible linkers usually separate domains without interfering with the discrete functions of each domain and can act to facilitate protein stability. Such linkers are often disordered coiled regions that are flexible and have the potential of interacting with other proteins or other regions of the same protein to affect morphological change (Reddy Chichili *et al.*, 2013). Flexible linkers are often enriched in proline and glycine, with prolines thought to prevent nonspecific interactions and glycines endowing the region with flexibility.

For NlpE, the flexible linker is 29-amino acid long starting from M99 and extending to M127 (M99LRE**G**N**P**IESQFNYTLEAAQSSL**P**MT**P**M127). In this study, we showed that further deleting the flexible linker in a C-terminally truncated version of NlpE

resulted in destabilization, since NlpE Δ100-216 was not detected by western blotting (Fig.3-4 and Fig.3-5). This observation indicates that the flexible linker not only links two β-barrel domains but also plays an important role in stabilizing the NlpE protein, specifically the N-terminus. NlpE Δ100-108, NlpE Δ108-115 and NlpE Δ108-112-NlpE mutants in which the flexible linker was shortened failed to signal the Cpx pathway when overexpressed or upon surface adhesion (Fig.3-3 and Fig.3-6). The flexible linker is long enough to allow the NlpE carboxyl-terminus to span the envelope and reach periplasmic or inner membrane components (Egan *et al.*, 2014; Hirano *et al.*, 2007). Since our data implicate both the C-terminus and the flexible linker as being essential in signaling, one model is that adhesion may lead to a reorientation of the N- and C-terminal domains, mediated by the flexible linker, that permits the C-terminus of NlpE to interact with important signaling complexes, such as CpxA. A similar model has recently been proposed to allow lipoprotein LpoB to span the periplasm and interact with PBP1B and regulate its activity in the inner membrane (Egan *et al.*, 2014).

In an attempt to investigate the role of the NlpE amino-terminal domain, constructs were made that contained the lipid modified N-terminal region fused to the periplasmic C-terminus with or without the flexible linker, but lacking the N-terminus. However, none of these constructs resulted in the production of stable proteins (data not shown). Therefore, the amino-terminus, in addition to being necessary to anchor the protein to the outer membrane, also appears to be involved in stabilizing the carboxyl-terminus. Mutation of conserved amino acids in the N-terminus revealed that L43 and T90 are required for signaling the Cpx pathway

when NlpE is overexpressed and upon surface adhesion (Fig.3-3). Previous studies showed that when NlpE is localized to the periplasm by virtue of a lack of lipid modification, it failed to activate the Cpx pathway when overexpressed (Hirano *et al.*, 2007). Taken together, these studies suggest that the amino-terminus of NlpE performs some signaling function that requires proper lipid modification and localization. As shown in Fig.3-3B, L43 is located in the β2 strand and Y90 is located in the β6 strand of the lipocalin-resembling amino-terminus, and both face toward the interior of the β –barrel. This localization, coupled with the signaling phenotypes of the mutants, suggests that L43 and Y90 could be involved in ligand binding that contributes to signal transduction, perhaps through conformational changes mediated by binding and releasing of ligand upon surface adhesion.

In this study, the assay used to examine the activity of the Cpx pathway upon surface adhesion was modified from one previously performed in the Silhavy lab (Otto and Silhavy, 2002). In their experiment, 1mL of overnight culture was added to 3g of hydrophobic glass beads and cultured without shaking for 2h to allow adhesion, after which the activity of the Cpx pathway in planktonic and adherent cells was measured by β-galactosidase assay using CpxR-regulated *cpxR-lacZ* and *cpxP-lacZ* reporters. In this study, a luminescent *cpxP-luxCDABE* reporter was used to measure the activity of the Cpx pathway. Initially, a time-course adhesion assay performed revealed that the activation of the Cpx pathway at 6h after adhesion is a better representation, with less error, resulting in smaller standard deviations. This is mostly likely due to increased numbers of cells adhering to the surface after longer adherence times. This conclusion is supported by the fact that induction of Cpx

activity was observed in planktonic cells repeatedly isolated from the supernatant of the glass bead filled samples upon adherence (Otto and Silhavy, 2002).

Another topic that will require further experimentation is the precise cellular localization of the NlpE mutants constructed. In this study, the expression levels of the various NlpE mutants were examined in the total membrane fraction. It is possible that mislocalizing NlpE to the inner membrane could also abolish activation of the Cpx pathway upon surface adhesion. Therefore, in the future, the expression level of NlpE mutants in outer membrane fractions should be examined to rule out this possibility.

Interestingly, four truncated NlpE mutants constructed, Δ126-216, Δ100-108, Δ108-115 and Δ108-112 showed decreased *cpxP-lux* expression compared to wild-type and *ΔnlpE* mutant strains (Fig.3-6). Whether or not these mutants decrease the basal activity of the Cpx pathway should be further determined. This phenotype might reflect a previously unidentified role for NlpE in controlling basal activity of the Cpx response, and therefore be of interest for future study.

In summary, our results suggest that NlpE signaling requires the collaboration of all three regions of the protein - the carboxyl-terminus, amino-terminus and the unstructured flexible linker in between. Our results, and previous studies, are consistent with a model in which an alteration in ligand binding by the N-terminus may stimulate movement of the N- and C-termini relative to one another, in a fashion that requires the flexible linker, leading to an integral signaling activity that is performed by the C-terminal domain. One possibility could be that the C-terminal domain becomes reoriented upon adhesion such that it is capable of interacting

with CpxA and/or other signal transduction complexes in the periplasm or inner membrane.

3.4 Tables and Figures

Table 3-1 NlpE mutants constructed in this study

Mutants	Ability to induce the Cpx pathway when overexpressed	Expression level	Method used
120-236	Decreased	Not affected	Cloning
100-236	Decreased	Decreased	Cloning
100-108	Decreased	Not affected	Cloning
108-115	Decreased	Not affected	Cloning
108-112	Decreased	Not affected	Cloning
N2D	Not affected	Not affected	Site-directed mutagenesis
F121A	Not affected	Not tested	Site-directed mutagenesis
Y133A	Not affected	Not tested	Site-directed mutagenesis
D138G	Not affected	Not tested	Site-directed mutagenesis
C211S	Not affected	Not tested	Site-directed mutagenesis
C145S	Not affected	Not tested	Site-directed mutagenesis
C31SDDC35S	Not affected	Not tested	Site-directed mutagenesis
L43G	Decreased	Not affected	Site-directed mutagenesis
Y90A	Decreased	Not affected	Site-directed mutagenesis
E103A	Decreased	Not affected	Site-directed mutagenesis
M127A	Decreased	Not affected	Site-directed mutagenesis
E103I	Decreased	Not affected	Random mutagenesis
M127I	Decreased	Not affected	Random mutagenesis
Frame shifting	Decreased	Not tested	Random mutagenesis
T9A	Decreased	Decreased	Random mutagenesis
T9S	Decreased	Decreased	Random mutagenesis

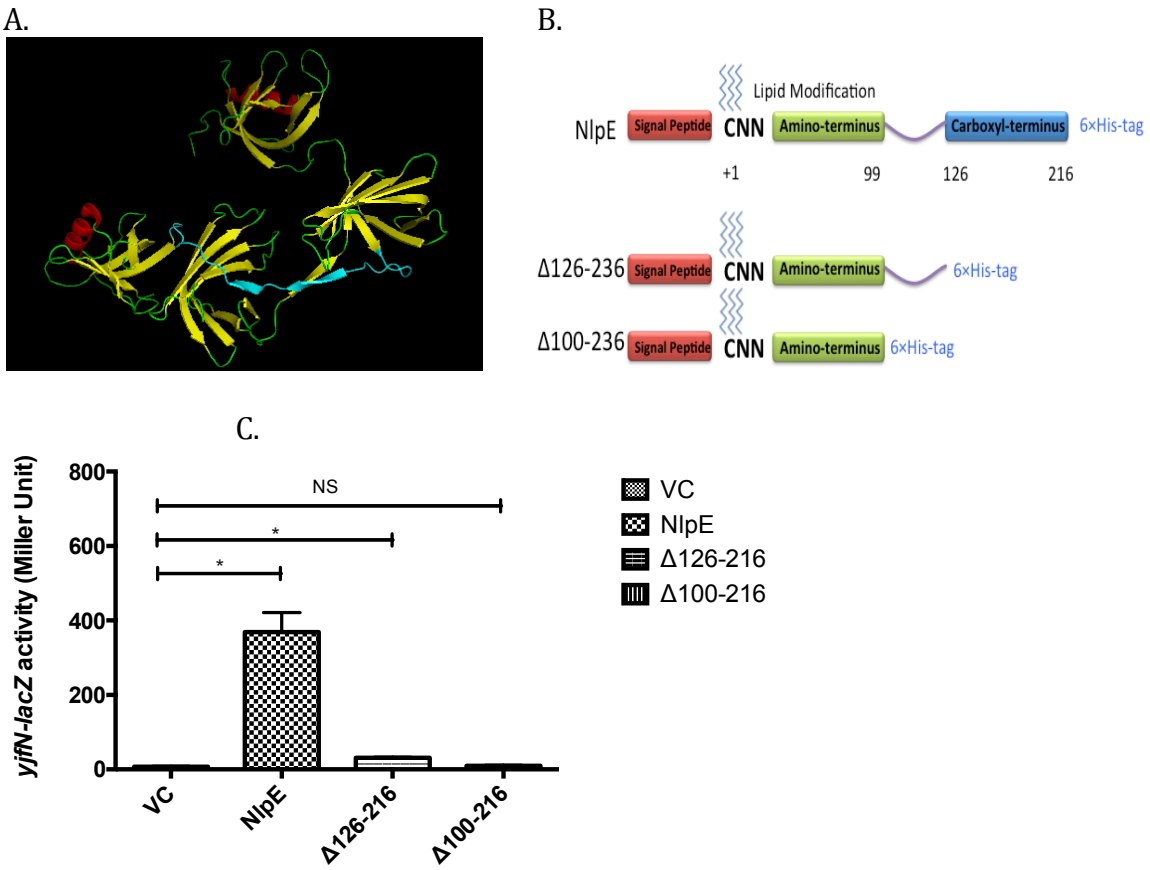


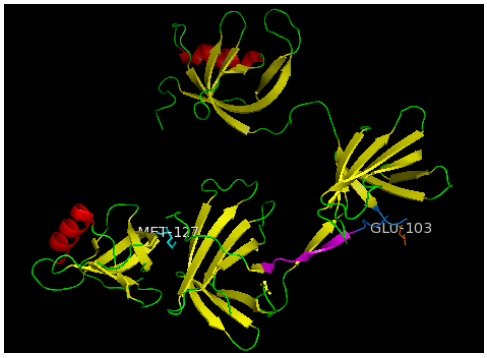
Figure 3-1 The NlpE carboxy-terminus is critical for signaling the Cpx pathway when overexpressed

A. Cartoon representation of an NlpE dimer with β-strands labeled as yellow, α-helices shown in red and the loop regions colored green. The flexible linker (100-125) connecting the amino- and carboxyl-terminal domains is shown in cyan. The NlpE sructure was obtained from the Protein Structures Database (2Z4H) and modified using Pymol (http://pymol.org).

B. Schematic representations of NlpE and the truncated mutants that were constructed.

C. β-galactosidase activity of a Cpx-regulated *yjfN-lacZ* reporter gene upon overexpression of NlpE, NlpE Δ100-216 and NlpE Δ126-216. SL195, an MC4100 derivative carrying a chromosomal CpxR regulated *yjfN-lacZ* reporter, was transformed with pET-22b (vector control, VC) or various NlpE overexpression plasmids. All strains were sub-cultured into fresh LB with ampicillin and cultured at 37°C. IPTG was added 1H after subculture to a final concentration of 1mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8 and lysed using chloroform and 1% SDS. The β -galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 plate reader after addition of ONPG to the cell lysate. Experiments were performed three times, and each experiment contained triplicate experimental replicates; the mean and standard deviation are shown. NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

A.



B.

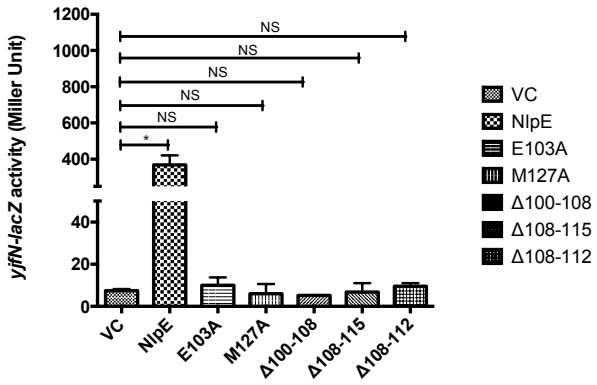
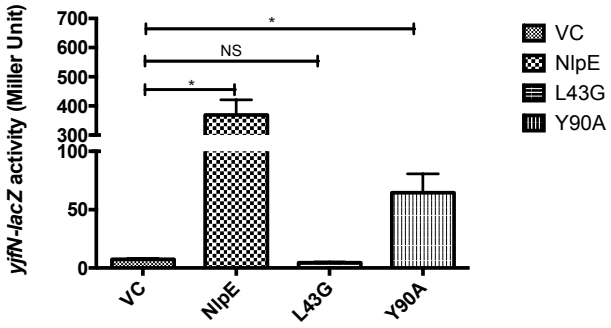


Figure 3-2 The role of the NlpE flexible linker in signaling.

A. Cartoon representation of an NlpE dimer with β-strands shown in yellow, α-helices labeled as red, and loops depicted in green. M127 is labeled in cyan. E103 is shown in orange. Regions that were deleted in the linker in NlpE Δ100-108 and Δ108-115 are labeled as blue and magenta respectively. The NlpE structure was obtained from the Protein Structures Database (2Z4H) and modified using Pymol (http://pymol.org).

B. β-galactosidase activity of the Cpx pathway upon overexpression of NlpE, NlpE E103A, NlpE M127A, NlpE Δ100-108, NlpE Δ108-115 and NlpE Δ108-112. SL195 was transformed with pET-22b (vector control, VC) based NlpE overexpression plasmids. All strains were sub-cultured into fresh LB with ampicillin and cultured at 37°C. IPTG was added 1h after subculture to a final concentration of 1 mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8, lysed using chloroform and 1% SDS and the β -galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 Plate reader after addition of ONPG. Experiments were performed three times, with three experimental replicates in each experiment; the mean and standard deviation are shown. NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

A.



B.

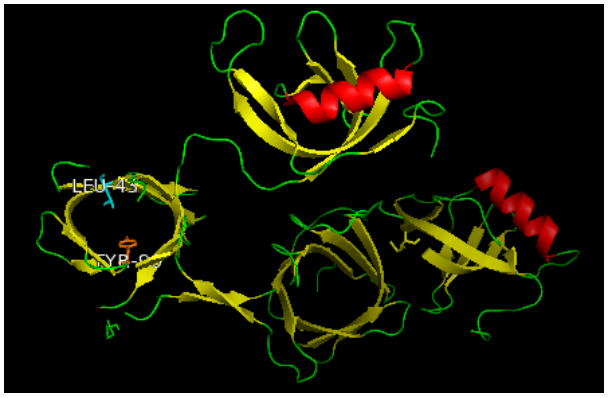


Figure 3-3 N-terminal aa in NlpE are involved in signaling.

A. β-galactosidase activity produced from a Cpx-dependent *yjfN-lacZ* reporter gene upon overexpression of NlpE, NlpE L43G and NlpE Y90A. SL195 was transformed with pET-22b (VC) or pET-22b based NlpE overexpression plasmids. All strains were sub-cultured into fresh LB with ampicillin and cultured at 37°C. IPTG was added 1h after subculture to a final concentration of 1mM to induce NlpE overexpression. Cells were collected when the OD600 reached 0.6-0.8. Cells were lysed using chloroform and 1% SDS and the β -galactosidase activity was measured using a Perkin Elmer Wallac Victor2 1420 Plate reader after addition of ONPG. Experiments were performed in triplicate three times; the mean and standard deviation are shown. The β-galactosidase activity and standard deviation of three experimental replicates per sample is given by each bar and respective error bar above. NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

B.Cartoon representation of an NlpE dimer with the β-strands labeled as yellow, α-helices shown in red and loops indicated in green. L43 is labeled in cyan, while Y90 is labeled in orange. The NlpE structure was obtained from the Protein Structures Database (2Z4H) and modified using Pymol (http://pymol.org).

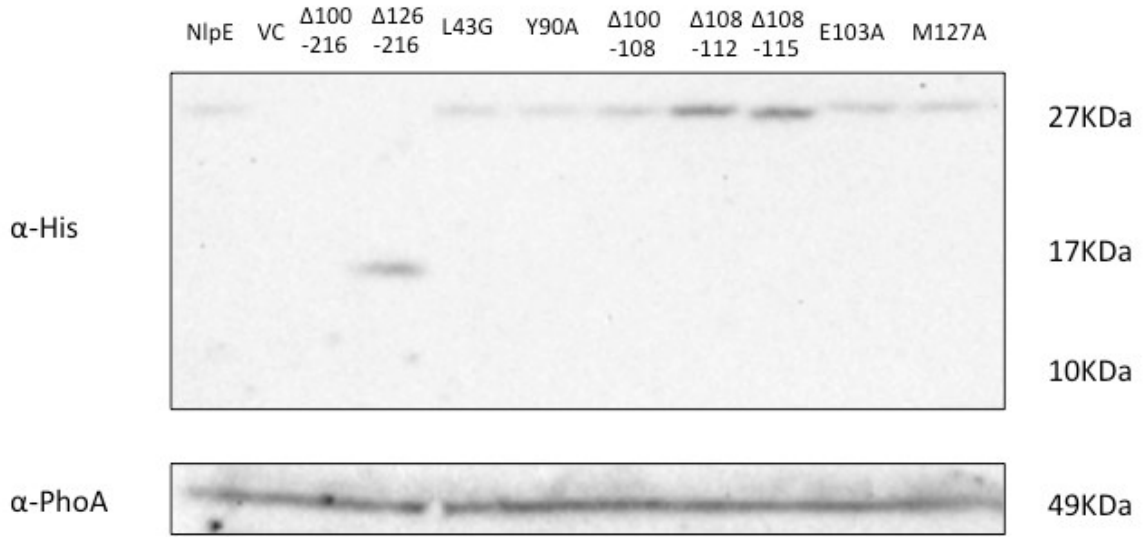


Figure 3-4 Overexpression level of NlpE mutants in *E.coli*

Western blotting results showing the expression level of NlpE mutants in total membrane fractions prepared from MC4100 harboring pET-22b based NlpE overexpression plasmids. Bacterial strains were sub-cultured into 5 mL fresh LB in 1:100 dilutions and grown at 37 °C. To induce protein overexpression, IPTG was added to the culture to a final concentration of 0.1 mM 1h after subculture. Equal numbers of cells were collected when the OD_{600} reached 0.6-0.8. Cells were re-suspended in PBS and lysed by sonication. The resulting cell lysates were centrifuged at 9391 g at 4 °C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet whole cell membranes. The resulting pellets were solubilized in 100mM Tris-HCl (pH 8.0, with 2% Triton X-100). Solubilized membrane samples of each strain were electrophoresed on 12% SDS-PAGE gels, followed by western blotting. NlpE was visualized using a His-tag antibody. PhoA was visualized using a PhoA antibody as loading control.

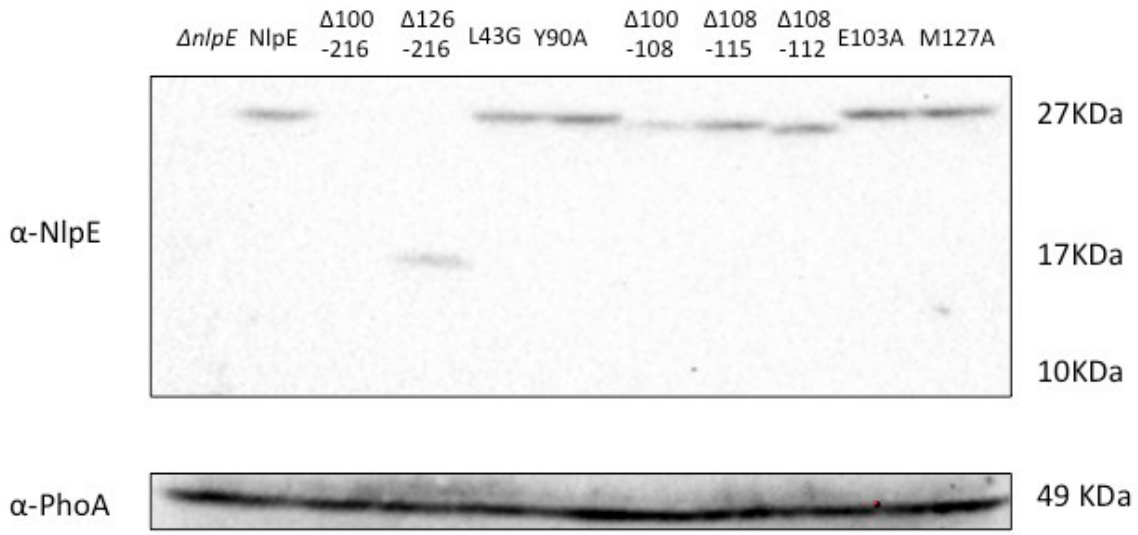
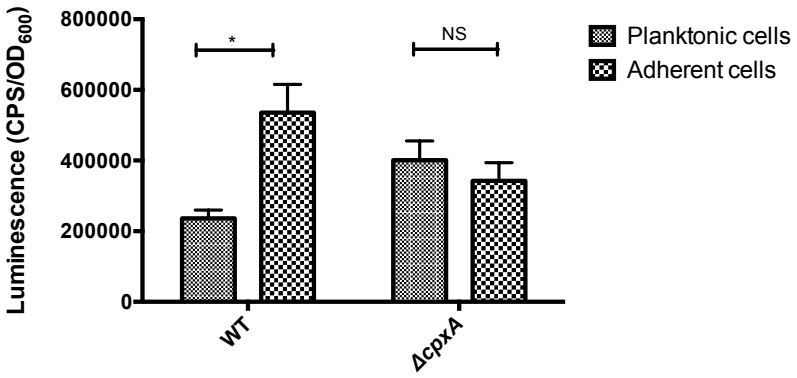


Figure 3-5 Expression level of NlpE chromosomal mutants

Western blotting results showing the expression levels of chromosomal NlpE mutants in total membrane fractions. Bacterial strains were sub-cultured into 10 mL fresh LB at 1:100 dilution and grown at 37°C until the OD_{600} reached 0.6-0.8. Equal numbers of cells were collected. Cells were re-suspended in PBS and lysed by sonication. The resulting cell lysate were centrifuged at 9391 g at 4°C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet whole cell membranes. The resulting pellets were solubilized in 100 mM Tris-HCl (pH 8.0, with 2% Triton X-100). NlpE in solubilized membrane samples of each strain was visualized by 12% SDS-PAGE followed by western blotting. NlpE was visualized using α-NlpE antibody. PhoA was visualized using α-PhoA antibody as loading control.

A.



B.

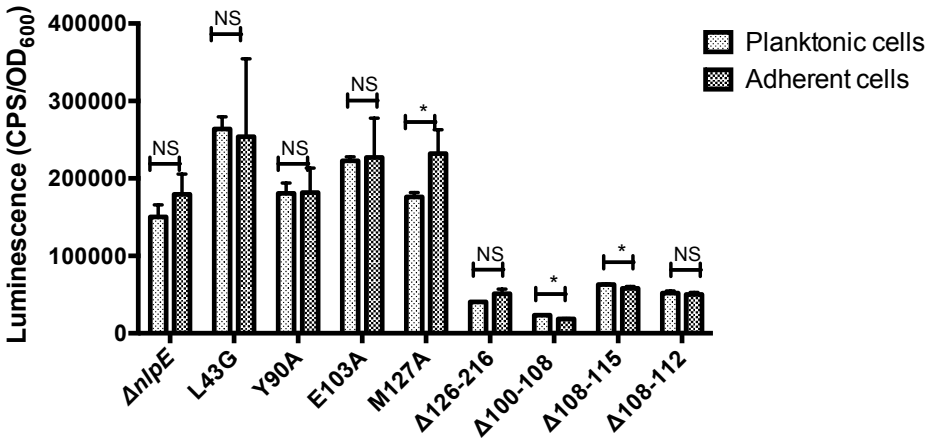


Figure 3-6 NlpE mutations that negate activation of the Cpx response upon NlpE overexpression also impact the ability of NlpE to signal adherence.

A. Activity of the Cpx pathway upon surface adherence in MC4100 wildtype (WT) and *ΔcpxA* mutant (*ΔcpxA*) strains. B. Activity of the Cpx pathway upon surface adherence in constructed various *nlpE* mutants. Acid-washed glass beads were used to increase the surface area that *E. coli* cells were able to adhere to. The glass beads were treated with trichloroethylene (TCE) and dimethyldichlorosilane (DDS) to make them hydrophobic. Overnight cultures of each strain were added to hydrophobic glass beads and incubated for 6h without shaking to allow adhesion. Planktonic cells were harvested from LB medium. Glass beads were washed with fresh LB medium before adhered cells were collected after vortexing. The activity of the Cpx pathway in planktonic cells and adhered cells was analyzed by measuring luminescence produced from a Cpx-regulated *cpxP-lux* reporter gene. Experiments were performed in triplicate at least three times. NS indicates not statistically significant between strains (P>0.05, one-way ANOVA test); * indicates a statistically significant difference between strains (P<0.05, one-way ANOVA test).

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4 Investigation of NlpE Interacting Proteins and Their Involvement in Signaling the Cpx Two-component System

4.1 Introduction

The bacterial envelope is crucial for survival since it is where bacteria encounter environmental changes. The gram-negative bacterial envelope consists of the inner membrane, the outer membrane and the periplasm in between (Silhavy, Kahne, & Walker, 2010). In *E. coli,* the Cpx two-component system regulates an envelope stress response. It consists of the inner membrane sensor kinase CpxA and the response regulator CpxR. CpxA senses a variety of signals in the envelope such as alkaline pH, misfolded pilus subunits PapE and PapG, changes in inner membrane composition and overexpression of an outer membrane lipoprotein NlpE (Vogt & Raivio, 2012). The auxiliary regulator NlpE was first identified in a screen for over-expressed genes that could suppress the toxicity of a tripartite LamB-LacZ-PhoA fusion protein in the periplasm. Overexpression of NlpE was found to increase the levels of the periplasmic serine protease DegP by activating the Cpx two-component system (Snyder, Davis, Danese, Cosma, & Silhavy, 1995). Further research revealed that NlpE is also involved in sensing adhesion to hydrophobic surfaces in *E. coli*. In response to surface adhesion, NlpE activates the Cpx pathway and it also contributes to surface adhesion itself (Otto & Silhavy, 2002). In EHEC, NlpE senses adhesion to hydrophobic surfaces and also to Caco-2cells and signals the Cpx two-component system (Shimizu, Ichimura, & Noda, 2015). However, the function or molecular mechanisms by which outer membrane lipoprotein NlpE accomplishes this signaling role remain unclear. Considering that NlpE is anchored to the outer membrane through its amino-terminus lipid modification, it is a mystery how NlpE senses surface adhesion and signals the Cpx pathway. Our goal in this study was to

determine if other proteins might interact with NlpE in *E. coli* to facilitate surface sensing. We used a pull-down assay with purified NlpE to identify potential interacting partners and identified the abundant outer membrane protein OmpA. The interaction between NlpE and OmpA *in vivo* was further confirmed by co-immunoprecipitation and formaldehyde cross-linking. We hypothesized that the interaction of NlpE and OmpA is required for the NlpE to sense surface adhesion. Indeed, the activation of the Cpx pathway was decreased upon adhesion when OmpA was mutated. Accordingly, we propose a model in which NlpE interacts with surface adhesin OmpA at the outer membrane to sense adherence.

4.2 Results

4.2.1 NlpE Interacts with Outer Membrane Protein OmpA as Revealed in Pull Down Assays

In order to identify proteins that interact with NlpE, we conducted an *in vitro* pull-down assay. For this purpose, we planned to purify NlpE by polyhistidine (His) affinity purification. Because NlpE is a lipoprotein and the N-terminal lipoprotein signal peptide is cleaved by prolipoprotein signal peptidase during lipid modification, we constructed a recombinant NlpE protein containing a His-tag at its carboxyl-terminus. We found that full length NlpE containing the lipid modification site (highly hydrophobic) did not bind to the cobalt resin and therefore, a soluble cytoplasmic NlpE construct (cytoNlpE) with a carboxyl-terminal His-tag was made by deleting the lipoprotein signal peptide. This protein was successfully purified. Purified His-tagged NlpE was immobilized on cobalt resin and used as bait to

capture proteins from a Triton X-100 solubilized *E. coli* membrane lysate. As shown in Fig.4-1, two additional bands corresponding to 30kDa and 48kDa were detected when the His-tagged cytoNlpE was applied to the cobalt resin but not in its absence. To analyze protein identity, the bands of interest were cut out of the gel and sent for mass-spectrometry analysis (by Alberta Proteomics and Mass Spectrometry Facility). Mass spectrometry results revealed that the 48kDa band was enriched in LpdA (Table.4-1). LpdA is a lipoamide dehydrogenase that loosely associates with the inner membrane on the cytoplasmic side. The interaction of NlpE and LpdA is likely not physiologically relevant since NlpE is localized to the outer membrane *in vivo*. This interaction might have been detected due to the loss of anchoring of the cytoNlpE His-tagged construct to the outer membrane and its re-localization to the cytoplasm. Mass spectrometry results revealed that the 30kDa band was enriched in OmpA (Table. 4-2). OmpA is an abundant outer membrane protein that has been shown to function as an adhesin and invasin in *E. coli* (Prasadarao et al., 1996; Torres and Kaper, 2003; Lower et al., 2005; Hillman et al., 2013; Gaddy et al., 2009; Martinez et al., 2014; Kim et al., 2010; Bartra et al., 2012). Our pull-down assay results suggest that NlpE may interact with OmpA at the outer membrane. We set out to verify this interaction *in vivo*.

4.2.2 Co-immunoprecipitation Confirms an NlpE:OmpA Interaction

The pull-down assay indicated the possibility of an NlpE and OmpA interaction. Therefore, we sought to verify that NlpE does interact with OmpA in the cell using an *in vivo* co-immunoprecipitation assay. Membrane extracts of MC4100, MC4100 *ΔnlpE* and MC4100 overexpressing NlpE from the pCA-NlpE plasmid were applied to

NlpE antibody-immobilized on protein A agarose beads. Immuno-precipitated protein complexes were visualized using 12% SDS-PAGE and the presence of NlpE and OmpA was visualized by western blotting using anti-NlpE and anti-OmpA antibodies respectively. As shown in Fig.4-2, OmpA was co-immunoprecipitated with NlpE in wildtype MC4100, and both proteins were detected in the eluates at higher levels when NlpE was overexpressed (Fig.4-2). In contrast, PoxB, a peripheral membrane protein, was not detected in the eluate from the co-immunoprecipitation. These results support our initial observations and suggest that NlpE and OmpA interact in a physiologically significant manner in the cell.

4.2.3 Cross-linking Reveals a Complex Formed by NlpE and OmpA

To further verify the existence of a complex between NlpE and OmpA, formaldehyde cross-linking experiments were also performed. Considering that chromosomal NlpE is expressed at very low levels (Fig.4-3), both MC4100(pCA-NlpE) and a Δ*ompA*(pCA-NlpE) were used for cross-linking experiments (Kitagawa *et al.*, 2005). As a negative control, we also included an MC4100 *ΔnlpE* mutant. For all tested strains, cells were harvested at mid-log phase, re-suspended in PBS buffer and treated with or without formaldehyde for 15min. The presence of NlpE and OmpA protein or an NlpE:OmpA protein complex in the membrane was visualized by SDS-PAGE and western blotting with anti-NlpE and anti-OmpA antibodies. In the presence of the cross linker formaldehyde, a complex corresponding in size to smaller than 250kDa was recognized by both anti-NlpE and anti-OmpA antibodies in wildtype MC4100 (Fig.4-3). Upon deletion of OmpA or NlpE, the complex disappeared (Fig.4-3). Determination of the accurate size and composition of the

250kDa complex will require further investigation by other techniques such as size-exclusion chromatography analysis of cross-linked species and/or immunoprecipitation of the complex, followed by mass spectrometry. None-the-less, these data further support the conclusion that NlpE interacts with OmpA, and further suggest that this complex contains at minimum, more than one monomer of each protein.

4.2.4 OmpA Overexpression Induces the Cpx Pathway in a CpxA and NlpE-dependent Manner

Our results indicate that NlpE and OmpA interact. Interestingly, previous studies showed that OmpA increases biofilm formation on hydrophobic surfaces by repressing cellulose synthesis through activation of the Cpx pathway in *E. coli* (Ma and Wood, 2009). Later, another study indicated that overexpressed OmpA is partially responsible for the induction of the Cpx pathway in an EPEC *Δhfq* mutant (Vogt and Raivio, 2014). Together, these findings led us to investigate the role of OmpA in Cpx signaling. We hypothesized that changes in an NlpE:OmpA complex at the outer membrane could be involved in signaling adhesion. To test our hypothesis, TR50, an MC4100 strain with a chromosomal *cpxP-lacZ* reporter gene, was transformed with the OmpA overexpression plasmid pCA-OmpA (Kitagawa *et al.*, 2005). The activity of the Cpx pathway upon OmpA overexpression was tested by measuring β-galactosidase produced from the Cpx-regulated *cpxP-lacZ* reporter gene. As shown in Fig.4-4, OmpA overexpression induced the Cpx pathway 6-fold compared to the vector control. To elucidate whether or not the activation of the Cpx pathway upon OmpA overexpression required the sensor kinase CpxA, the

activity of the Cpx pathway upon OmpA overexpression was assessed when *cpxA* was mutated. In a *ΔcpxA* mutant, overexpression of OmpA showed no activation of the Cpx pathway compared to the vector control (Fig.4-4). This result indicates that OmpA overexpression activates the Cpx pathway in a CpxA-dependent manner. Since our data indicate that OmpA and NlpE form a complex, we asked whether induction of the Cpx response by OmpA overexpression requires NlpE. To do this, we measured the activitiy of the Cpx pathway in an *ΔnlpE* mutant when OmpA was overexpressed. We found that in an *ΔnlpE* mutant, activation of the Cpx pathway upon OmpA overexpression was abolished (Fig.4-4). We conclude that NlpE is required for sensing OmpA overexpression and signaling the Cpx pathway in *E. coli*. Notably, this is the only other NlpE–dependent Cpx activating signal that has been identified, besides adhesion, to date. We also determined whether induction of the Cpx response by NlpE overexpression requires OmpA by measuring the activity of the Cpx pathway in both wildtype and *ΔompA* mutant strains when NlpE was overexpressed. As shown in Fig.4-5, activation of the Cpx pathway upon NlpE overexpression when *ompA* is mutated was not affected. These results demonstrate that while the ability of OmpA to induce the Cpx response is dependent on NlpE, NlpE can induce the pathway in the absence of OmpA.

4.2.5 OmpA is Required for Sensing Surface Adhesion

Based on the above results, we hypothesized that OmpA is required for NlpE to sense surface adhesion. If this is true, then mutating *ompA* will abolish activation of the Cpx pathway upon surface adhesion. The involvement of OmpA in sensing surface adhesion was investigated. Acid-washed glass beads were used to increase

the surface area that *E. coli* cells had available for adherence. The glass beads were treated with trichloroethylene (TCE) and dimethyldichlorosilane (DDS) to make them hydrophobic. Overnight cultures of MC4100 wildtype, *ΔnlpE* mutant, *ΔompA* mutant and *ΔnlpE ΔompA* double mutant strains were added to hydrophobic glass beads and incubated for 6h without shaking to allow adhesion. Planktonic cells were harvested from the LB medium. Glass beads were washed with fresh LB medium before adhered cells were collected by vortexing. The activity of the Cpx pathway in planktonic cells and adhered cells was analyzed by measuring expression of a Cpx-dependent luminescent *cpxP-lacZ* reporter gene. In wildtype *E. coli*, the Cpx pathway showed more than 2.5-fold induction in adhered cells compared to planktonic cells (two sample t-test, P =0.0008), as previously observed (Otto and Silhavy, 2002). In the *ΔnlpE* mutant, the activity of the Cpx pathway in adherent vs. planktonic cells is not significantly different (two sample t-test, p=0.1636). In the *ΔompA* mutant, the activity of the Cpx pathway in adherent cells and planktonic cells was also not significantly different (two sample t-test, p=0.379). Similarly, the activation of the Cpx pathway upon adherence was abolished in a *ΔnlpE ΔompA* double mutant (Fig. 4-7). These data suggest that both NlpE and OmpA play a role in signaling adhesion to the CpxA sensor kinase.

4.3 Discussion

In this study, we sought to study NlpE function through investigating its interacting protein(s) partners in *E. coli*. The pull-down assay we performed indicates that OmpA interacts with NlpE *in vitro*. The interaction is further confirmed by co-immunoprecipitation and cross-linking studies on whole cells. Further characterization highlights the role of NlpE in sensing OmpA abnormalities in the cell, since OmpA overexpression signals the Cpx pathway in an NlpE-dependent manner. This result further reinforces the idea that NlpE serves as an outer membrane sensor of the Cpx pathway. Preliminary adherence assays showed that activation of the Cpx pathway upon surface adhesion requires OmpA, in addition to NlpE. Based on the results in this study, we propose that CpxA utilizes the outer membrane protein NlpE as a sensor of specific envelope stress signals at outer membrane. How these signals are relevant to the mostly inner membrane adaptations controlled by the Cpx response is an intriguing area that remains to be investigated.

4.3.1 NlpE Interacts With Outer Membrane Adhesin OmpA

To investigate the functions and molecular mechanisms of signaling used by NlpE, we set out to identify proteins interacting proteins. We first performed a pull down assay using a C-terminally His-tagged, soluble version of NlpE and cobalt resin *in vitro*. As shown in Fig.4-1, a band corresponding to 30kDa was identified specifically in the presence of purified His-tagged NlpE. Further mass spectrometry revealed that the band is enriched in OmpA, indicating a specific interaction between NlpE and OmpA *in vitro*. However, it is possible that, since our pull down assay used a

soluble NlpE construct that is folded in the cytoplasm and missing its acyl chain anchor to the outer membrane, this interaction was not relevant under physiological conditions. Thus, to verify the interaction of NlpE and OmpA, we turned to the "gold standard" for studying protein interaction *in vivo,* co-immunoprecipitation. Membrane extract of MC4100 was applied to immobilized NlpE antibody to capture NlpE containing protein complexes. The presence of NlpE and OmpA in a co-immunoprecipitated protein complex was visualized by western blotting using NlpE and OmpA antibody. OmpA was detected in co-immunoprecipitated eluates only in the presence of NlpE (Fig.4-2). This shows that NlpE interacts with OmpA *in vivo* in *E. coli*. Further formaldehyde cross-linking experiments provided us with additional confirmation of an NlpE:OmpA complex. In the presence of NlpE, a cross-linked complex that was detected by both NlpE and OmpA antibodies was identified (Fig.4-3). However, the accurate size and composition of the complex requires further investigation using size-exclusion chromatography and mass spectrometry. The molecular weight of NlpE is 27kDa and OmpA is 37kDa. Considering the previous study showing OmpA forms a homodimer of 74kDa in *E. coli* (Marcoux *et al.*, 2014), the complex must involve more than one OmpA dimer and NlpE monomer.

OmpA is the major component of the outer membrane of *E. coli*. It plays diverse roles including maintaining outer membrane integrity and contributing to adhesion and biofilm formation (Gaddy *et al.*, 2009). Due to its important cellular roles, it is also a target of the immune response, colicins, and bacteriophage (Achouak *et al.*, 2001). OmpA structure has been extensively studied. Currently, there are two models of OmpA structure; a larger porin with 14-transmembrane regions and a

smaller eight-stranded outer membrane porin (Sugawara and Nikaido, 1994; Nikaido, 2003) with a globular C-terminus that resides in the periplasm and interacts with peptidoglycan (Ishida *et al.*, 2014; Pautsch and Schulz, 1998; Arora *et al.*, 2001).

In a previous study, another outer membrane lipoprotein, RcsF, was reported to monitor β-barrel protein assembly by interacting with outer membrane porins OmpC, OmpF and OmpA (Konovalova *et al.*, 2014; Cho *et al.*, 2014). RcsF is an outer membrane lipoprotein that functions as an auxiliary regulator of the Rcs phosphorelay (Konovalova *et al.*, 2014). The Rcs phosphorelay is an atypical two-component system with an extra phosphotransfer protein. Cross-linking experiments revealed that the RcsF flexible linker is threaded through the lumen of OmpC and OmpF and interacts with loops mapped to the same region of the lumen of OmpC and OmpF (Konovalova *et al.*, 2014). However, OmpA also adopts a smaller pore that is not large enough to accommodate RcsF, suggesting that RcsF may interact only with the large porin form of OmpA or in a different manner (Konovalova *et al.*, 2014). Interestingly, proper RcsF and OmpA interaction is formed during outer membrane protein assembly and requires a functional Bam complex. An OmpA-RcsF cross-linked complex could be detected when RcsF was present during OmpA refolding *in vitro* in the presence of detergent. In contrast, the OmpA-RcsF cross-linked complex was not detected when RcsF was mixed with folded OmpA (Konovalova *et al.*, 2014). Differently from the RcsF-OmpA interaction, NlpE was found to interact with folded OmpA *in vitro* as revealed by our pull down

assay (Fig.4-1). This indicates that NlpE interacts with OmpA in a different manner from RcsF.

The known roles of NlpE and OmpA, together with their cellular locations and interaction raise a model of surface sensing that is likely to have physiological relevance. Indeed, our preliminary results suggest that this is the case.

4.3.2 OmpA and NlpE Function and Signaling are Connected

Previous studies showed that OmpA activates the Cpx pathway in an EPEC *Δhfq* mutant and during biofilm formation upon hydrophobic surfaces (Ma and Wood, 2009; Vogt and Raivio, 2014). We have expanded that role here. As shown in Fig.4-4, OmpA overexpression activates the Cpx pathway 6-fold. Further, this signaling requires a functional CpxA, as well as NlpE. When NlpE is absent, the Cpx pathway is blind to OmpA overexpression. We propose that NlpE functions as a sensor of OmpA abnormalities at the outer membrane and communicates this to CpxA, thus transferring signals from the outer membrane to the inner membrane. NlpE is not responsible for sensing most Cpx sensed signals since an *nlpE* mutant still responds to most inducing cues such as misfolding of pilus subunits PapE and PapG, cell growth, and alkaline pH (DiGiuseppe and Silhavy, 2003). Here, we report another inducing signal, OmpA overexpression, which requires functional NlpE in *E. coli*. In addition, our data provide evidence in support of a model in which NlpE senses surface adhesion through its interaction with adhesin OmpA.

The role of OmpA and its homologs as adhesins/invasins has been well established in several different species including *E. coli K1*, EHEC, *Rickettsia conorii*, *Acinetobacter baumannii*, *Acinetobacter nosocomialis*, *Coxiella burnetii*, *Cronobacter*

sakazakii and *Yersinia pestis* (Torres and Kaper, 2003; Prasadarao *et al.*, 1996; Hillman *et al.*, 2013; Gaddy *et al.*, 2009; Kim *et al.*, 2010; Martinez *et al.*, 2014; Bartra *et al.*, 2012). Also, OmpA mediates *E. coli* adhesion to abiotic surfaces through direct binding, as revealed by atomic force microscopy (Lower *et al.*, 2005). The unique bond formed between a silicon nitride surface and *E. coli* cells expressing OmpA detected by atomic force microscopy was abolished when cells were treated with proteinase K or lacking OmpA overexpression. Combined with the data presented here, these studies contribute to a model in which NlpE senses surface adhesion through direct interaction with OmpA. Perhaps changes in this interaction trigger signaling and subsequent activation of the Cpx two-component system.

4.4 Tables and Figures

Table 4-1Mass spectrometry results of pull-down assay (48 kDa band)

Accession	Description	Score*	Coverage *	# Unique Peptides*	# Peptides*	# PSMs*
P0A910	Outer membrane protein A OS=*Escherichia coli* (strain K12) GN=*ompA* PE=1 SV=1 - [OMPA_ECOLI]	406.33	69.08	19	19	170
P40710	Lipoprotein NlpE OS=*Escherichia coli* (strain K12) GN=*nlpE* PE=1 SV=1 - [NLPE_ECOLI]	294.37	78.39	16	16	145
C4ZUG9	30S ribosomal protein S3 OS=*Escherichia coli* (strain K12 / MC4100 / BW2952) GN=*rpsC* PE=3 SV=1 - [RS3_ECOBW]	266.61	51.50	10	10	100

*Score: Sequest score
Coverage: Percent coverage of the protein observed
Unique Peptides: Unique peptides identified that only occur in the protein identified
Peptides: All unique peptide plus peptides that may be common between two or more proteins
PSMs: Number of peptide spectral matches

Table 4-2 Mass spectrometry results of pull-down assay (30 kDa band)

Accession	Description	Score *	Coverage *	# Unique Peptides *	# Peptides *	# PSMs *
P40710	Lipoprotein NlpE OS=*Escherichia coli* (strain K12) GN=*nlpE* PE=1 SV=1 - [NLPE_ECOLI]	514.72	74.58	16	16	230
P0A9P0	Dihydrolipoyl dehydrogenase OS=*Escherichia coli* (strain K12) GN=*lpdA* PE=1 SV=2 - [DLDH_ECOLI]	382.57	45.99	21	21	132
P0CE47	Elongation factor Tu 1 OS=*Escherichia coli* (strain K12) GN=*tufA* PE=1 SV=1 - [EFTU1_ECOLI]	125.18	62.69	17	17	49

*Score: Sequest score

Coverage: Percent coverage of the protein observed

Unique Peptides: Unique peptides identified that only occur in the protein identified

Peptides: All unique peptide plus peptides that may be common between two or more proteins

PSMs: Number of peptide spectral matches

Table 4-3 Mass spectrometry results of co-immunoprecipitated proteins

Replicate 1:

Accession	Description	Score*	Coverage*	# Unique Peptides*	# Peptides*	# PSMs*
P0A911	Outer membrane protein A OS=Escherichia coli O157:H7 GN=*ompA* PE=3 SV=1 - [OMPA_ECO57]	186.30	47.11	13	13	67
P06996	Outer membrane protein C OS=Escherichia coli (strain K12) GN=*ompC* PE=1 SV=1 - [OMPC_ECOLI]	123.37	42.23	13	13	67
P07014	Succinate dehydrogenase iron-sulfur subunit OS=Escherichia coli (strain K12) GN=*sdhB* PE=1 SV=1 - [SDHB_ECOLI]	61.23	52.52	10	10	33
P39180	Antigen 43 OS=Escherichia coli (strain K12) GN=*flu* PE=1 SV=3 - [AG43_ECOLI]	49.60	18.96	14	14	22
P0ABJ9	Cytochrome bd-I ubiquinol oxidase subunit 1 OS=Escherichia coli (strain K12) GN=*cydA* PE=1 SV=1 - [CYDA_ECOLI]	27.19	14.75	8	8	9
P02931	Outer membrane protein F OS=Escherichia coli (strain K12) GN=*ompF* PE=1 SV=1 - [OMPF_ECOLI]	27.10	15.75	6	6	12
P0AC49	Fumarate reductase iron-sulfur subunit OS=Escherichia coli O157:H7 GN=*frdB* PE=3 SV=2 - [FRDB_ECO57]	22.31	24.18	5	5	8
P69805	Mannose permease IID component OS=Escherichia coli (strain K12) GN=*manZ* PE=1 SV=2 - [PTND_ECOLI]	17.34	20.85	5	5	9
P40710	Lipoprotein NlpE OS=Escherichia coli (strain K12) GN=*nlpE* PE=1 SV=1 - [NLPE_ECOLI]	16.95	19.92	5	5	6

*Score: Sequest score

Coverage: Percent coverage of the protein observed

Unique Peptides: Unique peptides identified that only occur in the protein identified

Peptides: All unique peptide plus peptides that may be common between two or more proteins

PSMs: Number of peptide spectral matches

Replicate 2:

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs
P0A911	Outer membrane protein A OS=Escherichia coli O157:H7 GN=*ompA* PE=3 SV=1 - [OMPA_ECO57]	186.30	47.11	13	13	67
P06996	Outer membrane protein C OS=Escherichia coli (strain K12) GN=*ompC* PE=1 SV=1 - [OMPC_ECOLI]	123.37	42.23	13	13	67
P07014	Succinate dehydrogenase iron-sulfur subunit OS=Escherichia coli (strain K12) GN=*sdhB* PE=1 SV=1 - [SDHB_ECOLI]	61.23	52.52	10	10	33
P39180	Antigen 43 OS=Escherichia coli (strain K12) GN=*flu* PE=1 SV=3 - [AG43_ECOLI]	49.60	18.96	14	14	22
P0ABJ9	Cytochrome bd-I ubiquinol oxidase subunit 1 OS=Escherichia coli (strain K12) GN=*cydA* PE=1 SV=1 - [CYDA_ECOLI]	27.19	14.75	8	8	9
P02931	Outer membrane protein F OS=Escherichia coli (strain K12) GN=*ompF* PE=1 SV=1 - [OMPF_ECOLI]	27.10	15.75	6	6	12
P0AC49	Fumarate reductase iron-sulfur subunit OS=Escherichia coli O157:H7 GN=*frdB* PE=3 SV=2 - [FRDB_ECO57]	22.31	24.18	5	5	8
P69805	Mannose permease IID component OS=Escherichia coli (strain K12) GN=*manZ* PE=1 SV=2 - [PTND_ECOLI]	17.34	20.85	5	5	9
P40710	Lipoprotein NlpE OS=Escherichia coli (strain K12) GN=*nlpE* PE=1 SV=1 - [NLPE_ECOLI]	16.95	19.92	5	5	6
P0A908	MltA-interacting protein OS=Escherichia coli (strain K12) GN=*mipA* PE=1 SV=1 - [MIPA_ECOLI]	15.65	16.53	3	3	4

*Score: Sequest score
Coverage: Percent coverage of the protein observed
Unique Peptides: Unique peptides identified that only occur in the protein identified
Peptides: All unique peptide plus peptides that may be common between two or more proteins
PSMs: Number of peptide spectral matches

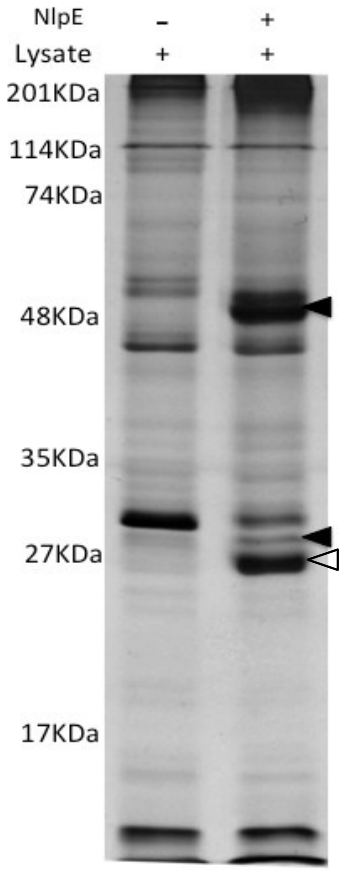

Figure 4-1 *In vitro* pull-down assay using His-tagged soluble NlpE

Pull-down assays were carried out using purified, His-tagged NlpE and cobalt resin. Cell lysates were prepared from *E. coli* MC4100 and mixed with recombinant His-tagged NlpE immobilized on cobalt resin (Lane 2) or plain cobalt resin as a control (Lane 1). Eluted, pulled-down proteins were subjected to SDS-PAGE analysis followed by silver staining. Positions of molecular mass standards are indicated on the left. Protein pulled down by His-tagged NlpE is indicated by closed arrowheads. Purified His-tagged NlpE is indicated by the open arrowhead.

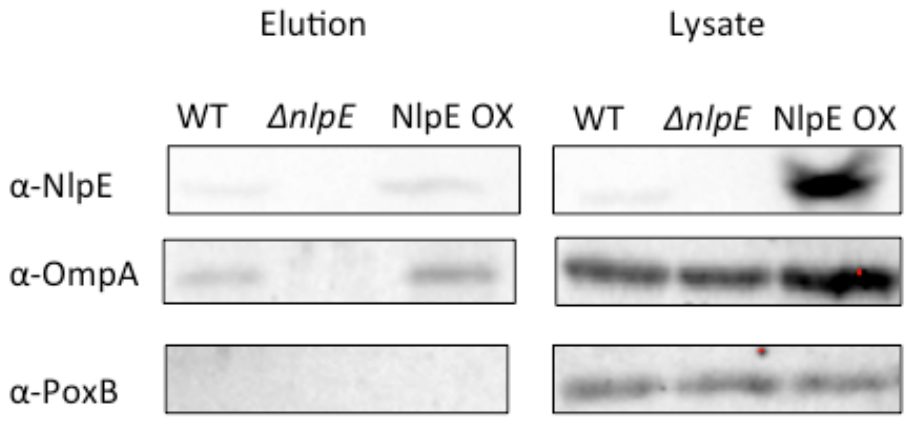

Figure 4-2 Co-immunoprecipitation of NlpE and OmpA in *E. coli*

Strains shown are wildtype *E. coli* MC4100 (WT), MC4100 *ΔnlpE* mutant (*ΔnlpE*) and MC4100 overexpressing NlpE from the pCA-NlpE plasmid (NlpE OX). Membrane extracts of each strain tested were mixed with NlpE-antibody cosslinked to protein A agarose resin, eluates were analyzed by SDS-PAGE followed by western blotting with anti-NlpE, anti-OmpA and anti-PoxB antibodies (left side, elution). Membrane extracts used for co-immunoprecipitation are shown on the right (lysate).

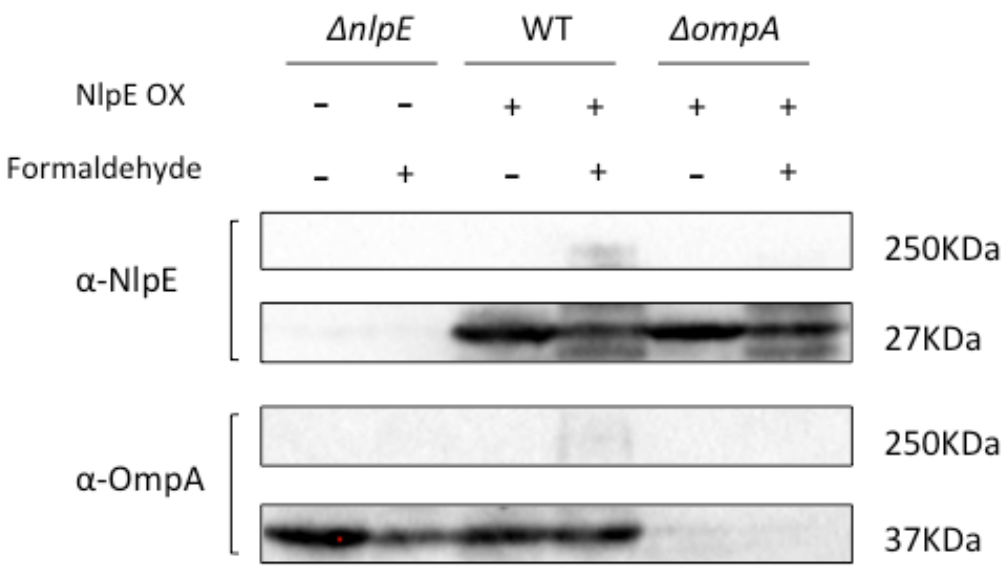

Figure 4-3 *In vivo* cross-linking reveals an NlpE-OmpA complex in *E. coli*

E. coli MC4100 wild-type and *ΔompA* strains overexpressing NlpE from pCA-NlpE, along with MC4100 *ΔnlpE* mutant were harvested at mid-log phase, re-suspended in PBS buffer and treated with or without formaldehyde for 15min. Then membrane extracts of each sample were collected and resolved on 12% SDS-polyacrylamide gel followed by western blotting. NlpE and OmpA were visualized with anti-NlpE and anti-OmpA antibodies respectively. The presence or absence of NlpE overexpression and formaldehyde treatment is indicated above the figure. The strains shown are wildtype (WT), *ΔompA* mutant (*ΔompA*) *and ΔnlpE* mutant (*ΔnlpE*).

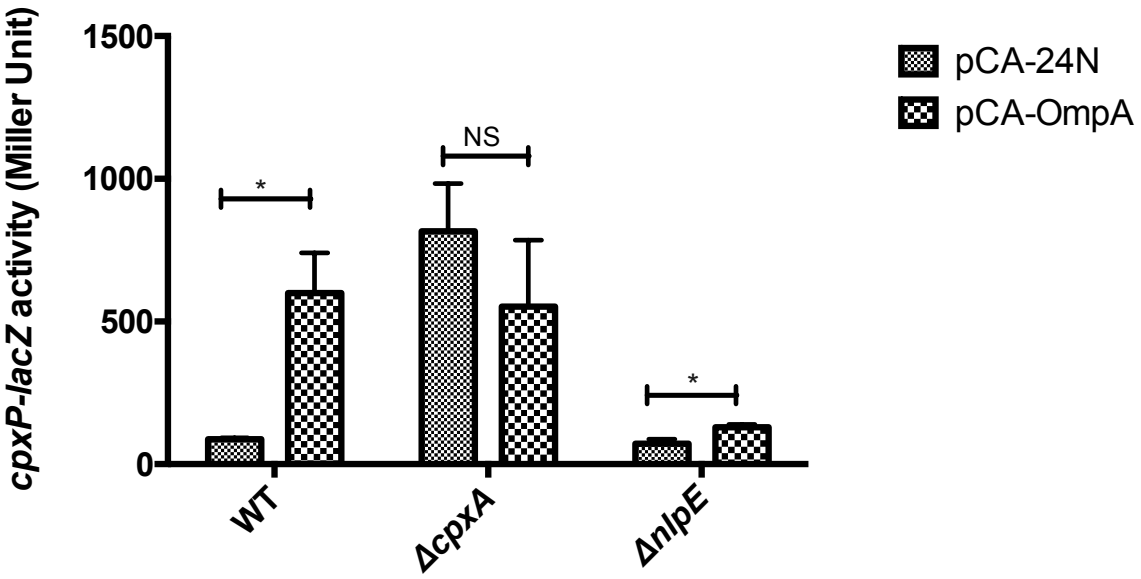

Figure 4-4 NlpE is required for sensing OmpA overexpression

β-galactosidase activity was measured in strains TR50, TR50*ΔcpxA*, and TR50*ΔnlpE* upon overexpression of OmpA. TR50 (WT), TR50*ΔcpxA* (*ΔcpxA*) and TR50*ΔnlpE* (*ΔnlpE*) transformed with pCA-24N or pCA-OmpA were sub-cultured into fresh LB with chloramphenicol and cultured at 37°C or 30°C (TR50 *ΔcpxA* strains). IPTG was added 1h after subculture to a final concentration of 1mM to induce OmpA overexpression. Cells were collected when the OD_{600} reached 0.6-0.8. Cells were lysed using chloroform and 1% SDS and β -galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 Plate reader after addition of ONPG. Experiments were performed in triplicate and done three times each; the mean and standard deviation are shown. NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

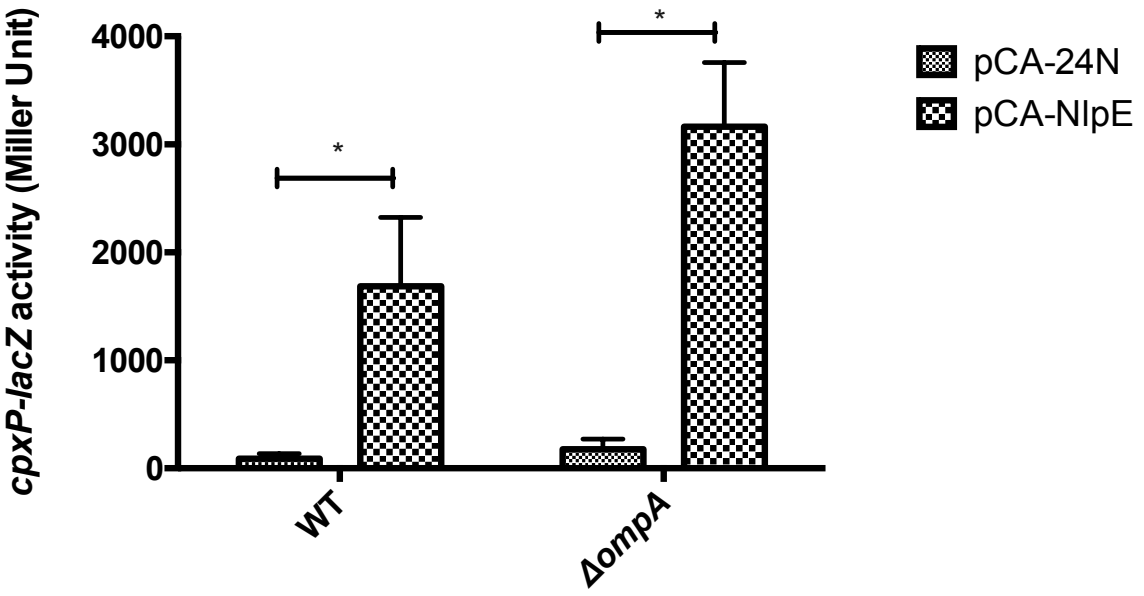

Figure 4-5 OmpA is not required for sensing NlpE overexpression

β-galactosidase activity was measured in strains TR50 and TR50*ΔompA* upon overexpression of NlpE. TR50 (WT) and TR50*ΔompA* (*ΔompA*) transformed with pCA-24N or pCA-NlpE were sub-cultured into fresh LB with chloramphenicol and cultured at 37°C. IPTG was added 1h after subculture to a final concentration of 0.1 mM to induce NlpE overexpression. Cells were collected when the OD_{600} reached 0.6-0.8. Cells were lysed using chloroform and 1% SDS and β -galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 Plate reader after addition of ONPG. Experiments were performed in triplicate and done three times each; the mean and standard deviation are shown. * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

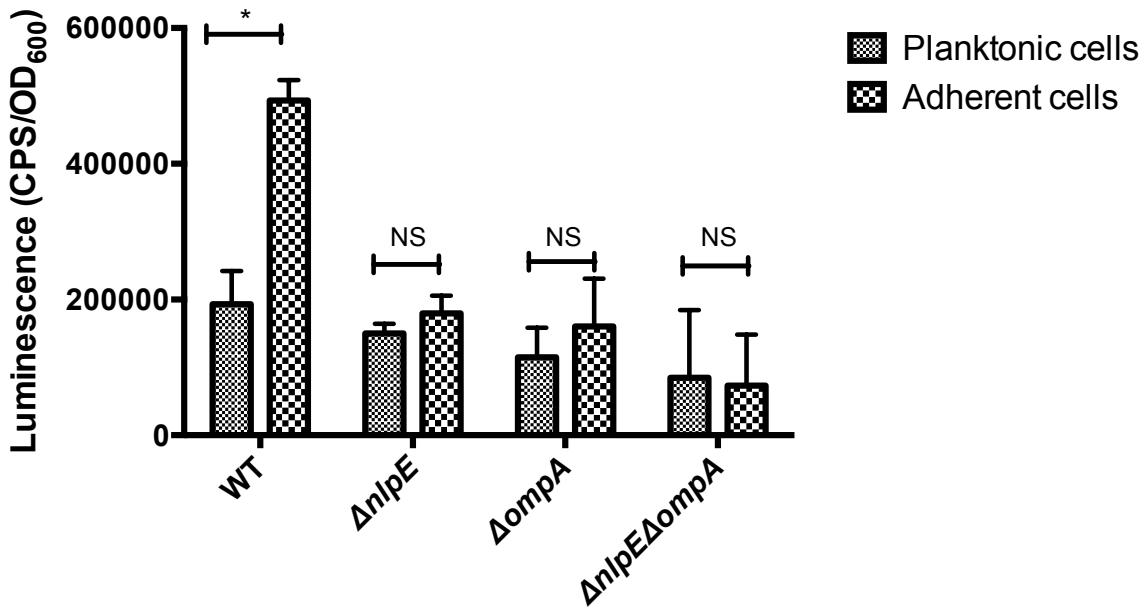

Figure 4-6 OmpA and NlpE are required for sensing surface adhesion

Adhesion assay results of MC4100 wildtype (WT), Δ*nlpE* mutant (*ΔnlpE*), *ΔompA* mutant (*ΔompA*) and *ΔnlpEΔompA* double mutant (*ΔnlpEΔompA*). Acid-washed glass beads were used to increase surface area that *E.coli* cells adhere to. The glass beads were treated with trichloroethylene (TCE) and Dimethyldichlorosilane (DDS) to make them hydrophobic. Overnight culture of each strin was added to hydrophobic glass beads and was cultured for 6h without shaking to allow adhesion. Planktonic cells were harvested from LB medium. Glass beads were washed by fresh LB medium before adhesion cells were collected by vortexing. The activity of the Cpx pathway in planktonic cells and adhesion cells were analyzed by measuring luminescent of a *cpxP-lux* reporter. Experiments were performed in triplicate at least three times. NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

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5 Concluding Remarks

In the laboratory, most studies in bacteria are performed in well-controlled conditions using planktonic cultures. However, bacteria predominantly exist as adherent cells to biotic and abiotic surfaces in the environment (Costerton *et al.*, 1987). Moreover, surface adhesion of bacteria often leads to the development of more complicated, structured communities called biofilms (Costerton *et al.*, 1987). Biofilms can cause huge problems when developed within indwelling medical devices not only because of the protection of the extracellular matrix but also due to the differing bacterial behaviors of adhered, as compared to planktonic cells, including altered growth characteristics and resistance to antimicrobial treatments (Hathroubi *et al.*, 2016; Stewart, 2002; Costerton *et al.*, 1999). For pathogens, establishment of initial contact is critical for colonization and infection in the host. Adherent cells experience structural and physiological changes compared to planktonic cells. Upon surface adhesion, bacteria exhibit a number of adaptations to characteristics including outer membrane protein composition (Otto *et al.*, 2001), decreased respiration (Geng *et al.*, 2014), activation of iron starvation responses (Zhang and Normark, 1996), pathogenesis (Kansal *et al.*, 2013) and signal transduction (Moorthy *et al.*, 2016). Therefore, it is of importance to study how microbes detect surfaces, in order to better understand the problematic characteristics of biofilms, as well as to manipulate them. Compared to the actual adhesins involved, less is known about sensing surface adhesion.

Based on the results of this thesis research, we are proposing a surface-sensing mechanism for the outer membrane lipoprotein NlpE that invokes direct interaction with surface adhesin OmpA. How does NlpE sense surface adhesion through its

interaction with OmpA? Two models have been proposed for OmpA structure. The first model is a two-domain structure with an amino-terminal membrane spanning domain and a soluble periplasmic carboxyl-terminus that interacts with peptidoglycan (Ishida *et al.*, 2014; Pautsch and Schulz, 1998; Arora *et al.*, 2001). The second one is a large porin with 14-transmembrane domains (Sugawara and Nikaido, 1994; Nikaido, 2003). Both conformations of OmpA can be purified from the outer membrane with about 2-3% of OmpA forming a larger pore structure (Ishida *et al.*, 2014). In addition, the structural transition from small to large pore was observed when the temperature reached 24-39°C (Zakharian and Reusch, 2003). However, the first model is more supported by the fact that amino-terminal of OmpA (residues 1-171) can still take out most of OmpA function in cells (Smith *et al.*, 2007) and the carboxyl-terminus is conserved in many OmpA-like proteins (Park *et al.*, 2012; Teixeira *et al.*, 2015; Oliveira *et al.*, 2011). The smaller pore model proposes that the amino-terminal β-barrel domain is composed of eight membrane-spanning β-strands connected by four long extracellular loops and three short periplasmic turns. The four extracellular loops are thought to mediate adhesion to host cells. Further, differences in the sequence of the extracellular loops raise the possibility of OmpA binding to different substrates (Smith *et al.*, 2007; Prasadarao *et al.*, 1996). This binding could subsequently cause conformational changes in OmpA structure in response to mechanical stimuli and lead to an alteration in the interaction of OmpA with NlpE. Therefore, by sensing conformational changes of adhesin OmpA, NlpE could sense mechanical stimulation during surface adhesion.

An OmpA:NlpE complex may additionally act to exclude NlpE from activating the Cpx pathway inappropriately in the absence of an adhesion signal.

NlpE is an outer membrane lipoprotein with an amino-terminal lipocalin-resembling β-barrel domain, a carboxyl-terminal β-barrel domain that shares similarity to an oligonucleotide/oligosaccharide binding fold (OB binding motif) and a flexible linker in between (Hirano *et al.*, 2007)(Fig.3-1). It is probable that the lipid modification is essential to activate the Cpx pathway upon adherence, since a periplasmic version of NlpE fails to induce the Cpx response upon overexpression, and we show here that all *nlpE* mutations that prevent activation of Cpx upon overexpression also negate surface sensing (Fig.3-1, 3-2, 3-3, 3-6). One important feature of the targeting conferred by lipid modification and outer membrane targetting of NlpE may be to facilitate its interaction with OmpA. Flexible linkers and OB binding motifs in other proteins have been implicated in protein interactions (Murzin, 1993), and it seems possible that these domains in OmpA may similarly contribute to the interaction of NlpE and OmpA. Our mutational analysis supports this model, since both regions are critical for signaling the Cpx pathway upon surface adhesion (Fig.3-6). Additional mechanistic insights could be uncovered by a more detailed characterization of how NlpE and OmpA interact, such as has been done with RcsF and OmpA (Konovalova *et al.*, 2014). One simple follow-up experiment that could be done is to determine whether OmpA overexpression leads to Cpx response induction in the set of *nlpE* mutant strains constructed here (Chapter 3). We demonstrated that OmpA overexpression only activates the Cpx pathway when NlpE is present (Fig.4-4), and so the impact of OmpA overexpression

in the various mutants could reveal information about which parts of NlpE are necessary for a functional interaction with OmpA.

Bacterial cells employ multiple adhesins at different stages during adherence. What physiological impact might the sensing of surface adhesion through an NlpE:OmpA complex have on the cell? The outer membrane protein OmpA is one of the most abundant proteins in *E. coli* with a copy number of 100,000 copies/cell (Koebnik *et al.*, 2000). In contrast, NlpE is present at much lower abundance; therefore, its interaction with OmpA to facilitate surface sensing need not be involved in, or affect, other demonstrated functions of OmpA. In addition, OmpA mediated surface adhesion has been shown in many models, including EPEC and EHEC (Torres *et al.*, 2005; McWilliams and Torres, 2014), and mediates both specific receptor-mediated adhesion and nonspecific binding to abiotic surfaces (Lower *et al.*, 2005). By sensing OmpA activity/conformational states, NlpE could be generally responsive to all adhesion mediated by OmpA, regardless of the attributes of the surface. The involvement of OmpA in the initial attachment of cells to surfaces indicates that the Cpx pathway may play a role in mediating cellular adaptation to to early stages of an altered microbial lifestyle that occurs in attached communities.

How does NlpE convey the surface attachment signal to CpxA? NlpE is anchored to the outer membrane through its amino-terminal lipid modification, while the sensing domain of CpxA is localized to the periplasmic side of the inner membrane. NlpE L43 is located in the β2 strand, while Y90 is located in the β6 strand of the lipocalin-resembling amino-terminus; both are facing toward the interior of the β–barrel (Fig. 3-3B). A reasonable hypothesis is therefore that L43 and Y90 could be

involved in ligand binding, alterations of which may contribute to signaling by altering the orientation of the amino- and carboxyl-terminus upon surface adhesion. Alternatively, perhaps these mutations define a ligand binding site that allows signaling to the Cpx response on exposure to an as yet undefined activating signal. The flexible linker and the demonstration in this work that it plays an essential role in signal transduction would support such a model (Fig.3-2, 3-6). The flexible linker of NlpE, when stretched, is predicted to be long enough to span the periplasm and bring the carboxyl-terminus of NlpE in close proximity to the inner membrane (Hirano *et al.*, 2007). It is thus physically possible for NlpE to interact with inner membrane proteins. The carboxyl-terminal β-barrel domain shares structural similarity to the OB binding motif that mediates protein-protein interaction and ligand binding (Murzin, 1993). Accordingly, we consider that one possibility is that NlpE signals CpxA through direct interaction with the C-terminal OB fold upon adhesion. One caveat to this is that CpxA was not identified in the pull-down assays performed in this study. The His-tagged NlpE construct used in our *in vitro* pull-down assay consisted of the soluble domains of NlpE missing the N-terminal lipid modification and secretion sequences, and ultimately folded in the cytoplasm instead of the periplasm. This could hinder proper folding and/or association with important cofactors that might only happen after secretion across the inner membrane, possibly impacting proper protein:protein interactions. In the future, the possible interaction of NlpE and CpxA could be investigated using an *in vivo* co-immunoprecipitation assay where NlpE anti-sera is incubated with various cell fractions to pull down NlpE and the interacting proteins, which could be identified

through SDS-PAGE and MS analysis. It is also possible that NlpE causes some physiological defect in the inner membrane upon adhesion that is sensed by the Cpx pathway. This idea is supported by the finding that overexpression of NlpE decreases cell survival in *E. coli*, especially in a *cpxR* mutant (unpublished observation). Alternatively, NlpE may communicate surface adhesion to CpxA via another envelope protein(s) or protein complex that is anchored in the inner membrane and subsequently interacts with CpxA. In light of the results presented in Appendix B, we view this is a real possibility (see Appendix B).

This thesis has focused on defining mechanism for how NlpE may act as a sensor of surface adhesion at the outer membrane. Another equally important question is why there is a need for Cpx response activation under these conditions. Does activation of the Cpx response contribute to adaptation upon surface adhesion? What is the physiological relevance of activation of the Cpx pathway upon surface adhesion? Activation of the Cpx response leads to changes in the regulation of a vast regulon, mostly consisting of genes encoding inner membrane proteins in *E. coli* (Raivio *et al.*, 2013; Price and Raivio, 2009). Perhaps Cpx induction contributes to the changes in membrane protein profile that are known to occur in adherent cells compared to planktonic cells (Otto *et al.*, 2001). First, the activation of the Cpx pathway could up-regulate proteins that are critical for surface adhesion. It was shown that treatment with tetracycline that inhibits new protein synthesis abolished the activation of the Cpx pathway upon surface adhesion and impacted adherence similarly to *cpxR* and *nlpE* mutants (Otto and Silhavy, 2002). This observation indicates that synthesis of an unknown, Cpx-regulated protein(s) is

required to establish adhesion efficiently. Such proteins could directly contribute to initial cellular binding properties or mediate later adaptive responses that stabilize the contact of the attached cell with the surface. The activation of the Cpx pathway also contributes to protein folding and degradation in the envelope by up-regulation of periplasmic chaperones and proteases, including SurA, PpiA, DsbA, DegP, Spy and Skp (Pogliano *et al.*, 1997; Quan *et al.*, 2011). The activation of the Cpx pathway could therefore also combat potential protein damage caused by surface adhesion to make sure the envelope functions properly. A further role of the Cpx response upon adherence could be to regulate cellular energization and metabolism, since it is known to directly control the expression of membrane bound complexes including succinate dehydrogenase, NADH dehydrogenase and cytochrome *bo* oxidase (Mahoney and Silhavy, 2013; Raivio *et al.*, 2013). Intriguingly, it has been shown that adherence leads to a decrease in respiration and metabolism, and it will be interesting to determine if this adaptation is dependent on the Cpx response, and if so, why it might be necessary (Geng *et al.*, 2014). Finally, the Cpx response has also been implicated in regulating pathogenesis in a wide variety of organisms (see Vogt and Raivio, 2012). For example, activation of the Cpx pathway decreases expression of the EPEC type III secretion system, encoded by the locus of enterocyte effacement (LEE) pathogenicity island, at post-transcriptional and transcriptional levels (MacRitchie *et al.*, 2012). The Cpx response further both facilitates assembly of the EPEC type IV BFP required for initial adherence to the intestinal epithelium, and inhibits transcription the *bfp* operon encoding the structural and assembly genes (Vogt *et al*. 2010). Thus, another role of NlpE-mediated activation of the Cpx

response upon adherence could be to limit the expression of virulence determinants in pathogens to the correct time and place during infection.

Altogether, the work presented in this thesis has resulted in a detailed model for the molecular mechanism by which the outer membrane lipoprotein NlpE, in conjunction with at least one other protein, OmpA, senses adherence and communicates this event to the Cpx signal transduction apparatus located in the cytoplasmic membrane. It provides us with a wealth of new hypotheses to test and the beginnings of the basic understanding that will be required to manipulate cell behavior and develop potential treatments to control biofilm formation.

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Appendix A A Genetic Screen for Envelope Inhibitors of the Cpx Pathway in *Escherichia coli*[1]

[1] This work was completed in collaboration with Ph.D Julia Wong; Randi Guest constructed strain RG140 (EPEC E2348/69 *ΔcyoABCDE*)

A1. Introduction

Two-component systems are the most common strategy that bacteria employ to sense environmental stresses (Stock *et al.*, 2000). In gram-negative bacteria, sensor kinases sense extracellular signals and phosphorylate a cognate response regulator, which subsequently mediates a cellular response (Stock *et al.*, 2000). Two-component systems may additionally be controlled by auxiliary regulators other than the sensor kinase and the response regulator (Buelow and Raivio, 2010). Auxiliary regulators endow two-component systems connections to other cellular regulatory pathways and the ability to sense an expanded repertoire of stimuli by regulating both the sensor kinase and response regulator in different cellular compartments ((Buelow and Raivio, 2010; Cho *et al.*, 2014). The identification and study of auxiliary regulators often provides additional insights into the role and physiological relevance of a given two-component system.

The Cpx envelope stress response is a two-component system consisting of the sensor kinase CpxA and the response regulator CpxR (Dong *et al.*, 1993; Vogt and Raivio, 2012). In *E. coli* the Cpx pathway has been shown to sense a variety of signals including alkaline pH, inner membrane lipid composition changes, NlpE overexpression and surface adhesion through NlpE, and the presence of misfolded pilus subunits (including PapE and PapG)(Mileykovskaya and Dowhan, 1997; Danese *et al.*, 1998; Lee *et al.*, 2004; Danese and Silhavy, 1998). The Cpx pathway is inhibited when envelope stresses are absent. In its "off" state, CpxA functions as a phosphatase that dephosphorylates CpxR and keeps it inactive. In the presence of inducing cues, CpxA autophosphorylates itself at the conserved site of

autophosphorylation, H248, and subsequently phosphorylates CpxR. Phosphorylated CpxR is active and regulates the transcription of its downstream targets (Raivio and Silhavy, 1997). The auxiliary regulator CpxP is a periplasmic chaperone that also inhibits activity of the Cpx pathway through direct binding to the sensor kinase CpxA (Raivio *et al.*, 1999b; Tschauner *et al.*, 2014; Zhou *et al.*, 2011). When overexpressed, CpxP greatly decreases the activity of the Cpx pathway, even under Cpx-activated conditions (Raivio *et al.*, 1999b). CpxP is additionally one of the strongest regulated targets of the Cpx pathway when it is active. This likely reflects its requirement to mediate cellular stresses, probably through its chaperone activity and modulation of Cpx pathway activity (Danese and Silhavy, 1998). However, the activity of the Cpx pathway is elevated only slightly and can be further activated by other inducing cues when *cpxP* is mutated (Raivio *et al.*, 1999a). Therefore, we hypothesized that there are other mechanisms that inhibit the Cpx pathway in addition to CpxP. In this work, we sought out new feedback inhibitors of the Cpx response by screening a subset of *E. coli* envelope proteins that are CpxR regulated. Specifically, we set out to identify inhibitors that decreased Cpx pathway activity in a CpxA-dependent manner, in order to elucidate molecular mechanisms that keep the Cpx pathway off.

We reasoned that such inhibitors might function similarly to CpxP in that they could be regulated by the Cpx pathway and envelope localized. We used a microarray expression profiling study that characterized the Cpx regulon to identify potential candidates (Raivio *et al.*, 2013). About 176 envelope-localized proteins are either up- or down-regulated when the Cpx pathway is activated by NlpE overexpression

in both EPEC and MC4100. Each of these genes encoding these candidate proteins was tested for its effect on Cpx pathway activity when overexpressed. To do this, we looked for variation in the color of colonies on a lactose-MacConkey indicator plate of a strain carrying a chromosomal copy of the Cpx-regulated *cpxP-lacZ* reporter gene upon transformation with plasmids overexpressing each of the candidate genes. Out of 176 candidates tested, two novel multi-copy inhibitors of the Cpx pathway were identified. NuoF, a subunit of NADH dehydrogenase I, and EfeB, a heme peroxidase of the EfeU ferrous iron transporter (Leif *et al.*, 1995; Cao *et al.*, 2007). Further characterization of NuoF and EfeB was described in Julia Wong's PhD thesis.

In addition, five envelope proteins-CyoA, CyoC, FkpA, YidQ and EmrA-were shown to activate the Cpx pathway in a CpxA-dependent manner when overexpressed. CyoA and CyoC encode subunits II and III of cytochrome *bo* oxidase, respectively (Nakamura *et al.*, 1997). FkpA encodes a heat shock periplasmic prolyl isomerase (Arié *et al.*, 2001). YidQ encodes a σ^E-regulated outer membrane protein with unknown function (Dartigalongue *et al.*, 2001). EmrA encodes the membrane fusion protein of the EmrAB-TolC efflux pump (Lomovskaya and Lewis, 1992). Though the molecular mechanisms of these newly identified, multi-copy effectors of the Cpx pathway awaits further investigation, recognition of their roles in signaling the Cpx pathway provides us with a link to different physiological aspects of the envelope.

A2. Materials and Methods

A.2.1 Bacterial Strains and Growth Condition

All strains and plasmids used in this study are listed in Table A-1. Bacteria were cultured in LB broth at 37°C with shaking at 225 rpm. MC4100 *ΔcpxA* and *ΔcpxR* strains were cultured at 30°C with shaking at 225 rpm. IPTG was added to a final concentration of 0.1 mM when protein overexpression was needed. Chloramphenicol was added to a final concentration of 25 ug/mL. Kanamycin was added to a final concentration of 50 ug/mL. MacConkey agar (BD) was made following the manufacturer's instructions with supplementation of 25 ug/mL chloramphenicol and 0.1 mM IPTG.

A.2.2 Strain Construction

The EPEC E2348/69 *Δ cyoABCDE* mutant was constructed by allelic exchange as previously described (Edwards *et al.*, 1998) (by PhD candidate Randi Guest). Transformation was carried out following standard molecular techniques (Sambrook and Russell, 2001).

A.2.3 Genetic Screen for Novel Inhibitors of the Cpx Pathway

To initiate the screen for novel inhibitors of the Cpx pathway, candidates were first identified based on a previously published microarray expression prifoling study characterizing the Cpx regulon upon NlpE overexpression in *E. coli* (Raivio *et al.*, 2013). Genes whose expression was regulated at least 2-fold were cross-referenced to the Ecocyc database to determine their cellular localization (Keseler *et al.*, 2011). In total, 176 CpxR-regulated genes that are known or predicted to localize in the

envelope were identified and screened. The methodology of the genetic screen was designed as previously described (Wong *et al.*, 2013). For each candidate, the overexpression plasmid from the ASKA library (Kitagawa *et al.*, 2005) was extracted and transformed into TR50, a derivative of *E. coli* strain MC4100 carrying a chromosomal *cpxP-lacZ* reporter. Four single colonies of the resulting transformants were screened for changed Cpx activity based on color variation on lactose MacConkey plates supplemented with 0.1 mM IPTG to induce expression from the plasmid. Bright red colonies indicated high levels of *lacZ* transcription and pink or white colonies indicated low levels of *lacZ* expression. TR50(pCA-24N) was used as the vector control. TR50(pCA-YlbF) was used as a positive control. Overexpression of YlbF-cytoplasmic oxamate carbamoyltransferase- was previously revealed to strongly inhibit Cpx activity on MacConkey plates (unpublished observation). The CpxA and CpxR dependency of changed Cpx pathway activity upon overexpression of each candidate gene was tested in MC4100 strains carrying *ΔcpxA* or *ΔcpxR* mutations in addition to the *cpxP-lacZ* reporter gene. The inhibitory phenotype of candidates that showed changed Cpx activity was further confirmed by β-galactosidase assays to quantify Cpx pathway activity.

A.2.3 β-galactosidase Assay

β-galactosidase activity was measured in microtiter plates as previously described (Silhavy *et al.*, 1984). Briefly, single colonies of each strain to be tested were inoculated into 2 mL LB broth supplemented with appropriate antibiotics overnight, shaking at 225 rpm at 37°C and then sub-cultured to 2 mL of fresh LB broth under the same conditions the following day. For induction of protein overexpression from

the ASKA library IPTG-inducible promoter, IPTG was added to a final concentration of 0.1 mM 1h after subculture. Cells were collected when the OD_{600} reached 0.6-0.8 and re-suspended in freshly prepared buffer Z (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM$MgSO_4{\cdot}7H_2O$; containing 270 µL β-mercaptoethanol). Then, 250 µL of cell mixture were transferred to a 96-well polystyrene plate. The OD_{600} was read in a 96-well using a Perkin Elmer Wallac Victor^2 1420 plate reader. The remaining cells were lysed using chloroform and 0.1% SDS for 10min and the cellular debris were removed by centrifugation. The lysed cell mixture was added to 1X Z-buffer in 96-well plates (5 µL lysed cell mixture, 195 µL 1X Z-buffer for *cpxP-lacZ* reporter), and 50 µL 10mg/mL ONPG (o-nitrophenyl β–D-galactopyranoside)(Sigma) was added to initiate the reaction. The A_{420} was read 20 times over approximately 30 minutes in the plate reader and Miller Units were calculated (Zhang and Bremer, 1995). Experiments were done in triplicate, three times.

A.2.5 Luminescence Assay

The activity of the Cpx pathway in EPEC E2348/69 strains was assessed by measuring the luminescence produced from Cpx-dependent luminescent reporter genes. The luminescence assay was performed as previously described (MacRitchie *et al.*, 2008). Briefly, strains were cultured overnight in LB at 37°C with aeration at 225 rpm, then sub-cultured 1:100 into fresh LB and grown at 37°C with aeration for 1 h. IPTG was added to a final concentration of 0.1 mM. Then 200 µL of culture was transferred to a black, clear-bottom 96-well plate (Corning). The plate was cultured at 37°C for 8 h. After every hour, absorbance at 600 nm and luminescence (counts

per second, CPS) were measured using a Perkin Elmer Wallac 420 multilabel plate reader. The final bioluminescence (CPS/OD_{600}) activity was calculated by optimizing the corrected CPS (CPS sample-CPS blank control) by the corrected OD_{600} (OD_{600} sample-OD_{600} blank control). Assays were performed at least three times in triplicate.

A3. Results

A3.1 Novel Multi-copy Inhibitors of the Cpx Two-component System Identified

To initiate our screen for novel inhibitors of the Cpx two-component system, we first compiled a list of candidates based on two criteria: a) each gene was known to be Cpx-regulated, and b) each gene encoded an envelope localized protein. Previously, our lab performed a microarray expression profiling study to investigate the Cpx regulon upon NlpE overexpression (Raivio *et al.*, 2013). Genes that were either up- or down-regulated by greater than 2-fold as revealed by the microarray study were identified. Among these, 176 envelope-localized proteins were screened. First, the overexpression plasmid of each candidate gene from the ASKA library was purified and transformed into TR50, a MC4100 strain carrying a chromosomal *cpxP-lacZ* reporter gene. The resulting transformants were patched onto MacConkey plates supplemented with IPTG and tested for color variation relative to the transformants carrying the pCA24-N vector control. For the initial screen, in total 25 candidates were shown to consistently decrease the activity of the Cpx pathway. To make sure that observed color variation upon overexpression of each candidate reflected signaling through the Cpx pathway; the dependency of this phenotype on

CpxA was tested. To do this, the activity of the Cpx-dependent reporter gene *cpxP-lacZ* was determined upon overexpression of each candidate in wild type and *ΔcpxA* strains, as quantified by β-galactosidase assay. Two proteins, NuoF and EfeB, were shown to inhibit the Cpx pathway in a CpxA dependent manner (Fig A-1). In wild type TR50, overexpression of NuoF resulted in a 24-fold decrease of Cpx pathway activity and overexpresson of EfeB led to a 30-fold decrease. Both proteins strongly inhibited the Cpx pathway upon overexpression. When *cpxA* was mutated, however, inhibition of the Cpx response upon overexpression of NuoF and EfeB was almost completely negated, resulting in only a 1.2-fold decrease. These results indicate that the inhibition of the Cpx response upon NuoF and EfeB overexpression requires the sensor kinase CpxA.

A3.2 Novel Multi-copy Activators of the Cpx Two-component System Identified

During the screen for novel inhibitors of the Cpx pathway, we observed darker red colonies on MacConkey plates indicating increased Cpx pathway activity upon overexpression of candidate genes. In total, 16 out of 176 proteins screened showed increased Cpx pathway activity. To identify potential activators that specifically activate the Cpx pathway through the sensor kinase, the CpxA dependency for activation of the Cpx pathway was tested. We identified five multi-copy activators of the Cpx pathway. As shown in Fig.A-2, in wild type strains, CyoC, CyoA, FkpA, YidQ and EmrA overexpression activated the Cpx pathway approximately 4-fold, 8-fold, 3.5-fold, 4-fold and 4-fold, respectively. When *cpxA* was mutated, activation of the Cpx pathway upon by each of these genes was diminished to less than 2-fold (Fig. A-2). These data indicate that the envelope localized proteins CyoC, CyoA, FkpA, YidQ

and EmrA all function as multi-copy activators of the Cpx pathway in a CpxA-dependent manner.

A3.3 Activation of the Cpx Two-component System Upon CyoA Overexpression is Not Dependent on a Functional Cytochrome *bo* Complex

CyoA and CyoC encode subunits II and III of the cytochrome *bo* oxidase complex encoded by *cyoABCDE* in *E. coli* (Nakamura *et al.*, 1997). Other components of the cytochrome *bo* oxidase, CyoB, CyoD and CyoE have no effect on Cpx pathway activity when overexpressed (data not shown). In order to test whether or not activation of the Cpx pathway upon CyoA and CyoC overexpression required a functional cytochrome *bo* complex, activity of the Cpx pathway in EPEC E2348/69 and an EPEC E2348/69 *ΔcyoABCDE* mutant upon CyoA and CyoC overexpression was examined. EPEC E2348/69 and E2348/69 *ΔcyoABCDE* mutant transformed with the *cpxP-lux* reporter construct on plasmid pJW25 and with either pCA-24N, pCA-CyoC or pCA-CyoA. Activity of the Cpx pathway was examined by measuring luminescence produced from the *cpxP-luxCDABE* reporter cloned into plasmid pJW25. (Fig.A-3) In wild type EPEC, overexpression of CyoC did not activate the Cpx pathway., while overexpression of CyoA showed about 4-fold induction of the Cpx pathway. When *cyoABCDE* was mutated, overexpression of CyoA showed about 2.5-fold induction of the Cpx pathway. This result suggests that activation of the Cpx pathway upon CyoA overexpression doesn't require a functional cytochrome *bo* complex.

A4. Discussion

To fully understand how the Cpx pathway is maintained inactive in the absence of envelope stress we performed a screen for novel envelope-localized inhibitors of the Cpx two-component system in *E. coli*. In total, two novel multi-copy inhibitors of the Cpx response were identified, NuoF and EfeB. NuoF is the soluble cytoplasmic subunit of the NADH dehydrogenase I complex (Leif *et al.*, 1995). Microarray analysis indicates that NuoF is strongly depressed by the Cpx pathway when it is activated (Raivio *et al.*, 2013). The NADH dehydrogenase I complex catalyzes electron flow from NADH to the quinone pool and generates electrochemical gradient in the process. The NuoF is the very subunit that binds to NADH and NADPH in the cytoplasm (Friedrich, 1998). Overexpression of NuoF could lead to abnormal binding to NADH and therefore change the NADH/NAD^+ ratio in the cell, which subsequently triggers metabolite signals that Cpx pathway senses. EfeB is a heme-containing peroxidase of the EfeUOB ferrous iron transporter, however, the EfeUOB is cryptic in *E. coli* K-12 strain due to a frame-shift mutation (Cao *et al.,* 2007). Thus, the inhibition of the Cpx response by EfeB does not require functional ferrous iron transporter. During proper ferrous iron transport, EfeB reduces the ferric iron to ferrous iron (Cao *et al.*, 2007). Without functional EfeUOB ferrous iron transporter, excess EfeB could abnormally bind to its substrate and sequester iron in the cell. Further characterization of the inhibiting effect of NuoF and EfeB was described in more detail in PhD. Julia Wong's thesis.

In the course of screening for inhibitors, five novel multi-copy activators of the Cpx two-component system were also identified. CyoA and CyoC encode for subunits II

and III of the cytochrome *bo* terminal oxidase, respectively (Nakamura *et al.*, 1997). All components of the cytochrome *bo* complex are strongly repressed when the Cpx pathway is active (Raivio *et al.*, 2013). Cytochrome *bo* is one of the three major terminal oxidases in the respiratory chain of *E. coli* (Anraku and Gennis, 1987). It is predominantly expressed under high oxygen conditions and contributes to proton motive force across the inner membrane (Puustinen *et al.*, 1991). Our results may suggest that perturbation to the complex upon CyoA or CyoC overexpression acts as a Cpx inducing cue, which would subsequently act to feedback inhibit the expression of the entire complex. Previous study showed that resistance to antimicrobials hydroxyurea and aminoglycoside is conferred by repression of succinate dehydrogenase, NADH dehydrogenase, cytochrome oxidase and the EfeUOB ferrous iron transporter through the Cpx pathway (Mahoney and Silhavy, 2013). Therefore, by responding to excess CyoA and CyoC subunits, the Cpx pathway could contribute to resistance to antimicrobials hydroxyurea and aminoglycoside. FkpA encodes a heat shock periplasmic peptidyl-prolyl cis-trans isomerase (PPIase) that has chaperone activity (Arié *et al.*, 2001). FkpA is up regulated by 2-3-fold when the Cpx pathway is activated (Raivio *et al.*, 2013). FkpA overexpression could lead to mis-folding of itself or other proteins in the envelope that activates the Cpx pathway accordingly. YidQ encodes a σ^E-regulated outer membrane protein with unknown function (Dartigalongue *et al.*, 2001). When the Cpx response is activated upon NlpE overexpression, transcription of YidQ is slightly elevated (Raivio *et al.*, 2013). Recently, it is reported that periplasmic chaperone Skp could catalyze the insertion of outer membrane proteins to the inner membrane when outer membrane protein

assembly went wrong (Grabowicz *et al.*, 2016). In case of YidQ overexpression, it could abnormally be integrated to the inner membrane and subsequently activate the Cpx pathway. The activation of the Cpx pathway in turn up regulates sRNA CpxQ that represses Skp transcription and combat stresses in the inner membrane (Chao and Vogel, 2016).

EmrA encodes the membrane fusion protein for the EmrB multidrug efflux pump (Lomovskaya and Lewis, 1992). EmrA is in the periplasm and interacts with TolC to form the efflux pump (Borges-Walmsley *et al.*, 2003). EmrA is down regulated by about 0.5-fold when the Cpx pathway is activated (Raivio *et al.*, 2013). Previous study showed that metabolite accumulation resulting from mutation of the common outer membrane efflux pump component TolC activates the Cpx pathway in *E. coli* (Rosner and Martin, 2013; Taylor *et al.*, 2014). Thus, one hypothesis to explain the Cpx activating phenotype of EmrA overexpression could be that it disrupts the Emr efflux pump, leading to a similar accumulation of metabolites in the periplasm that generates an envelope stress cue. In turn, the Cpx pathway up regulates expression of efflux pumps to mediate stresses in the envelope (Acosta *et al.*, 2015; Taylor *et al.*, 2014).

Our identification of novel multi-copy auxiliary regulators, both activators and inhibitors of the Cpx pathway, provide us with more clues to the Cpx two-component system. These identified multi-copy effectors of the Cpx pathway are components that are involved in diverse cellular functions such as metabolism, energy production, toxic compound efflux, and protein folding in the envelope (Table A-2). Recognition of their roles in signaling the Cpx pathway provides us with

link to different physiology aspects in the envelope. It is consistent with the diverse roles played by the Cpx pathway in the cell. The molecular mechanisms involved await further investigation.

A5. Tables and Figures

Table A-1 Bacterial strains and plasmids used in this study

Strain	Description	Resource or Reference
TR50	MC4100 *λRS88* [*cpxP-lacZ*]	(Raivio & Silhavy, 1997)
JSW10	TR50/pCA-24N	This study
JSW2	TR50/pCA-NlpE	This study
JLW609	TR50/pCA-YlbF	This study
JLW770	TR50/pCA-NuoF	This study
JLW771	TR50/pCA-EfeB	This study
JSW18	TR50/pCA-CyoA	This study
JSW17	TR50/pCA-CyoC	This study
JSW249	TR50/pCA-FkpA	This study
JSW250	TR50/pCA-EmrA	This study
JSW251	TR50/pCA-YidQ	This study
RM53	TR50 *ΔcpxA*	Raivio lab
JSW252	RM53/pCA-24N	This study
JLW699	RM53/pCA-YlbF	This study
JLW 877	RM53/pCA-NuoF	This study
JLW 878	RM53/pCA-EfeB	This study
JSW253	RM53/pCA-CyoA	This study
JSW254	RM53/pCA-CyoC	This study
JSW255	RM53/pCA-FkpA	This study
JSW256	RM53/pCA-EmrA	This study
JSW257	RM53/pCA-YidQ	This study
TR69	MC4100 *λRS88* [*degP-lacZ*] *cpxR::spc*	This study
JLW773	TR69/pCA-24N	This study
JLW774	TR69/pCA-YlbF	This study
JLW780	TR69/pCA-NuoF	This study
JLW782	TR69/pCA-EfeB	This study
E2348/69	EPEC O127:H6 strain	(Levine et al., 1978)
NLP94	E2348/69/pJW25	(Price & Raivio, 2009)
JSW258	NLP94/ pCA-24N	This study
JSW259	NLP94/ pCA-CyoA	This study
JSW260	NLP94/ pCA-CyoC	This study
RG140	E2348/69 *ΔcyoABCDE/pJW25*	Raivio Lab
JSW261	RG140/pCA-24N	This study
JSW262	RG140/pCA-CyoA	This study
JSW263	RG140/pCA-CyoC	This study

Plasmids	**Description**	**Resource or Reference**
pCA-24N	Empty vector used for ASKA library	(Kitagawa et al., 2005)
pCA-CyoA	CyoA overexpression plasmid from	(2005)

	ASKA libray	
pCA-CyoC	CyoC overexpression plasmid from ASKA libray	(2005)
pCA-Fkpa	FkpA overexpression plasmid from ASKA libray	(2005)
pCA-YdiQ	YdiQ overexpression plasmid from ASKA libray	(2005)
pCA-EmrA	EmrA overexpression plasmid from ASKA libray	(2005)
pJW15	Low copy number plasmid with a promoterless *luxCDABE* reporter	(MacRitchie et al., 2008)
pJW25	Low copy number plasmid with a *cpxP-luxCDABE* luminescence reporter	(2008)

Table A-2 Summary of identified multi-copy activators and inhibitors of the Cpx two-component system

Protein	Function or description	Effect on the Cpx two-component system when overexpressed
CyoC	Subunit III of the Cytochrome *bo* Terminal Oxidase	Activator
CyoA	Subunit II of the Cytochrome *bo* Terminal Oxidase	Activator
FkpA	Peptidyl-prolyl cis-trans Isomerase	Activator
YidQ	Conserved outer membrane protein with unknown function	Activator
EmrA	Membrane fusion protein of the EmrB multidrug efflux pump	Activator
NuoF	NADH dehydrogenase complex I	Inhibitor
EfeB	Periplasmic heme-containing peroxidase enzyme; component of the cryptic EfeUOB ferrous iron transporter	Inhibitor

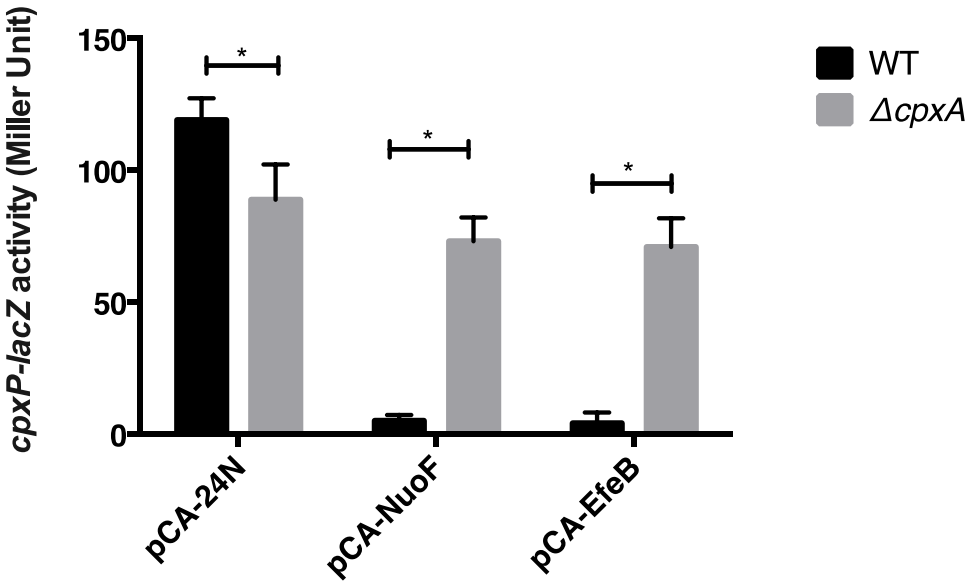

Figure A-1 NuoF and EfeB overexpression inhibit the Cpx pathway in a CpxA-dependent manner

Wild type TR50 containing a *cpxP-lacZ* reporter (black bar) and the TR50 *ΔcpxA* mutant (grey bar) were transformed with pCA-24N based NuoF and EfeB overexpression plasmids, sub-cultured into fresh LB supplemented with chloramphenicol, and grown at 37°C (wildtype strains) or 30°C (*ΔcpxA* strains). IPTG was added 1 h after subculture to a final concentration of 1 mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8 and lysed using chloroform and 1% SDS. β -galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 Plate reader after addition of ONPG to the cell lysate. Experiments were performed in triplicate three times; the mean and standard deviation are shown. * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

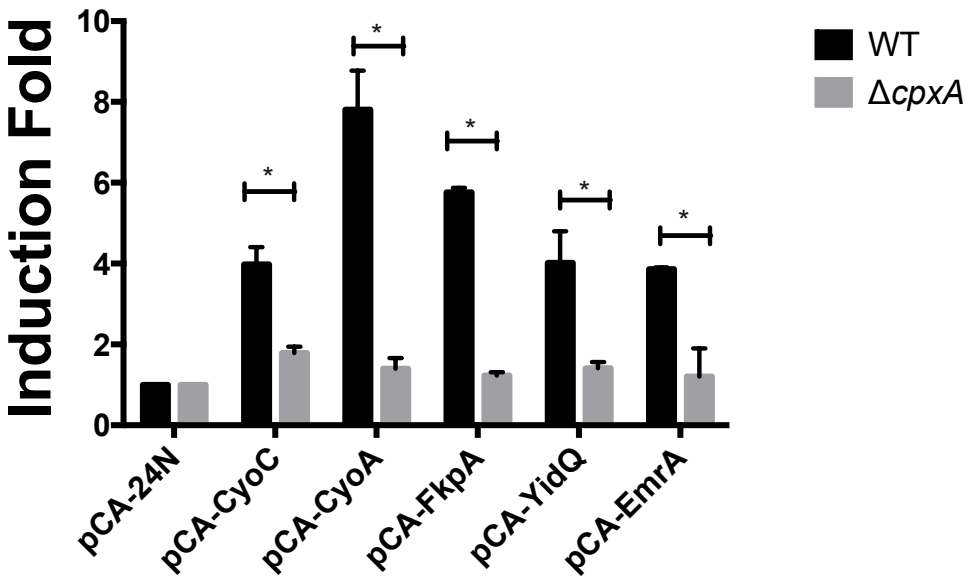

Figure A-2 Induction fold of the Cpx two-component system upon overexpression of multi-copy activators

Wild type TR50 carrying a *cpxP-lacZ* reporter (black bar) and a TR50 *ΔcpxA* mutant (grey bar) were transformed with pCA-24N based CyoC, CyoA, FkpA, YidQ and EmrA overexpression plasmids, sub-cultured into fresh LB with chloramphenicol and grown at 37°C (wildtype strains) or 30°C (*ΔcpxA* strains). IPTG was added 1 h after subculture to a final concentration of 1 mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8, lysed using chloroform and 1% SDS, and thehe β -galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 Plate reader after addition of ONPG to the cell lysate. Induction fold was calculated by normalizing the β–galactosidase activity to that of the vector control group. Experiments were performed in triplicate three times; the mean and standard deviation are shown. * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

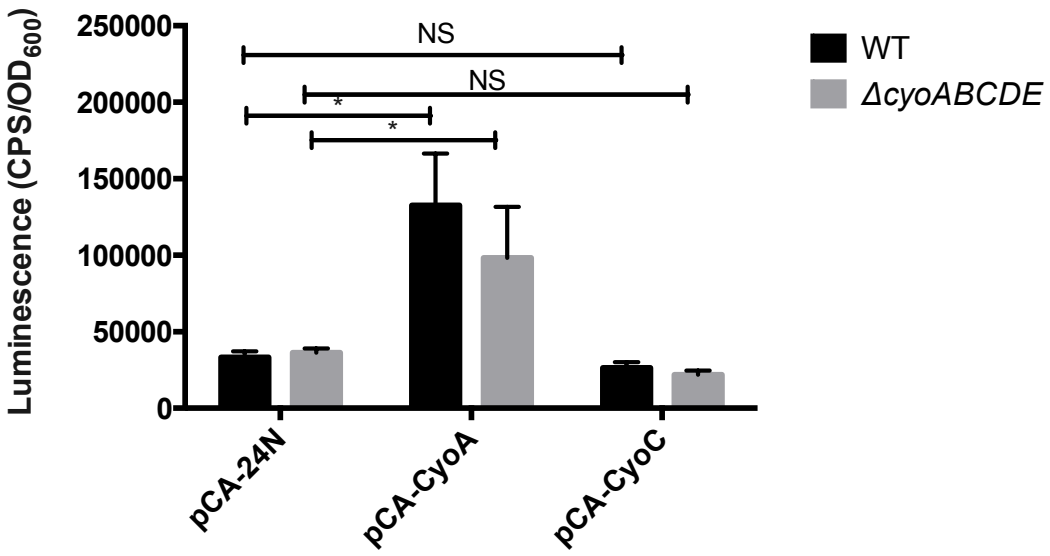

Figure A-3 Activation of the Cpx two-component system upon CyoA overexpression does not require a functional Cytochrome *bo* oxidase complex

EPEC E2348/69 and an E2348/69*ΔcyoABCDE* mutant transformed with pJW25 and pCA-24N based CyoC and CyoA overexpression plasmids were sub-cultured into fresh LB with chloramphenicol and kanamycin. Strains were cultured at 37°C for an hour before IPTG was added to a final concentration of 0.1 mM to induce protein overexpression. Then luminescence (cps, counts per second) and OD_{600} was measured hourly for 8 hours using a Perkin Elmer Wallac Victor^2 1420 Plate reader. The final bioluminescence (CPS/OD_{600}) activity was calculated by optimizing the corrected CPS (CPS sample-CPS blank control) by the corrected OD_{600} (OD_{600} sample-OD_{600} blank control). Assays were performed at least three times in triplicate. The mean and standard deviation from the 3 h reading from one experiment are shown. NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

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Appendix B Role of Trans-envelope Complexes in Cpx Signal Transduction

B1. Introduction

The envelope of gram-negative bacteria consists of the outer membrane, inner membrane and the periplasm in between (Silhavy *et al.*, 2010). Protein quality control in the envelope is critical for bacterial survival. DegP encodes a periplasmic serine protease that degrades misfolded envelope proteins and also functions as a chaperone (Ortega *et al.*, 2009; Huber, and Bukau, 2008). DegP is essential for bacterial survival under harsh conditions such as high temperature (Huber, and Bukau, 2008). Its expression is up-regulated by both the Cpx and σ^E envelope stress responses (Danese and Silhavy, 1997; Danese *et al.*, 1995). In a study investigating the effects of lipoprotein overexpression, Pal was shown to strongly induce DegP expression (Miyadai *et al.*, 2004). Pal is an abundant outer membrane lipoprotein that contributes to membrane integrity through its interaction with peptidoglycan and also inner membrane protein TolA, since mutations in *pal* or *tolA* result in increased sensitivity to SDS and elevated formation of outer membrane vesicles (Koebnik, 1995; Mizuno, 1979; Cascales *et al.*, 2002; Lazzaroni *et al.*, 1999; Lazzaroni and Portalier, 1992; Santos *et al.*, 2015; Yeh *et al.*, 2010). The interaction of Pal and TolA connects the outer membrane to the inner membrane and therefore forms a trans-envelope complex. This interaction requires another periplasmic protein, TolB, and energization by interaction with inner membrane proteins TolQ and TolR, which are cabable of harvesting the proton motive force (Walburger *et al.*, 2002; Germon *et al.*, 2001; Cascales *et al.*, 2001). The effect of Pal overexpression on transcription of *degP* is reminiscent of another protein, NlpE. NlpE is also an outer membrane lipoprotein, and similarly induces *degP* transcription upon

overexpression, through its activation of the Cpx envelope stress response (Snyder *et al.*, 1995). Given the effects of Pal overexpression on DegP levels, in this study, we sought to investigate whether the Cpx pathway contributes to this upregulation, and also whether other components of the Tol-Pal complex were involved.

Another protein complex, TonB-ExbBD, drew our attention because of its homology to the Tol-Pal complex. TonB interacts with outer membrane receptors in a PMF-dependent manner, similar to how TolA (homologue of TonB) interacts with Pal (Ollis *et al.*, 2009; Germon *et al.*, 2001; Cascales *et al.*, 2001). The TonB-ExbBD complex energizes the transport of macromolecular substrates such as siderophores and vitamin B12 across the outer membrane (Skare and Postle, 1991; Shultis *et al.*, 2006). Thus, we also investigated whether the TonB-ExbBD trans-envelope complex might impact Cpx signal transduction, because of its homology with the Tol-Pal complex (Cascales *et al.*, 2001). Strikingly, we found that overexpression of each component of the Tol-Pal and TonB-ExbBD complexes induced the Cpx pathway in a CpxA-dependent manner. These results indicate that the Cpx envelope stress response senses changes caused by the stoichiometric imbalance in the Tol-Pal and TonB-ExbBD complexes and/or accumulation of misfolded components of both complexes. Because the Tol-Pal and TonB-ExbBD protein complexes affected the magnitude of the Cpx response, we also investigated whether the presence or absence of these complexes altered other Cpx signal transduction events. Unexpectedly, we found that a functional TonB-ExbBD complex is required for the activation of the Cpx pathway upon NlpE overexpression, in response to misfolded pilus subunit PapE, and also by metal-limited conditions in *E. coli*. Additionally, we

showed that mutation of *tonB* suppressed NlpE toxicity in *E. coli*. Although the molecular mechanism is unclear, our results link the TonB-ExbBD complex to physiological changes that lead to Cpx activation.

B2. Materials and Methods

B2.1 Bacterial Strains and Growth Conditions

All bacterial strains used in this study are listed in Table B-1. Strains were cultured in LB broth at 37°C with aeration at 225 rpm with the exception of the Δ*cpxA* mutant, which was cultured at 30°C. For alkaline pH inducing conditions, 100 mM sodium phosphate buffer was used to buffer LB broth to pH 5.0 and 8.0. To prepare metal-limited LB broth, 100 mL 10X concentrated stock of LB broth was treated with 5 g Chelex-100 resin (Bio-Rad) with stirring for 5-6 h and then dialyzed against a 10 fold volume of MilliQ water using a 3000-5000 MW dialysis tube (Fisher) overnight. In addition, all glassware used for metal chelating experiments was thoroughly washed with 1 M HNO_3 (sigma) and rinsed with MilliQ water to avoid metal contamination.

B2.2 Strain Construction

Transformation and P1 transduction were carried out using standard protocols (Sambrook and Russell, 2001). MC4100 mutants carrying mutations in *tolA, tolQ, tolR, tonB, exbB* or *exbD* were constructed by P1 transduction using the corresponding mutants from the Keio library (Baba *et al.*, 2006). The kanamycin cassette in the resulting mutants was flipped out by FRT-mediated recombination using the pFlp2 plasmid as previously described (Hoang *et al.*, 1998).

B2.3 β-galactosidase Assay

β-galactosidase activity was measured in microtiter plates as previously described (Silhavy *et al.*, 1984). Single colonies of each strain tested were inoculated into 2 mL LB broth supplemented with appropriate antibiotics overnight and sub-cultured at a 1:00 dilution to 2 mL LB broth the following day. For induction of protein overexpression from the pCA24-N IPTG-inducible promoter, IPTG was added to a final concentration of 0.1 mM 1 h after subculture. Cells were collected when the OD_{600} reached 0.6-0.8 and resuspended in freshly prepared Z buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM$MgSO_4{\cdot}7H_2O$; containing 270 µL β-mercaptoethanol). Then 250 µL of cell mixture were transferred to a 96-well polystyrene plate. The OD_{600} was read in a 96-well using a Perkin Elmer Wallac Victor^2 1420 plate reader. The remaining cells were lysed by vortexing for 1min after the addition of 1 drop each of chloroform and 0.1% SDS and then letting the culture rest for 10min. The cellular debris was removed by centrifugation. The lysed cell mixture was added to 1X Z-buffer in 96-well plates (5 µL lysed cell mixture, 195 µL 1X Z-buffer for strains carrying the *cpxP-lacZ* reporter), and 50 µL of 10 mg/mL ONPG (o-nitrophenyl β–D-galactopyranoside)(Sigma) was added to initiate the reaction. The A_{420} was read 20 times over approximately 30 min in the plate reader and Miller Units were calculated (Zhang and Bremer, 1995). Experiments were done in triplicate, three times.

B2.4 Membrane Preparation

Membrane extracts were prepared as previously described (Lobos and Mora, 1991). Overnight cultures of bacterial strains were sub-cultured into 5 mL fresh LB at 1:100 dilution and grown at 37°C with aeration at 225 rpm to an OD_{600} of 0.6-0.8. To induce protein overexpression from the pCA-24N IPTG-inducible promoter, IPTG was added to the culture to a final concentration of 0.1 mM 1 h after subculture. Equal numbers of cells were collected by adjusting cell volumes to a standard OD_{600} and resuspending in1 mL PBS, followed by sonication. The resulting cell lysates were centrifuged at 9391 g at 4°C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet whole cell membranes. The resulting pellets were solubilized in 50 µL 100 mM Tris-HCl (pH 8.0, with 2% Triton X-100).

B2.5 Western Blotting

Electrophoresis and Western blotting were performed as previously described (Raivio, Popkin, & Silhavy, 1999). Blots were incubated with primary antisera anti-NlpE (raised in this study) and anti-PhoA (Abcam) at 1:10,000 dilutions in 2% (wt/vol) skimmed milk for 1 h with agitation at room temperature. Blots were then washed with wash buffer (1 M Tris, 0.9 M NaCl with 0.2% vol/vol tween 20) for 30 mins three times. NlpE antiserum was raised in rabbits against purified NlpE protein by Rockland Immunochemicals Inc (See Chapter 2). The secondary antibody used was an anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma) at a concentration of 1:25,000 in 2% (wt/vol) skimmed milk for 1 h with agitation at room temperature. Blots were washed with wash buffer (1 M Tris, 0.9 M NaCl with

0.2% vol/vol tween 20) for 30 mins three times. Blots were developed with the chemiluminescent Immun-Star AP Substrate Pack (Bio-Rad) following the manufacturer's directions and imaged using a Chemi-Doc Imager (Bio-Rad).

B2.6 Growth Curves

Single colonies of each tested strain were inoculated in 2 mL LB with appropriate antibiotics and cultured at 37°C with aeration at 225 rpm. The following day, the overnight culture was diluted 1:100 into fresh LB and grown for 1h at 30°C with aeration at 225 rpm. Then IPTG was added to the culture to a final concentration of 1 mM. After mixing the samples, 200 µL of culture was transferred into a clear 96-well microtiter plate (Corning). The plate was cultured at 37°C with aeration at 225 rpm. Optical density at 600nm was measured every 2 h for 8 h.

B3. Results

B3.1 Components of the Pal-Tol Complex Induce the Cpx Pathway When Overexpressed

As previously published, Pal overexpression strongly induces DegP transcription in *E. coli* (Miyadai *et al.*, 2004). DegP is regulated by both the Cpx two-component system and the σ^E stress response (Danese and Silhavy, 1997; Danese *et al.*, 1995). Therefore, we first sought to examine whether Pal overexpression activated *degP* transcription via the Cpx pathway. The activity of the Cpx response was measured upon Pal overexpression by using a CpxR-regulated *cpxP-lacZ* reporter instead of the *degP-lacZ* reporter previously described. The expression of *cpxP* is not controlled by the σ^E response. TR50, an MC4100 strain carrying a chromosomal

cpxP-lacZ reporter, was transformed with plasmid pCA-Pal that overexpresses Pal. The activity of the Cpx pathway upon Pal overexpression was examined by β-galactosidase assay. In a wildtype strain, overexpression of Pal induced the Cpx pathway to 13-fold in comparison to the vector control (Fig.B-1A). Whether the induction of the Cpx pathway upon Pal overexpression was CpxA dependent was also tested by examining expression of β-galactosidase in a TR50 Δ*cpxA* mutant transformed with plasmid pCA-Pal. As shown in Fig.B-1B, the activation of the Cpx response upon Pal overexpression was abolished when CpxA was mutated. Thus, Pal overexpression activates the Cpx pathway in a CpxA-dependent manner, similarly to NlpE. This activation likely contributes to the previously observed increase in *degP* expression upon Pal overexpression, although we did not test this.

Pal is the outer membrane component of the envelope spanning Tol-Pal complex (Cascales *et al.*, 2000; Bouveret *et al.*, 1995). Pal interacts with the periplasmic component TolB and the inner membrane component TolA. As a whole, the Tol-Pal complex contributes to maintaining membrane integrity, OM invagination, and proper chemoreceptor localization (Gerding *et al.*, 2007; Santos *et al.*, 2014). Since Pal overexpression induced the Cpx response, we also tested whether the overexpression of other components of the Pal-Tol complex had a similar effect. Plasmids expressing each component of the Tol-Pal complex, including TolA, TolB, TolQ, and TolR were transformed into TR50. The activity of the Cpx pathway upon overexpression of each component of the Tol-Pal complex was examined by β-galactosidase assay. As shown in Fig.B-1, TolA overexpression strongly induced the Cpx pathway to 13-fold in comparison to the vector control. Overexpression of TolB,

TolQ and TolR showed 4-fold, 2.6-fold and 3.5-fold induction of the Cpx pathway respectively. The CpxA dependence of this activation was determined by repeating this experiment in a Δ*cpxA* mutant strain background. Similar to Pal, activation of the Cpx pathway upon TolA, TolB, TolQ and TolR overexpression was abolished when *cpxA* was mutated (Fig.B-1B). Together, our results show that the Cpx response senses perturbations of the Tol-Pal protein complex. This might occur through CpxA-mediated detection of altered Tol-Pal function that could be linked to the accumulation of misfolded components of the complex or changes caused by altered stoichiometry.

B3.2 Components of the TonB-ExbBD Complex Induce the Cpx Pathway When Overexpressed

Another well-studied trans-envelope complex, made up of TonB, ExbB, and ExbD, shares homology with the Pal-Tol complex (Cascales *et al.*, 2001). Since the Cpx response is sensitive to overexpression of components of the Pal-Tol complex, we sought to investigate whether or not the Cpx pathway senses overexpression of TonB, ExbB and/or ExbD as well. Plasmids expressing each component of the TonB-ExbBD complex, including TonB, ExbB and ExbD, were transformed into TR50 and a TR50 Δ*cpxA* mutant. The activity of the Cpx pathway upon overexpression of each component of TonB-ExbBD complex was examined by β-galactosidase assay. As shown in Fig.B-2A, overexpression of TonB, ExbB and ExbD resulted in 2-fold, 6-fold and 2-fold activation of the Cpx pathway, respectively. However, when CpxA was mutated, the activation of the Cpx pathway by overexpression of these proteins was abolished (Fig.B-2B). Thus, as for the components of the Pal-Tol complex, the Cpx

pathway is activated upon overexpression of TonB, ExbB and ExbD in a CpxA-dependent manner. Together, our results show that CpxA senses some cue associated with overexpression of components of the TonB-ExbBD complex. Whether this cue is related to TonB-ExbBD function, accumulation of misfolded proteins, changes caused by altered stoichiometry of the protein complex, or some combination of these, remains to be determined.

B3.3 Mutation of *tolA* Has a Small Effect on Induction of the Cpx Pathway by NlpE Overexpression

Pal is anchored to the outer membrane through its amino-terminal lipid modification. Its interaction with TolA provides a physical link to the inner membrane. We hypothesized that Pal overexpression might signal the Cpx pathway through its interaction with TolA. Then, the activation of the Cpx pathway upon Pal overexpression would be affected when *tolA* is mutated. To test this, TR50, an MC4100 strain carrying a chromosomal *cpxP-lacZ* reporter, and its *tolA* derivative were transformed with pCA-24N, pCA-NlpE and pCA-Pal. The activity of the Cpx pathway upon Pal and NlpE overexpression was examined by measuring β-galactosidase activity. As shown in Fig.B-3A, activation of the Cpx pathway upon Pal and NlpE overexpression was not affected in a *tolA* mutant compared to wildtype. Western blotting of membrane fractions revealed that overexpression of NlpE was not affected when *tolA* was mutated (Fig.B-3B).

B3.4 Mutation of *tonB* Diminishes Induction of the Cpx Pathway Upon NlpE Overexpression

We similarly wondered, given the activating effect of TonB, ExbB, and ExbD overexpression on the Cpx response, whether any of these proteins could be involved in other Cpx signaling events. To test this, we examined activation of the Cpx response upon NlpE overexpression in the presence of *tonB, exbB,* or *exbD* mutations, using a Cpx-regulated *cpxP-lacZ* reporter gene located on the chromosome of strain TR50. When *tonB* was mutated, overexpression of NlpE resulted in significantly diminished activation of the Cpx pathway, as compared to the wild type strain. NlpE overexpression led to a 3-fold activation of the Cpx pathway in a *tonB* mutant, while about 20-fold activation was observed in a wild type strain (Fig. B-4A). A similar trend was observed when *exbB* or *exbD* was mutated (Fig. B-4A). As with the overexpression of components of the TonB-ExbBD protein complex, these observations show that disruptions to this protein assembly impact Cpx signaling events. We considered several possible explanations. First, mutation of *tonB, exbB* or *exbD* could affect proper NlpE expression and insertion in the membrane, thus disrupting its ability to induce the Cpx response. A second explanation could be that the TonB-ExbBD complex is involved in communicating molecular inducing cues directly or indirectly to CpxA as part of a signaling complex. Yet another possibility is that alterations in expression of TonB, ExbB, or ExbD alter the generation of an undefined Cpx inducing, either increasing it (ie, upon overexpression) or decreasing it (upon mutation).

To distinguish amongst these possibilities, the protein level of NlpE in wildtype, *tonB, exbB* and *exbD* mutants were examined by western blotting of membrane fractions. As shown in Fig.B-4B, comparable levels of NlpE were detected in wildtype, *tonB, exbB* and *exbD* mutant strains. This result indicates that the protein expression and membrane location of NlpE is not affected when *tonB, exbB* or *exbD* is mutated. We note that it is still possible that the localization of NlpE to the outer membrane could be affected, as we only analyzed crude membrane fractions. This will need to be determined in the future. None-the-less, this observation suggests that the effect of alterations in the levels of individual proteins of the TonB-ExbBD complex on Cpx response activation upon NlpE overexpression is not due to an effect on its expression or localization, and support a model in which a functional TonB-ExbBD complex is either required for NlpE to signal the Cpx pathway when overexpressed. Alternatively, alterations in the TonB-ExbBD complex could be involved in modulating the level of a molecular signal that is generated upon NlpE overexpression.

B3.5 TonB is Required For Sensing Mis-folded Pilus Subunits and Metal-limited Conditions, But Not Alkaline pH

Given the impact of mutation of components of the TonB-ExbBD complex on Cpx response activation by NlpE overexpression, we sought to investigate whether or not TonB is also required for sensing other inducing cues of the Cpx pathway such as alkaline pH, misfolded pilus subunit PapE, and metal chelation. As shown in Fig.B-5A, in both wildtype and *tonB* mutant strains, activation of the Cpx pathway was observed when the TR50 strain carrying the *cpxP-lacZ* reporter gene was grown at

pH 8.0, as compared to when it was grown at pH 5.0. Conversely, the activation of the Cpx pathway when misfolded PapE was overproduced, as well as when bacteria were grown in metal-limited conditions was abolished when *tonB* was mutated (Fig.B-5B, 5C). Together, our results show that TonB is required for the activation of the Cpx pathway upon NlpE overexpression, in the presence of misfolded pilus subunit PapE, and in metal-limited conditions, but not for alkaline pH.

B3.6 Mutation of *tonB* Suppresses the Toxicity of NlpE Overexpression

During the course of these studies, we found that overexpression of NlpE is actually toxic (Fig. B-6). Given the diminished activation of the Cpx response when *tonB* was mutated, we wondered if TonB might also affect this phenotype. To test this, we monitored cell growth in wild type and *tonB* mutant strains after induction of NlpE overexpression. As shown in Fig.B-6, NlpE overexpression resulted in growth arrest in a wild type strain, as compared to the vector control. However, when *tonB* was mutated, no growth arrest upon NlpE overexpression was observed. These results suggest that TonB is somehow toxic in the presence of NlpE overexpression.

B4. Discussion

In this study, we investigated the link between two conserved trans-envelope complexes, Tol-Pal and TonB-ExbBD, with the Cpx envelope stress response. First, we showed that the previously described up-regulation of periplasmic serine protease and chaperone DegP upon Pal overexpression requires a functional Cpx pathway (Miyadai *et al.*, 2004). This finding led us to investigate the involvement of

the other members of the Tol-Pal complex, as well as the homologous TonB-ExbBD complex, with the Cpx two-component system.

Our results showed that overexpression of components of both the Tol-Pal and TonB-ExbBD complexes led to activation of the Cpx pathway in *E. coli.* In order to function properly, the stoichiometry of each complex is strictly regulated. For the Tol-Pal complex, the stoichiometric ratio of TolQ:TolR:TolA is about 4:2:1 (Cascales *et al.*, 2001). For the TonB-ExbBD complex, the stoichiometric ratio of TonB:ExbB:ExbD is about 1:7:2 (Higgs *et al.*, 2002). Therefore, one possibility is that overexpression of a single component of these complexes could lead to disruption of the normal stoichiometry and/or misfolded protein accumulation that is sensed by the Cpx pathway. Since these complexes are anchored in the inner membrane, it is probable that excess components that are not properly associated with functional complexes cause stress at this cellular location that is sensed by the Cpx pathway. The Tol-Pal complex has diverse cellular functions including maintaining outer membrane integrity and the proper localization of chemoreceptors, and as part of the invagination machinery for cell division (Santos *et al.*, 2014; Yeh *et al.*, 2010; Gerding *et al.*, 2007). TonB-ExbBD energizes transport of large macromolecular substrates such as vitamin B12 cofactors and siderophores for metal ion acquisition (Skare and Postle, 1991; Shultis *et al.*, 2006). In addition, proper functioning of the TonB-ExbBD and Tol-Pal complexes consumes proton motive force across the inner membrane, which is critical to maintain their proper cellular functions.

To study the potential involvement of the Pal-Tol and TonB-ExbBD complexes in signaling of the Cpx pathway in *E. coli*, we examined the effect of mutating individual

components on Cpx signaling. Deletion of the genes that encode the Tol-Pal complex had no affect on Cpx signaling, at least in response to overexpression of Pal and NlpE in *E. coli*. In contrast, an intact TonB-ExbBD complex is required for sensing metal limited conditions, misfolded PapE, and NlpE overexpression. One caveat to this conclusion is that we did not determine whether PapE expression and/or localization to the periplasm occurred in the same fashion in the *tonB* mutant as in a wild type strain. Accordingly, we cannot rule out the possibility that the reason PapE fails to signal the Cpx response in the *tonB* mutant is because its expression level is decreased.

Several explanations for the involvement of TonBExbBD in Cpx signaling can be envisioned. Under metal-limited conditions, the TonB-ExbBD system is up-regulated through the relief of repression by the ferric uptake regulator Fur (Young and Postle, 1994). Perhaps this leads to misfolding of the TonB, ExbB, and/or ExbD proteins, which leads to the activation of the Cpx pathway. In terms of NlpE overexpression, one possibility is that NlpE interacts directly with some part of the TonB-ExbBD complex that then generates a signal that is sensed by CpxA. Normally TonB interacts with outer membrane receptors through an interaction between a conserved TonB box and a β-sheet at the TonB carboxyl-terminus (Klebba, 2016). When excess NlpE is around, perhaps it could interact with TonB non-specifically. Notably, TonB was not detected in our pull down assay investigating proteins that interact with NlpE. However this could be due to the low abundance of the TonB-ExbBD complex under normal conditions. In future, a potential NlpE and TonB interaction could be investigated in a *fur* mutant, where the complex would be

present at higher levels. Another possibility could be that NlpE overexpression causes physiological changes in the envelope that require a functional TonB-ExbBD complex. This idea is supported by the fact that the toxicity of NlpE overexpression is not observed when *tonB* is mutated. The reason for the toxicity of NlpE is not known. Perhaps NlpE causes stress by sequestering some substance, maybe in the N-terminal domain which our results suggest binds a ligand, whose presence require TonB-ExbBD-dependent transport. The molecular details of how the TonB-ExbBD complex is involved in signaling the Cpx pathway await further investigation.

In this study, we studied the role of trans-envelope Tol-Pal and TonB-ExbBD complexes in signaling of the Cpx pathway. Our results showed that the TonB-ExbBD complex is involved in signaling of the Cpx pathway. Although the molecular detail remains unclear, it links Cpx activation by some inducing cues to another inner membrane localized pathway with a known physiological function and as such, this work may ultimately lead to insight into the molecular nature of a common Cpx inducing signal.

B5. Tables and Figures

Table B-1 Bacterial strains and plasmids used in this study

Bacterial strain	Description	Reference or source
TR50	MC4100 *λRS88* [*cpxP-lacZ*]	(Raivio and Silhavy, 1997)
RM53	TR50 *ΔcpxA*	Raivio lab collection
JSW10	TR50/pCA-24N	This study
JSW2	TR50/pCA-NlpE	This study
JSW21	TR50/pCA-Pal	This study
JSW20	TR50/pCA-TolA	This study
JSW23	TR50/pCA-TolB	This study
JSW14	TR50/pCA-TolQ	This study
JSW12	TR50/pCA-TolR	This study
JSW272	TR50/pCA-TonB	This study
JSW273	TR50/pCA-ExbB	This study
JSW274	TR50/pCA-ExbD	This study
JSW252	RM53/pCA-24N	This study
JSW275	RM53/pCA-TolA	This study
JSW276	RM53/pCA-TolB	This study
JSW277	RM53/pCA-TolQ	This study
JSW278	RM53/pCA-TolR	This study
JSW279	RM53/pCA-TonB	This study

JSW280	RM53/pCA-ExbB	This study
JSW281	RM53/pCA-ExbD	This study
JSW32	TR50 *ΔtolA*	This study
JSW31	TR50 *tolQ::Kan*	This study
JSW38	TR50 *tolR::Kan*	This study
JSW33	TR50 *ΔtonB*	This study
JSW50	TR50 *ΔexbB*	This study
JSW41	TR50 *exbD::Kan*	This study
JSW89	TR50 *ΔtolA*/pCA-24N	This study
JSW90	TR50 *ΔtolA*/pCA-NLpE	This study
JSW83	TR50 *ΔtonB*/pCA-24N	This study
JSW84	TR50 *ΔtonB*/pCA-NLpE	This study
JSW26	TR50 *ΔtonB*/pMMB66	This study
JSW27	TR50 *ΔtonB*/pHJ13	This study
Plasmid	Description	Reference or source
pCA-24N	Empty high copy number cloning plasmid	(Kitagawa *et al.*, 2005)
pCA-Pal	Pal overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-TolA	TolA overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-TolB	TolB overexpression	(Kitagawa *et al.*, 2005)

	plasmid from ASKA libray	
pCA-TolQ	TolQ overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-TolR	TolR overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-TonB	TonB overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-ExbB	ExbB overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-ExbD	ExbD overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pCA-NlpE	NlpE overexpression plasmid from ASKA libray	(Kitagawa *et al.*, 2005)
pMMB66	Expression plasmid	(Fürste *et al.*, 1986)
pHJ13	pMMB66-based PapE expression plasmid	(Lee *et al.*, 2004)

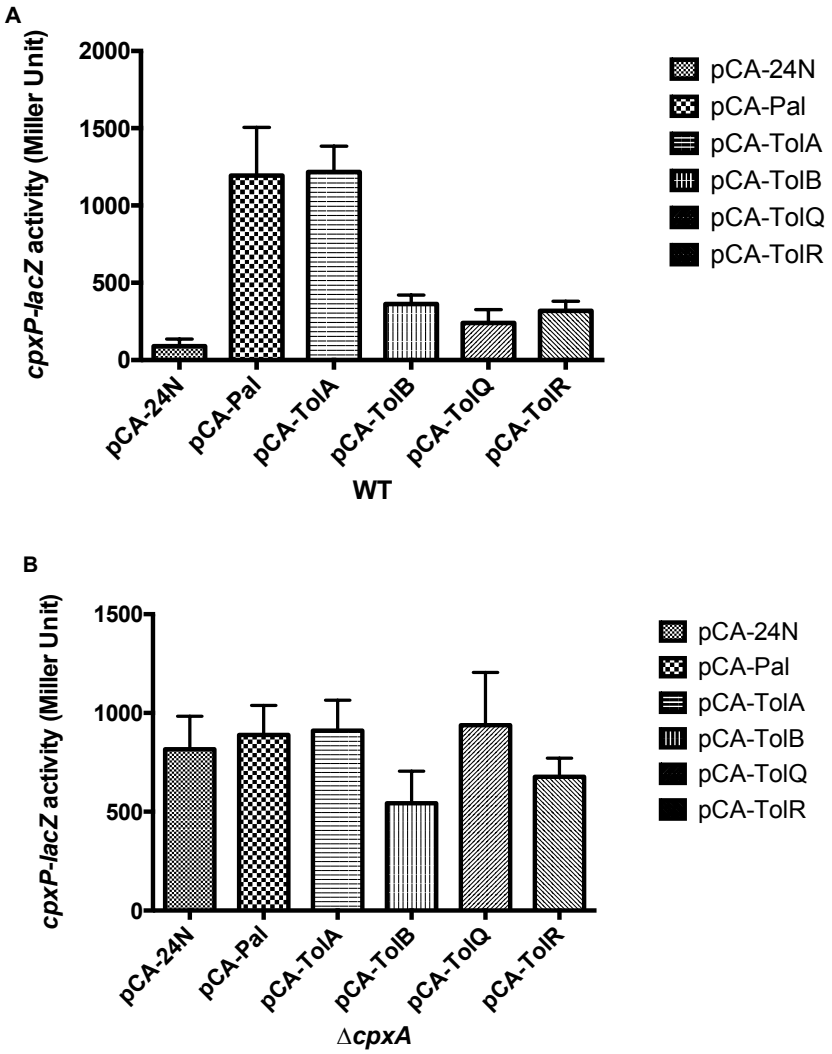

Figure B-1 Overexpression of components of theTol-Pal complex induces the Cpx pathway through the sensor kinase CpxA

β-galactosidase levels produced by TR50 upon overexpression of each of Pal, TolA, TolB, TolQ and TolR. B. β -galactosidase levels produced by a TR50 *ΔcpxA* mutant overexpressing Pal, TolA, TolB, TolQ, and TolR. Wildtype TR50 carrying a *cpxP-lacZ* reporter gene (A) and a TR50 *ΔcpxA* mutant (B) transformed with pCA-24N based Pal, TolA, TolB, TolQ and TolR overexpression plasmids were subcultured into fresh LB with chloramphenicol. TR50 strains were cultured at 37°C and TR50 *ΔcpxA* strains were cultured at 30°C. IPTG was added 1 h after subculture to a final concentration of 1mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8, lysed using chloroform and 1% SDS, and the β-galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 plate reader after addition of ONPG to the cell lysate. Experiments were performed in triplicate three times; the mean and standard deviation are shown.

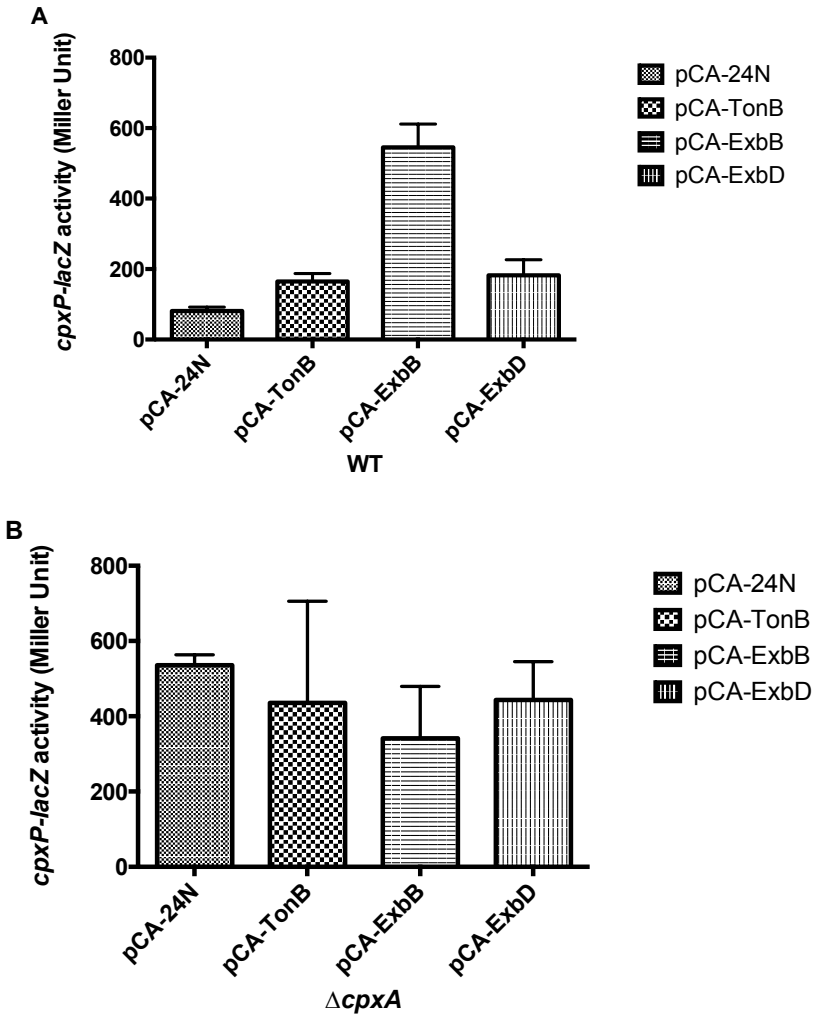

Figure B-2 Overexpression of Components of the TonB-ExbBD complex induces the Cpx pathway through the sensor kinase CpxA

A. β-galactosidase levels produced by TR50 upon overexpression of each of TonB, ExbB, or ExbD. B. β-galactosidase levels produced by a TR50 *ΔcpxA* mutant overexpressing TonB, ExbD, or ExbD. Wildtype TR50 carrying a *cpxP-lacZ* reporter gene (A) and a TR50 *ΔcpxA* mutant (B) transformed with pCA-24N based TonB, ExbB, or ExbD overexpression plasmids were subcultured into fresh LB with chloramphenicol. TR50 strains were cultured at 37°C and TR50 *ΔcpxA* strains were cultured at 30°C. IPTG was added 1 h after subculture to a final concentration of 1mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8, lysed using chloroform and 1% SDS, and the β-galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 plate reader after addition of ONPG to the cell lysate. Experiments were performed in triplicate three times; the mean and standard deviation are shown.

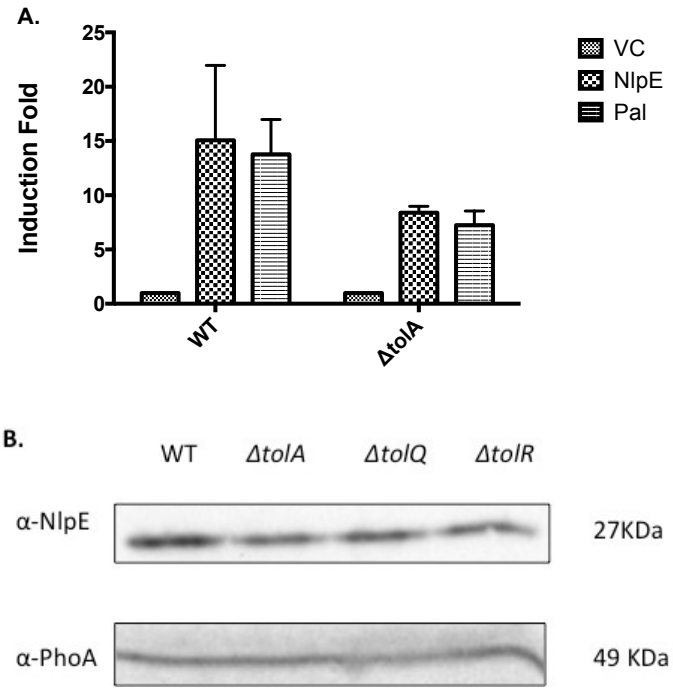

Figure B-3 Mutation of *tolA* has a mild affect on activation of the Cpx pathway upon overexpression of NlpE and Pal

A. Fold induction of the Cpx pathway by NlpE overexpression in TR50 wildtype (WT), *ΔtolA, ΔtolQ* and *ΔtolR* mutant strains. All TR50 strains were transformed with a pCA-24N based NlpE overexpression plasmid and overnight cultures were subcultured 1:100 into fresh LB with chloramphenicol and cultured at 37°C with shaking at 225 rpm. IPTG was added 1 h after subculture to a final concentration of 1 mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8, lysed using chloroform and 1% SDS, and the β-galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 plate reader after addition of ONPG to the cell lysate. The induction fold was calculated by normalizing the β–galactosidase activities to that of the vector control group. Experiments were performed in triplicate three times; the mean and standard deviation are shown.
B. Western blotting results showing the expression level of NlpE in wildtype (WT) *ΔtolA, ΔtolQ* and *ΔtolR* mutant strains in total membrane fractions. Bacterial strains were subcultured into 5 mL fresh LB at a 1:100 dilution and grown at 37°C with aeration for 1 h. IPTG was added to induce NlpE overexpression and grown until the OD_{600} reached 0.6-0.8 when equal numbers cells were collected and pelleted. Cells resuspended in PBS and lysed by sonication. The resulting cell lysate was centrifuged at 9391 g at 4°C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet whole cell membranes. The resulting pellets were solubilized in 50 mM Tris-HCl (pH 8.0, with 2% Triton X-100). Solubilized membrane samples of each strain were electrophoresed on 12% SDS-PAGE gels, followed by western blotting to detect NlpE and PhoA.

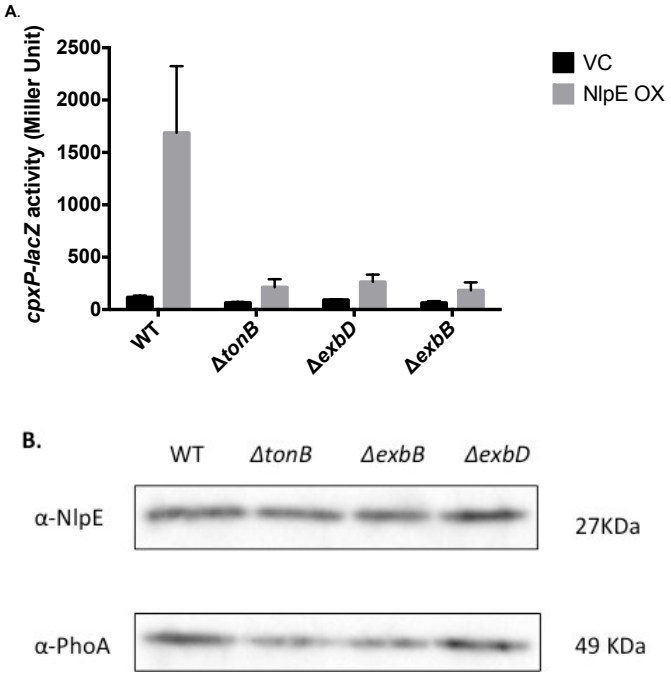

Figure B-4 Mutation of *tonB* impact activation of the Cpx pathway upon overexpression of NlpE

A. Fold induction of the Cpx pathway by NlpE overexpression in TR50 wildtype (WT), *ΔtonB, ΔexbB* and *ΔexbD* mutant strains. All TR50 strains were transformed with a pCA-24N based NlpE overexpression plasmid and overnight cultures were subcultured 1:100 into fresh LB with chloramphenicol and cultured at 37°C with shaking at 225rpm. IPTG was added 1h after subculture to a final concentration of 1 mM to induce protein overexpression. Cells were collected when the OD_{600} reached 0.6-0.8, lysed using chloroform and 1% SDS, and the β-galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 plate reader after addition of ONPG to the cell lysate. The induction fold was calculated by normalizing the β–galactosidase activities to that of the vector control group. Experiments were performed in triplicate three times; the mean and standard deviation are shown.
B. Western blotting results showing the expression level of NlpE in wildtype (WT) *ΔtonB, ΔexbB* and *ΔexbD* mutant strains in total membrane fractions. Bacterial strains were subcultured into 5mL fresh LB at a 1:100 dilution and grown at 37°C with aeration for 1h. IPTG was added to induce NlpE overexpression and grown until the OD_{600} reached 0.6-0.8 when equal numbers cells were collected and pelleted. Cells resuspended in PBS and lysed by sonication. The resulting cell lysate was centrifuged at 9391 g at 4°C for 5 min to remove unbroken cells and debris. The supernatant was transferred into a fresh eppendorf tube and centrifuged at 21130 g for 45 min to pellet whole cell membranes. The resulting pellets were solubilized in 50 mM Tris-HCl (pH 8.0, with 2% Triton X-100). Solubilized membrane samples of each strain were electrophoresed on 12% SDS-PAGE gels, followed by western blotting to detect NlpE and PhoA.

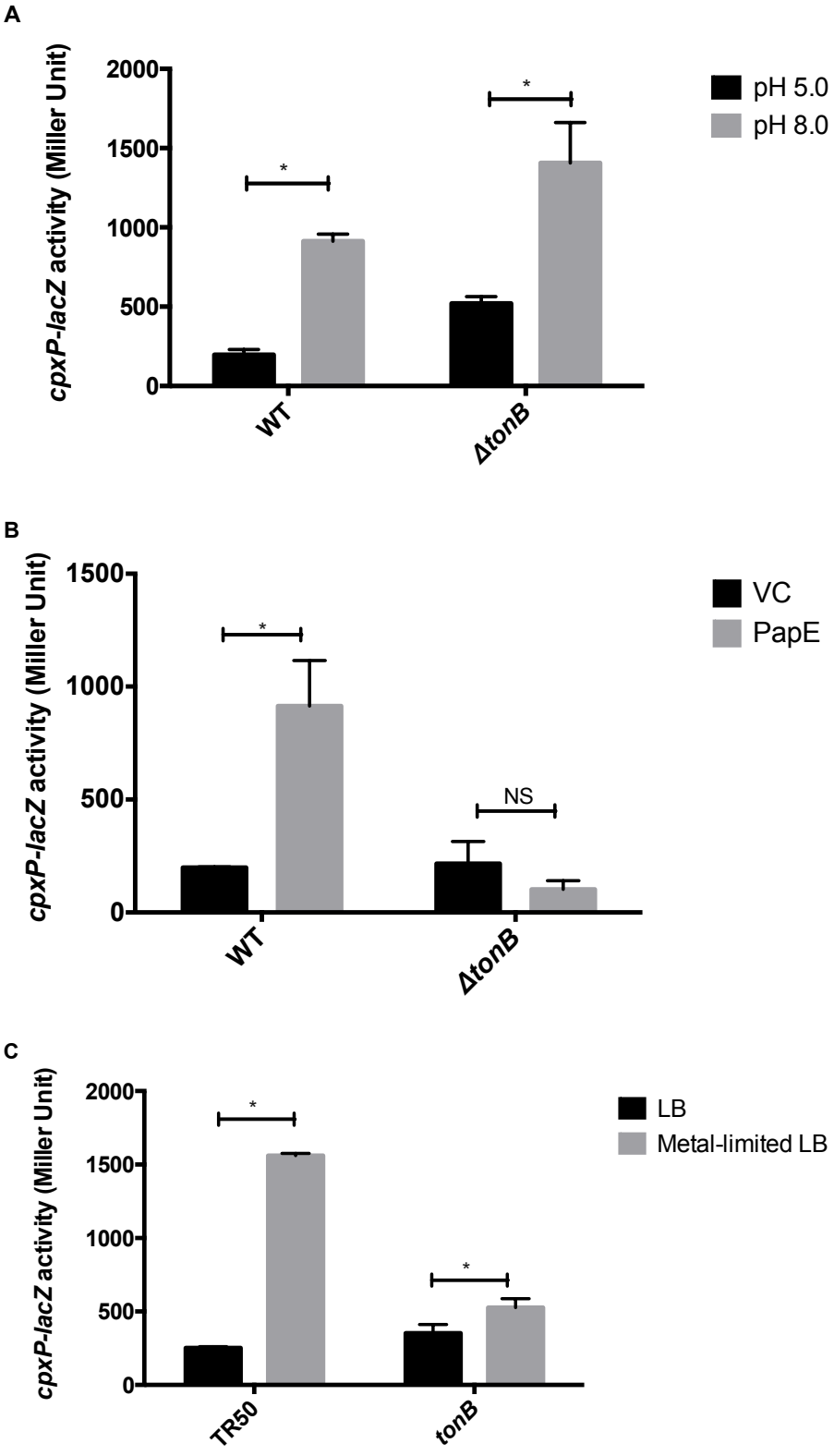

Figure B-5 TonB is required for sensing chelation and misfolded Pap subunits, but not alkaline pH

Levels of β-galactosidase produced from a *cpxP-lacZ* reporter gene in wildtype TR50 and Δ*tonB* strains in response to alkaline pH (A), PapE overexpression (B) and metal limitation (C). For alkaline pH induction of the Cpx pathway (A), cultures were grown at 37°C with aeration at 225 rpm to early log phase in LB at a pH of ~7.0, spun down for 10 minutes at 4500 g, resuspended in LB at pH 5.8 (Cpx off) or pH 8.0 (Cpx induced), and then grown for an additional two hours. For PapE overexpression (B), TR50 was transformed with plasmid pMMB66 (VC) and pHJ13-PapE overexpression plasmid (PapE). IPTG was added 1h after subculture to a final concentration of 1 mM to induce overexpression of PapE, and cells were collected when the OD_{600} reached 0.6-0.8. For induction by metal limitation (C), cultures were grown to early logphase in LB, spun down for 10 minutes at 4000 rpm, resuspended in LB or metal-limited LB and grown an additional 4 h at 37°C with aeration at 225 rpm. Cells were pelleted and resuspended in Z-buffer, lysed using chloroform and 1% SDS, and the β-galactosidase activity was measured using a Perkin Elmer Wallac Victor^2 1420 plate reader after addition of ONPG. Experiments were performed in triplicate three times; the mean and standard deviation are shown.
NS indicates not statistically significant between strains ($P>0.05$, one-way ANOVA test); * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA test).

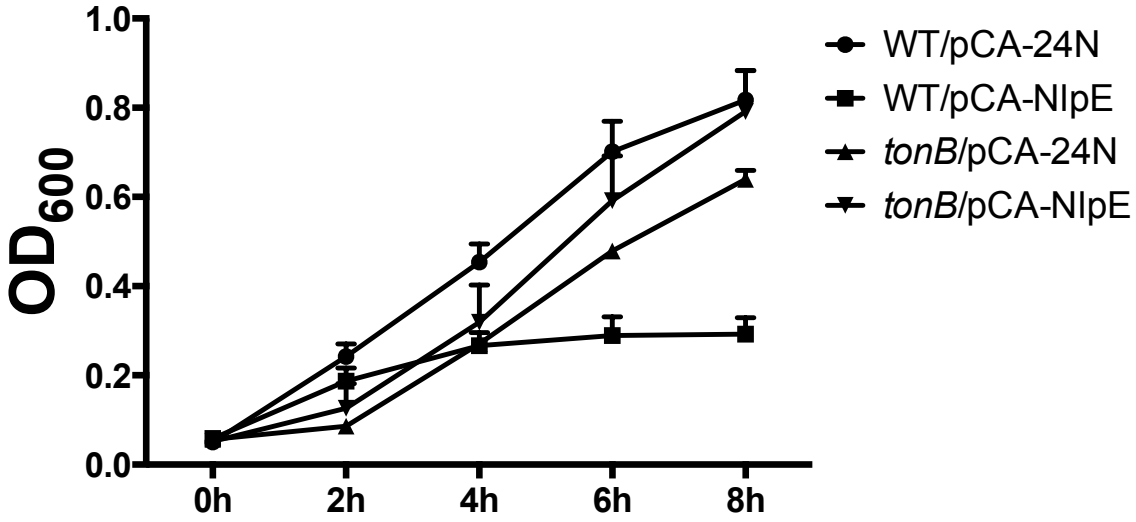

Figure B-6 TonB suppresses the toxicity of NlpE overexpression in *E. coli*

Growth curve of wildtype (WT) and *tonB* mutant strains in the absence and presence of NlpE overexpression from pCA-NlpE plasmid. IPTG was added to each tested strain 1 h after subculture and growth at 37°C with aeration at 225 rpm. Then, 200 µL of culture was transferred to a clear 96-well microtiter plate every 2 h for 8 H and the OD_{600} was measured.

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