The role of cysteine and the Cpx envelope stress response in alleviating zinc induced stress in *Escherichia coli*

by

Joseph Zimba

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences
University of Alberta

Abstract

Circumscribing the interior of Gram negative bacteria are two sets of layers: an inner and outer membrane. The periplasm and murein occupy the space between these membranes and acts as a site for protein folding and degradation. The bacterial envelope, as a whole, plays an essential role in the survival of the cell – providing structural support and as well carrying out cellular functions that are vital for the cell. Maintaining the envelope homeostasis is crucial for the cell's survival. Gram negative bacteria, such as *Escherichia coli*, employ envelope stress response systems, including the Cpx Two Component Signal Transduction System, that play a regulatory role of monitoring changes in the bacterial cell envelope and maintaining its integrity. The Cpx pathway is primarily induced by stress signals that affect the cell envelope such as alkaline pH, overexpression of specific membrane associated proteins, perturbations in membrane structure, and adhesion to abiotic surfaces. In response, the Cpx response regulates the expression of a number of genes involved in protein folding and degradation. Previous studies have demonstrated that the Cpx response is activated in the presence of excess external zinc, though the mechanism of activation and the direct effect that zinc has on *E. coli* is not fully understood. The objective of this thesis was to elucidate the mechanism that the Cpx response uses to sense external zinc and the role that the Cpx response has in mediating adaptation to zinc toxicity. Using luminescence and qPCR I provide results to show that the Cpx response senses the toxic effects of zinc in a CpxA dependent manner suggesting that zinc has an extracytoplasmic effect on *E. coli*. I also demonstrate that zinc has an effect on membrane permeability and provide evidence which suggests that the Cpx response could be sensing redox changes in the periplasm brought about by excess zinc such as the accumulation of cysteine, which is upregulated in response to excess zinc.

Dedication

I dedicate this thesis to my late parents Victoria and Simon Zimba. Dad wanted me to achieve more than he ever did, and mum had an unconditional love for me that gave me the courage to believe that I could overcome any obstacle that life could throw at me. “Mwabombeni mayo wandi pakunfyala, mayo nakula mayo” - thank you for giving birth to me mother, I am now grown.

Acknowledgments

I would like to begin by thanking Tracy for being the best mentor. Choosing the right supervisor played a particularly important role in my pursuit for graduate studies especially when it meant travelling across the world to become an international student in Canada. You took a chance on me, believed in me and I am here because of you. For that, I will forever be grateful! I would like to thank Martin Srayko for providing great insights that helped shape my thesis. Even though the majority of my thesis was not in your field, you always provided ideas that I probably would not have thought of on my own and I am especially happy that you were on my committee. I would like to thank Lisa Stein for being on my examining committee and for willing to share insights on my thesis.

I would like to thank Randi for inspiring me and being a source of knowledge and experience for me in times when I needed help. I am grateful to Julia, who even though we only had a brief encounter still gave great insights and ideas all the way from Australia. I would like to thank other members of my lab Sam and Rodrigo. We started this journey together as the three musketeers of the Raivio lab and you have been a source of support ever since. Laude and Junshu for being a delight in the lab. Yun for making my life easy and others that I have come across whose names I have not mentioned. I would like to thank members of the Dennis lab' especially Danielle for being so helpful.

Lastly thanks to my family and to everyone who's inspired, challenged and believed in me. You have all made my dreams come true. Thanks Wes!

Contents

List of Tables

List of Figures

List of Abbreviations

2CSTS: two-component signal transduction system

ATP: adenosine triphosphate

CAMP: cationic antimicrobial peptide

CPS: counts per second

cys: cysteine

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EPEC: enteropathogenic *E. coli*

EHEC: enterohemorrhagic *E. coli*

HK: histidine kinase

IM: inner membrane

IPTG: isopropyl-β-D-thiogalactopyranoside

LPS: lipopolysaccharide

OM: outer membrane

OMPs: outer membrane porins

PBS: phosphate buffer saline

PMF: proton motive force

RNA: ribonucleic acid

RR: response regulator

TLR4: toll-like receptor 4

Chapter 1: Introduction

1.1 *Escherichia coli*

Escherichia coli is one of the most characterised Gram-negative bacterial species. The K-12 strain is a choice for a lot of bacterial studies due to its amenability to genetic manipulation and the availability of information on the organism. In nature, the commensal strain of *E. coli*, lives in the gut of most mammals. Some strains of *E. coli* have developed the ability to cause infection. These pathogenic strains of *E. coli* are classified under two major groups based on whether the site of infection is localised within the intestines or occurs outside the intestines. Intestinal pathogens cause diarrhoea whereas extraintestinal pathogens cause urinary tract infections, sepsis and neonatal meningitis (Russo and Johnson, 2000; Johnson *et al.*, 2003). It is estimated that there are over 10^{21} *E. coli* strains in the human population alone and pathogenic *E. coli* strains are believed to be one of the major causes of infantile diarrheal deaths in the world (Conway and Cohen, 2015; Singh *et al.*, 2017).

Ingested *E. coli* faces a lot of environmental stress factors before it can colonise the intestines - stomach acid being one of the biggest challenges that *E. coli* must overcome before it can reach the intestines. Acid resistance mechanisms provide *E. coli* with the necessary protection for them to survive extreme pH (Zhao and Houry, 2010). Once in the colon, scavenging for nutrients becomes crucial for survival. The host immune system, antibiotics ingested by hosts, bile and other gut flora are still obstacles that *E. coli* has to face even after gaining enough nutrients for growth (Urdaneta and Casadesús, 2017; Palmela *et al.*, 2018). Yet, with all these deterrents in place, *E. coli* still manage to survive and successfully colonise the gut.

Understanding how *E. coli* navigate these complicated challenges can provide a foundation to uncovering how commensal organisms colonise the gut and give insight into how we can develop strategies to fight against pathogenic *E. coli* and other enteric pathogens as, to this day, infantile diarrhoeal deaths remain a major concern in the developing world.

1.2 Gram-negative bacterial cell envelope

The bacterial cell envelope is the first line of defence against antimicrobial agents that are present in the extracellular environment. The envelope also serves as a barrier that selectively controls the trafficking of nutrients from the extracellular environment into the cell and waste products out of the cell (Whitfield, Szymanski and Aebi, 2015). The cell envelope of Gram-negative bacteria is composed of a thin layer of peptidoglycan cell wall, surrounded by an outer membrane that forms the cell's exterior and a phospholipid bilayer that forms the inner membrane. The area occupied between both membranes is referred to as the periplasmic space (Dufresne and Paradis-Bleau, 2015). The inner and outer membranes differ in structure and function to suit the environments that they are in contact with. The inner membrane lies in direct contact with the cytoplasm and separates it from the periplasm. It is within this layer that membrane-bound functions, such as those carried out by eukaryotic organelles, occur. These include oxidative phosphorylation, lipid biosynthesis and protein translocation (Narita, 2011; Magalon, Arias-Cartin and Walburger, 2012; Arechaga, 2013; Kudva *et al.*, 2013). On the other hand, the outer membrane lies in direct contact with the extracellular environment, calling for functions that enable the selective prevention of toxic chemicals from permeating the cell (Zhang, Meredith and Kahne, 2013).

1.2.1 The outer membrane

Present only in gram-negative bacteria, the outer membrane is an asymmetric lipid membrane. Unlike the inner membrane, phospholipids are confined only to the inner leaflet of the outer membrane. The outer leaflet contains extracellular proteins and lipopolysaccharides (LPS) embedded at the periphery. LPS consists of an O-antigen polysaccharide of various lengths and a poly- or oligosaccharide region that is anchored in the outer membrane by a carbohydrate lipid moiety – lipid A (Kamio and Nikaido, 1976). Lipid A consists of two glucosamine units, an acyl chain and a phosphate (Sankaran and Wu, 1994). Accumulation of phosphate groups creates a strong negatively charged outer membrane that can interact with divalent cations such as Mg^{2+} and Ca^{2+}. This provides the stability and tightness to the outer membrane and gives the characteristic of resistance to external stress in Gram-negative bacteria (Clifton et al., 2015). Chelation of divalent cations from the outer membrane makes the gram-negative bacterial outer membrane more permeable and susceptible to external stress factors. Lipid A serves as the primary site in LPS that is involved in activating immune responses in mammalian hosts (Raetz and Whitfield, 2002). The lipid layer of the outer membrane is enriched with saturated fatty acids and phosphatidylethanolamine unlike the phospholipid layer of the inner membrane (Silhavy, Kahne and Walker, 2010).

The outer membrane houses, mainly, two classes of proteins: lipoproteins and outer membrane porins (OMPs) that allow the passive diffusion of solutes in and out of the cell. OMPs act as adhesion factors in virulence, channels for the uptake of nutrients, siderophore receptors and enzymes such as proteases and lipases (Rollauer et al., 2015). Lipoproteins contain lipid moieties that are attached to an amino-terminal cysteine residue embedded in the inner leaflet of the OM

(Sankaran and Wu, 1994). Though there are about 100 lipoproteins predicted to be present in *E. coli*, little is known about their function (Juncker et al., 2003; Miyadai et al., 2004). Integral OMPs differ from integral inner membrane proteins in that inner membrane proteins have α-helical transmembrane domains whereas integral OMPs span the outer membrane with amphipathic antiparallel β-strands that allow them to serve as channels (Fairman, Noinaj and Buchanan, 2011).

1.2.2 The peptidoglycan cell wall

Bacterial cell walls are composed of polymer peptidoglycan that consists of repeating units of the disaccharide N-acetyl glucosamine-N-acetyl muramic acid that are crosslinked by stretchable pentapeptides (Vollmer, Blanot and De Pedro, 2008). The rigidity of the cell wall provides the cell with its shape and protects the cell from lysis. The cell wall of gram-negative bacteria is 1-3 layers thick and is linked to the outer membrane via Braun's lipoprotein (Braun, 1975). The peptidoglycan is a target for some antibiotics, such as penicillin, which damage the peptidoglycan and cause the cell to lyse due to turgor pressure from the cytoplasm.

1.2.3 The inner membrane

The inner membrane (IM) maintains a tight control over the molecules that enter or exit the cell. IM proteins include integral IM proteins, lipoproteins and peripheral membrane proteins. Integral IM proteins span the IM with α-helical transmembrane domains, and lipoproteins are anchored to the outer leaflet of the IM. Around 25 % of all genes in the *E. coli* genome encode IM proteins (Luirink *et al.*, 2012). As a site for protein secretion, the inner membrane houses transporters that facilitate the secretion of essential envelope proteins while, at the same time, maintaining a tight control over the permeability of the inner membrane. Proteins destined for the

envelope are folded and inserted by the SecYEG translocon and YidC insertase that act independently yet cooperatively. Nascent proteins are channelled through a SecYEG cavity, of about 16 x 25 Å. The proteins travel from the cytoplasm to the periplasm, inner membrane or to chaperones that guide the proteins to their destination. The channel's cavity is closed off to the periplasm suggesting the need for the presence of substrate as a requirement to open the membrane cavity. The YidC insertase works in tandem with the SecYEG translocon to insert proteins into the IM and ensures that this occurs without compromising the barrier function of the IM (Breyton *et al.*, 2002).

The IM also houses components of the electron transport chain including NADH dehydrogenase I. Unlike NADH dehydrogenase II, the genes encoding for NADH I are expressed during both aerobic and anaerobic respiration. NADH dehydrogenase I is also involved in pumping protons from the cytoplasm to the periplasm to produce the proton motive force (PMF) (Friedrich, Dekovic and Burschel, 2016).

1.2.4 The periplasm

The periplasm is a highly viscous compartment that constitutes approximately 10% of the cell's volume (Van Wielink and Duine, 1990). Macromolecules and solutes that are imported into the cell have to pass through the periplasm in order to reach the appropriate transporters that actively import them into the cell. The periplasmic space is an oxidizing environment containing enzymes that catalyse the formation of disulphide bonds (Landeta, Boyd and Beckwith, 2018). The periplasm also houses several protein folding and degrading factors that are crucial for maintaining the cell's structure and ensuring that envelope-localised proteins are functional even in times of stress (Goemans, Denoncin and Collet, 2014). The sensing domains of many IM

signalling protein receptors, such as histidine kinases of most two component systems, detect signals from the periplasm. Devoid of ATP, the aforementioned activities occur without an obvious source of energy.

1.3 Bacterial envelope stress

The bacterial envelope plays an essential role in the survival of the cell – providing structural support as well as carrying out cellular functions that are vital for the cell. Most sensory proteins are housed in the envelope and nutrients are meticulously sieved through the envelope. The envelope is a target for many antimicrobial agents. Changes in environmental conditions that the cell may encounter in a mammalian host such as stomach acid, antibiotics or bile, combined with high internal turgor pressure can sometimes result in perturbations in the envelope, and the essential cellular processes that take place in this compartment. Therefore, monitoring, maintaining, and adapting the envelope structure and function is crucial for the cell's survival.

1.3.1 Overview of envelope stress response systems in enteric gram-negative bacteria

Envelope stress response systems play a regulatory role by monitoring changes in the bacterial cell envelope and maintaining its integrity. They are defined by their roles in responding to a number of threats to cell function, including misfolded proteins, disruptions in proton motive force and toxic compounds and their potential consequences on the envelope among other things. The σ^E response was among the first to be identified. It is known to respond to perturbations in the outer membrane and is essential at high temperatures (Raivio and Silhavy, 2001). The phage shock protein (Psp) response is known to sense perturbations to the proton motive force (Flores-Kim and Darwin, 2016). The bacterial adaptive envelope (Bae) response senses toxic compounds such as

indole and ethanol and regulates genes encoding multidrug transporters (Raivio, 2005). Collectively these envelope stress response systems, and others, form a set of signalling pathways that regulate genes to adapt to changes in envelope homeostasis.

1.3.2 Two Component Signal Transduction Systems (2CSTS)

Environmental changes that can affect bacterial metabolism and cause disruptions in the bacterial envelope. As such, bacteria need to respond in an appropriate manner in order to adapt to such environmental changes. 2CSTSs consisting of an inner membrane sensory kinase and a cytoplasmic response regulator (RR) serve as primary modes of action for bacteria to respond to such environmental stresses. 2CSTSs are found abundantly in eubacteria with a limited amount in archaea and eukaryotes. 2CSTSs regulate a subset of envelope stress responses. 2CSTSs consist of an inner membrane histidine kinase (HK) that, in the presence of inducing environmental signals, auto-phosphorylates by addition of a phosphoryl group on to a conserved histidine residue. The HK then transfers its phosphoryl group onto a conserved aspartate residue of RR which then acts as a transcription factor – either activating or repressing the expression of genes within its regulon. In the absence of inducing cues, the sensory kinase sometimes acts as a phosphatase that cleaves off the phosphate group from the RR thereby rendering it inactive. Some sensory proteins work with an auxiliary regulator that senses certain perturbations in the envelope so as to induce a specific response that maintains the envelope homeostasis (Capra and Laub, 2012).

1.3.3 The PhoPQ response

The PhoPQ response is a two-component system that responds to limiting extracellular Mg^{2+} and Ca^{2+} and cationic antimicrobial peptides (CAMPs). The system consists of an inner membrane sensor kinase PhoQ and its cognate response regulator PhoP. Under high Mg^{2+} concentrations, PhoQ exhibits phosphatase activity by dephosphorylating PhoP. In conditions of low Mg^{2+} or in the presence of CAMPs, PhoQ acts as a kinase and phosphorylates PhoP to activate the system. In *Salmonella*, the PhoPQ system regulates the expression of genes involved in modifying LPS as well as genes involved in Mg^{2+} transport including *mgtA* and *mgtB*. MgtA and MgtB constitute two out of the three Mg^{2+} transporters in *Salmonella*. It is believed that the PhoPQ response is activated under limiting Mg^{2+} conditions to recruit Mg^{2+} that is required to maintain stability of LPS in the outer membrane (Groisman, 2001). The PhoPQ system is well studied in *Salmonella* where it has been shown to be activated by CAMPs released by the host immune system most especially in phagocytes. This results in LPS modifications, particularly the lipid A portion that becomes modified by aminoarabinose and fatty acids, decreasing the outer membrane fluidity and negative charge. This in turn results in resistance to CAMPs and reduces the ability to induce host immunity through Toll-like receptor 4 (TLR4) (Hancock and McPhee, 2005).

1.3.4 The Conjugative plasmid expression (Cpx) 2CSTS

A genetic screening of mutations that negatively affected the expression of the conjugative F-plasmid in *E. coli* led to the discovery of the Cpx 2CSTS (McEwen and Silverman, 1980a). The mutant strains identified showed several phenotypes including temperature sensitivity and were auxotrophic to isoleucine and valine (McEwen and Silverman, 1980b, 1982). An interesting phenotype that was also observed was the mutant's resistance to aminoglycoside antibiotics and

colicins (Rainwater and Silverman, 1990). As most of the observed phenotypes affect the bacterial envelope, the Cpx 2CSTS became linked to bacterial envelope activity. Further exploration of the system showed that the *cpx* locus encoded a histidine kinase CpxA and a cognate response regulator CpxR (Dong *et al.*, 1993). With further studies, it was proposed that CpxA and CpxR made up a two-component signal transduction system that mainly responded to perturbations in the cell envelope.

And so, the Cpx 2CSTS can be described as an envelope stress mechanism that acts on misfolded proteins. It consists of the inner membrane sensor kinase: CpxA and a cytosolic response regulator CpxR. Activation of the Cpx 2CSTS involves phosphorylation of CpxR which acts as a transcription factor that either represses or activates the expression of genes within the Cpx regulon. In the absence of stress signals CpxA acts as a phosphatase, de-phosphorylating CpxR and therefore inactivating the Cpx 2CSTS (Paul N Danese *et al.*, 1995).

Some heterologous kinases and small-molecule phospho-donors such as acetyl-phosphate are capable of phosphorylating CpxR (P N Danese *et al.*, 1995). In such cases, the phosphatase activity of CpxA is crucial for keeping the Cpx 2CSTS inactive in the presence of such unwanted heterologous kinase activity (Raivio and Silhavy, 1997). Phosphorylated CpxR modulates the transcription of numerous genes encoding proteins of function in the IM and envelope such as; periplasmic chaperone proteins and protein degrading factors; genes encoding envelope localised protein complexes; genes involved in cell wall biogenesis; transporters; efflux pumps and genes of unknown function (Price and Raivio, 2009; Raivio, Leblanc and Price, 2013). CpxP acts as the periplasmic inhibitor of the Cpx response and its gene expression is heavily induced upon

activation of the Cpx 2CSTS (Raivio, Popkin and Silhavy, 1999; DiGiuseppe and Silhavy, 2003). For this reason, it is commonly used as a reporter for Cpx activity.

1.3.4.1 Inducing cues

The Cpx pathway is primarily induced by stress signals that affect the cell envelope such as alkaline pH, overexpression of specific membrane associated proteins, perturbations in membrane structure, and adhesion to abiotic surfaces (Snyder *et al.*, 1995; Danese and Silhavy, 1998; Hung *et al.*, 2001). Evidence of Cpx induction by overexpression of misfolded periplasmic proteins such as Pap subunits of the P pilin and the bundle forming pilus subunit BfpA pointed out the Cpx response was most likely activated in response to stress signals that would lead to protein misfolding (Nevesinjac and Raivio, 2005). These observations, taken together with the knowledge that activation of the Cpx pathway leads to an upregulation of genes encoding chaperones and proteases, gave rise to the idea that the Cpx response was most likely activated in response to stress signals that would lead to protein misfolding.

Alkaline pH strongly induces the Cpx response although, the exact mechanism and inducing cue generated from alkaline pH is still unknown. It is, however, believed that alkaline pH is likely to result in denatured proteins that subsequently activate the Cpx response (Danese and Silhavy, 1998). Other studies have demonstrated that perturbations in the membrane arising from a lack of phosphatidylethanolamine in the cell membrane result in the upregulation of the Cpx response, thereby inducing the expression of the periplasmic protease DegP (Mileykovskaya and Dowhan, 1997).

In another study, it was observed that the Cpx 2CSTS was activated by the accumulation of lipid II, an intermediate of enterobacterial common antigen synthesis, in the inner membrane. Enterobacterial common antigen is a glycolipid located on the outer leaflet of the outer membrane in members of the *Enterobacteriaceae* family. Omitting the glycolipid on its own did not induce the Cpx response but rather it was observed that accumulation of its intermediate: lipid II, resulted in sensitivity to bile salts and induction of the Cpx and σ^E responses. Although the exact mechanism involved in this activation is not known, it is possible that protein folding in the envelope may be the underlining reason for the activation of the Cpx response in this case as well (Danese *et al.*, 1998).

Adhesion to abiotic surfaces is another inducer of the Cpx pathway. While sensing of misfolded proteins likely occurs through the periplasmic portion of CpxA, sensing attachment occurs through the OM lipoprotein NlpE. It is believed that initial adhesion to a hydrophobic surface induces conformational changes in the OM lipoprotein NlpE which subsequently stimulates CpxA activation (Otto and Silhavy, 2002).

Characterization of the Cpx regulon in *Vibrio cholerae* revealed that the Cpx response affected genes that are involved in iron metabolism and acquisition (Acosta Amador, 2015). In a follow-up study, it was found that the Cpx response in *V. cholerae* was activated in a limited iron environment. Predominantly found in the form of iron sulphur clusters (Fe-S), iron is required for a variety of bacterial processes that includes respiration, photosynthesis and nitrogen fixation. Activation of the Cpx response in limited iron environments is believed to replenish iron cofactor levels to correct respiration defects (Acosta, Pukatzki and Raivio, 2015).

In *E. coli*, it has been shown that the Cpx response is activated in metal-limited LB and that this activation can be reversed by addition of exogenous zinc. The metal status of the cell, in this case, is sensed independently of the sensor kinase CpxA, suggesting that metal depletion activates the Cpx response in a cytoplasmic fashion (Wong, 2015).

1.3.4.2 The Cpx regulon

From its discovery, several investigations on the Cpx response have been carried out to further our understanding of its function. A microarray was conducted to identify genes that are regulated by the Cpx response and even more recent than that, RNA-Seq analysis was performed to further elucidate the overall function of the Cpx response (Raivio, Leblanc and Price, 2013; Raivio and Peng, unpublished findings) . It has been deduced from these studies that the majority of genes under the control of the Cpx response can be categorised into three major groups - genes encoding proteins that are a part of: envelope localised structures, inner membrane proteins or protein folding and degrading factors (Price and Raivio, 2009). Other studies have linked the response to biofilm formation and pathogenesis (Dorel, Lejeune and Rodrigue, 2006).

1.3.4.2.1 Envelope localised structures

Activation of the Cpx response leads to the down regulation of genes that encode several bulky structures localised within the envelope. These include pili, flagella and the type III secretion system. In many cases, the Cpx response represses the transcription of these genes directly or through the regulation of genes involved in pathogenesis (MacRitchie *et al.*, 2008; Vogt *et al.*, 2010). It is hypothesised that the Cpx response represses the transcription of these bulky envelope structures to reduce envelope stress by conserving energy that is consumed by such envelope

complexes. By so doing, the energy can be redirected towards re-folding/degrading misfolded envelope proteins or towards the expulsion of toxic metabolites that accumulate within the periplasm under stress conditions. In addition to this, repression prevents these structures from being otherwise misassembled during envelope stress which would exacerbate damage to the envelope.

1.3.4.2.2 Protein folding and degrading factors

Among the first genes characterised to be part of the Cpx regulon were those encoding proteins directly involved in protein folding and degradation in the envelope (*degP, dsbA* and *ppiA*) (P N Danese *et al.*, 1995; Danese and Silhavy, 1997; Pogliano *et al.*, 1997). DsbA is a bacterial disulphide bond oxidoreductase that is responsible for the folding of envelope proteins. It functions by introducing disulphide bonds that are crucial to the folding of envelope proteins. The resulting reaction leads to the reduction of DsbA. For continued functioning of the enzyme, electrons are transferred onto DsbB and consequently onto the respiratory chain via the quinone pool. Mismatched disulphide bonds can be corrected through the disulphide bond isomerase DsbC whose gene is also regulated by the Cpx response. DsbD receives electrons from the cytoplasmic thioredoxin TrxA, to maintain DsbC in its active reduced form (Figure 1.1) (Messens and Collet, 2006; Landeta, Boyd and Beckwith, 2018).

The Cpx response also highly regulates the gene encoding the DegP periplasmic endoprotease. Misfolded or damaged envelope proteins that are not corrected by protein folding factors end up being degraded by periplasmic proteases. DegP degrades misfolded proteins that accumulate within the periplasm as such proteins can be detrimental to the cell's survival (Pogliano

et al., 1997). The periplasmic inhibitor of the Cpx response: CpxP, also exhibits chaperone activity and targets misfolded proteins to DegP (Vogt and Raivio, 2012).

Overall, the Cpx response regulates protein folding and degrading factors to alleviate envelope stress that is brought about by misfolded proteins accumulating within the envelope. This includes oxidatively damaged proteins, unstable mutant proteins and protein aggregates.

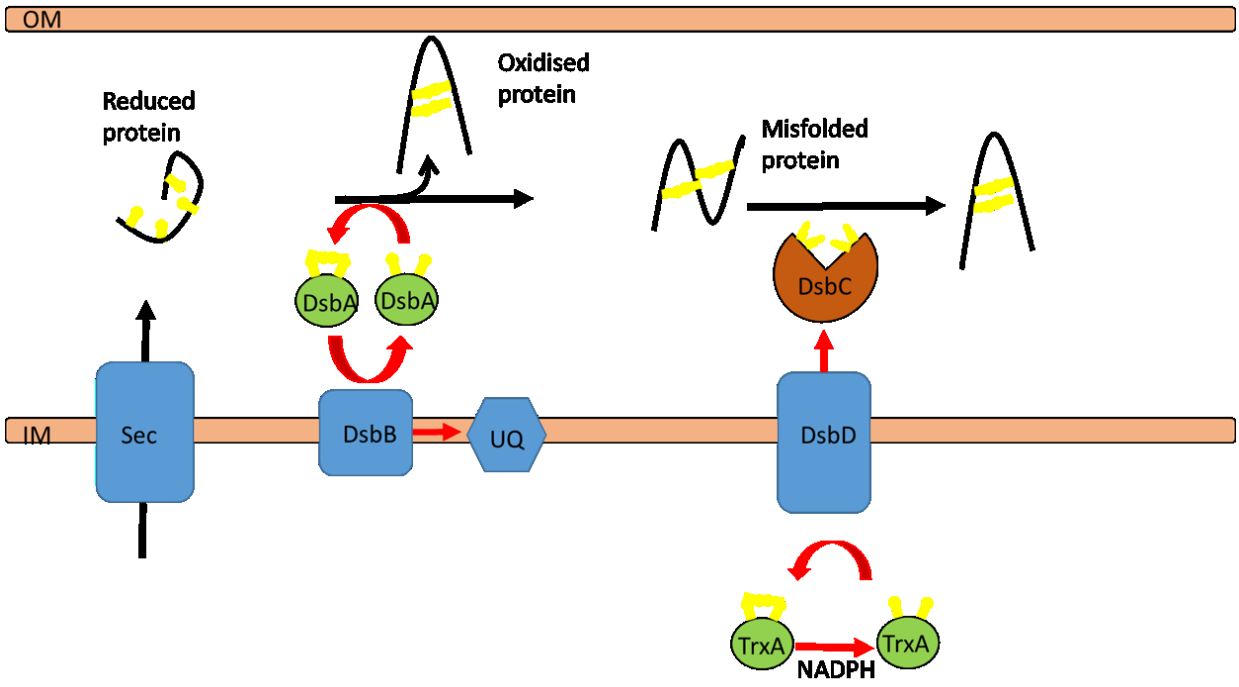

Figure 1.1: Disulphide bond formation. Proteins that require disulphide bonds for their proper folding are exported into the periplasm usually through the Sec pathway. DsbA introduces disulphide bonds into these proteins and as a result becomes reduced in the process. For proper functioning of DsbA, electrons are transferred onto the quinone pool via DsbB to keep DsbA in its oxidized form. Mismatched disulphide bonds can be corrected by DsbC or they end up being degraded. For this purpose, DsbC needs to be maintained in its reduced form and this is achieved through DsbD, which acquires electrons from the cytoplasmic thioredoxin, TrxA (Berkmen, 2012). Red arrows indicate electron flow and black arrows indicate products.

1.3.4.3 Role of the Cpx response in cellular and metabolic activities

1.3.4.3.1 Resistance to antimicrobials

The bacterial envelope is the first site of contact of antimicrobial agents. Bacteria are exposed to many toxic agents through the different environments that they encounter, and these agents include antibiotics that are taken for clinical or agricultural purposes, antimicrobial agents that are produced by other bacteria, toxic metabolites that are endogenously produced and the immune response produced by a mammalian host. The Cpx response, among other envelope stress response systems, has been associated with the role of mediating stress caused by these antimicrobial agents (Guest and Raivio, 2016).

Though the gram-negative envelope provides a highly selective barrier, antibiotics can gain access to the cell by self-promoted uptake involving nonspecific charge interactions at the outer membrane or through passive diffusion through porins. Entry of antibiotics into the cytoplasmic space can be achieved in a fashion that requires the proton motive force, ATP, and/or specific transporter proteins in the inner membrane (Chopra, 1988). The most commonly used antibiotics act by inhibiting protein synthesis, impeding cell wall assembly or by interfering with nucleic acids (Pucci and Bush, 2013). Activation of the Cpx response has been shown to increase resistance to aminoglycosides, a class of antibiotics that targets protein synthesis. Part of this observed phenotype has been linked to increased expression of *tolC* although deleting *tolC* does not completely abolish resistance suggesting that the Cpx response has an additional role in resistance to aminoglycosides (Weatherspoon-Griffin *et al.*, 2014). One study, unbeknownst at the time, showed that mutations in *cpxA* that resulted in resistance to aminoglycosides also lead to less

accumulation of aminoglycosides suggesting that the Cpx response leads to less uptake of this class of antibiotics (Bryan and Van den Elzen, 1976).

The Cpx response is also implicated in resistance to antibiotics through its ability to regulate genes that alleviate protein translocation stress that could be induced by antibiotics. It is believed that antibiotics that target translation elongation result in jammed translocators. This, in turn, leads to proteolytic degradation of these protein translocators such as SecY as secretion of most proteins occurs in a co-translational fashion. The Cpx pathway, in part, relieves this stress through its regulation of YccA, an inhibitor of the FtsH protease that is responsible for degrading components of the Sec translocon (van Stelten *et al.*, 2009).

1.3.4.3.2 Regulation of transport

Reduced proline and lactose uptake were among the first phenotypes to be observed in a *cpxA** mutant (Rainwater and Silverman, 1990). In *Vibrio cholerae*, the Cpx regulon is enriched with genes that are involved in iron uptake and metabolism. It has been demonstrated that the Cpx response is required for adaptation to low iron conditions when grown on non-fermentable sugars (Acosta, Pukatzki and Raivio, 2015). Deletion of certain efflux pumps also activates the Cpx response and this activation can be reversed by addition of exogeneous iron. The underlying implication of these results suggest that the Cpx response, in *V. cholerae*, maintains iron homeostasis by regulating iron acquisition factors and iron containing respiratory enzymes (Acosta, Pukatzki and Raivio, 2015).

In *E. coli*, the Cpx response downregulates the expression of several inner membrane transporters. These include the genes involved in the uptake of C4-dicarboxylic acids, galactose,

glucarate, and maltose (Raivio, Leblanc and Price, 2013). The *glpT* and *uphT* genes encoding glycerol-3-phosphate and glucose-6-phosphate uptake transporters, respectively, are directly repressed by CpxR and, interestingly, this observation has been implicated in resistance to fosfomycin (Kurabayashi *et al.*, 2014).

A microarray study undertaken to further characterise members of the Cpx regulon in *E. coli*, showed that overexpression of *nlpE* leads to an increase in the expression of genes involved in cysteine biosynthesis and cysteine/cystine transport. Among these genes includes the sulphate uptake genes *sbp*, *cysAWP* and the cystine importer *ydjN* (Raivio, Leblanc and Price, 2013).

1.3.4.3.3 Regulation of respiratory complexes

Regulation of respiratory complexes NDH-I and cytochrome bo3 by the Cpx response has been shown through microarray study and further confirmed though luminescent transcriptional reporters (Raivio, Leblanc and Price, 2013; Guest *et al.*, 2017). The results obtained suggest that the Cpx response decreases respiratory activity by repressing expression of the entire *nuo* and *cyo* operons. CpxR binding sites have been confirmed upstream of both *nuoA* and *cyoA* transcriptional start sites showing that regulation of these genes by the Cpx response is likely direct. It is hypothesised that respiration is toxic to the cell during conditions of envelope stress and/or assembly of these complicated complexes cannot happen in the presence of Cpx-inducing signals. Deletion of either the *nuo* or *cyo* operons decreases Cpx pathway activity suggesting that these complexes contribute to envelope stress sensed by CpxA. The implication of these results is that the activation of the Cpx response reduces respiration. To confirm this, the rate of oxygen consumption was determined in a *cpxA** mutant, in which the Cpx response is constitutively activated. Indeed, it was observed that oxygen consumption was reduced in this mutant supporting

the idea that activation of the Cpx response reduces expression of respiratory complexes (Guest *et al.*, 2017).

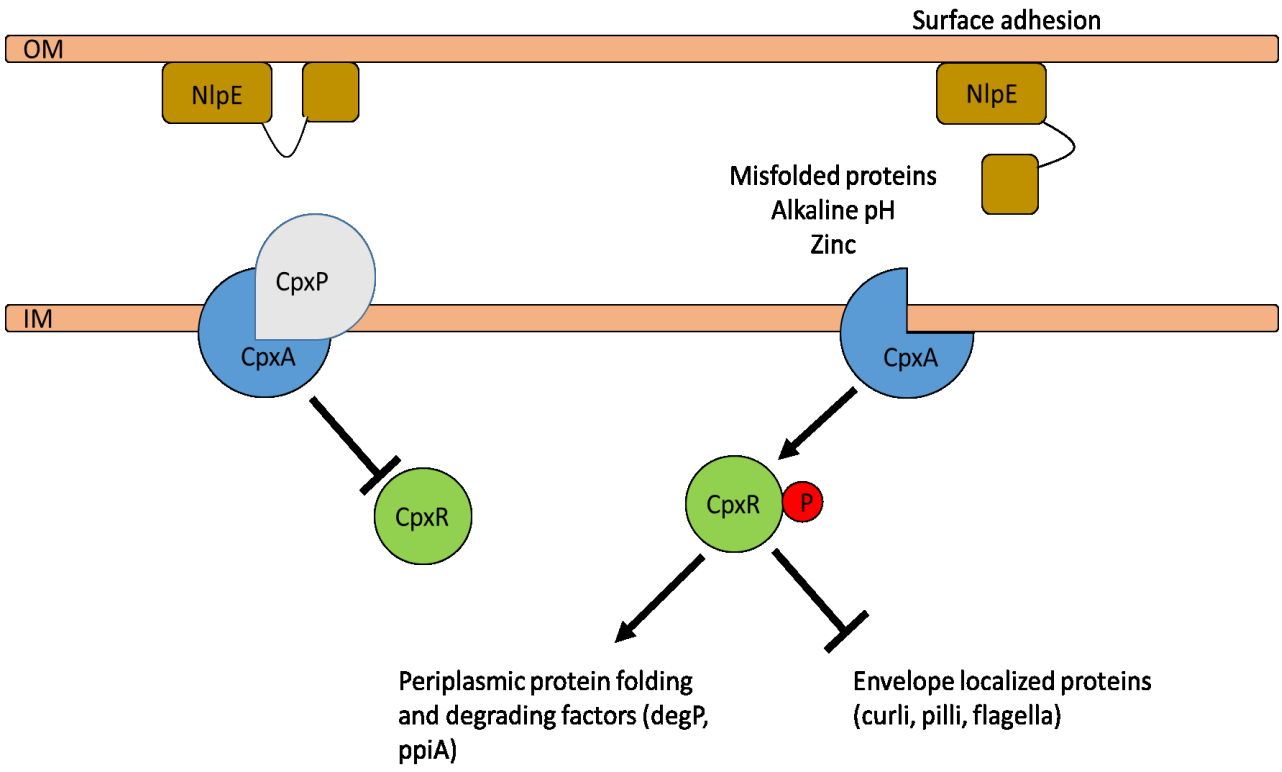

Figure 1.2: The Cpx envelope stress response. The Cpx envelope stress response consists of a histidine kinase, CpxA, and its cognate response regulator, CpxR. Under conditions that cause envelope stress such as the accumulation of misfolded proteins in the periplasm, CpxA acts as a kinase, phosphorylating CpxR which goes on to regulate protein folding and degrading factors. CpxR also represses the expression of curli and pili in order to alleviate envelope stress under these conditions. CpxP is an auxiliary regulator of the Cpx response and is believed to interact directly with CpxA. Activation of the response requires degradation of CpxP by the DegP protease. NlpE is a lipoprotein that is believed to sense surface adhesion and activate the Cpx response. Under non-inducing conditions, CpxA acts a phosphatase so as to dephosphorylate CpxR in the absence of stress (Vogt and Raivio, 2012).

1.4 Cysteine and the Cpx response

Cysteine is a proteinogenic amino acid with the chemical formula $HO_2CCH(NH_2)CH_2SH$. The sulphur in cysteine is an important atom that makes the amino acid highly reactive and distinguishes it from serine which has an oxygen atom in place of sulphur. As such, most sulphur containing cellular components such as methionine, thiamine, biotin, lipoic acid, coenzyme A and coenzyme M require cysteine as a precursor. Cysteine plays an important role in protein structure by forming disulphide bridges that help stabilise protein folding. It is also required for the biogenesis of [Fe - S] clusters and is found in the catalytic site of several enzymes. In addition to the aforementioned roles, cysteine and its derived proteins, including thioredoxin and glutathione, play an essential role in protecting against oxidative stress. Cysteine is known specifically to react with hydrogen peroxide to form cystine and water (Guédon and Martin-verstraete, 2006). Microarray data indicated that genes involved in cysteine biosynthesis are upregulated when the Cpx response is induced by *nlpE* overexpression (Price and Raivio, 2009). The role that cysteine could play in adapting to envelope stress will be explored later in this thesis.

1.4.1 Cysteine biosynthesis

1.4.1.1 Sulphate import and reduction to sulphide

Sulphate import in *E. coli* is facilitated by the periplasmic sulphate binding proteins, Sbp (sulphate binding protein) and CysP, the CysT and CysW permeases and an ATP-binding subunit, CysA (Figure 1.3). The *sbp-cysPTWA* cluster is essential to sulphur import as deletion of either *cysA*, *cysT*, or a double deletion of *sbp* and *cysP* results in cysteine auxotrophy (Kertesz, 2001).

The first step in assimilating inorganic sulphate involves the activation of sulphate by sulphate adenylyltransferase, in an ATP driven reaction that results in adenosine 5'-phosphosulfate and pyrophosphate. The enzyme consists of two subunits encoded by *cysD* and *cysN*. Adenosine 5'-phosphosulfate is then phosphorylated by the *cysC* encoded, adenosine 5'-phosphosulphate kinase which leads to the formation of 3'-phosphoadenosine 5'-phosphosulphate (PAPS). The third step involves cleaving of sulphite from PAPS by *cysH* encoded PAPS sulfotransferase. Sulphite is then reduced to sulphide by NADPH sulphite reductase whose α and β subunits are encoded by *cysJ* and *cysI* respectively (Guédon and Martin-verstraete, 2006).

1.4.1.2 Cysteine biosynthesis from reduced sulphide

In *E. coli*, serine is used as a precursor for cysteine biosynthesis. Serine is first acetylated by serine transacetylase encoded by *cysE*, to form *O*-acetyl-serine. This reaction requires acetyl-coenzyme A and is inhibited by cysteine via feedback inhibition as a way of controlling the intracellular concentration of cysteine. The final step in cysteine biosynthesis involves the catalytic conversion of *O*-acetyl-serine and sulphide to form cysteine and acetate. This reaction is catalysed by *O*-acetyl-serine-sulphydrylase. There are two *O*-acetyl-serine-sulphydrylase enzymes: CysK and CysM. In contrast to CysK, a mutant lacking CysM is still capable of growing on minimal media lacking cysteine showing that CysK is essential for cysteine biosynthesis (Guédon and Martin-verstraete, 2006).

1.4.2 Cysteine/Cystine trafficking

Cysteine can be toxic to the cell under high concentrations. To maintain cytosolic concentrations of cysteine below toxic levels, bacteria have developed systems to excrete cysteine.

Currently, three systems for cysteine secretion have been characterised in *E. coli*. YdeD and YfiK are involved in the secretion of OAS, cysteine and cysteine derivatives. Though the role that cysteine plays in the periplasm is not fully understood, it has been demonstrated that deletion of *ydeD* leads to increased susceptibility to hydrogen peroxide in *E. coli* (Figure 1.3). Once in the periplasm, cysteine can be oxidized to cystine by hydrogen peroxide, a product of the catalytic activity of superoxide dismutase (Takagi, 2010).

Cystine uptake from the periplasm has been extensively investigated in enteric bacteria. Two main transporters have been characterised including YdjN, a H^+ symporter, and FliY, a periplasmic protein of the cystine ABC transport system in *E. coli* encoded by *ydjN* and *fliY* respectively. *fliY* lies on the same operon as *fliA*, the major flagellar subunit, though there is evidence to suggest that *fliY* is not involved in motility. Deletion of *fliY* results in increased susceptibility to hydrogen peroxide and significant impairment of L-cystine uptake (Guédon and Martin-verstraete, 2006; Takagi, 2010).

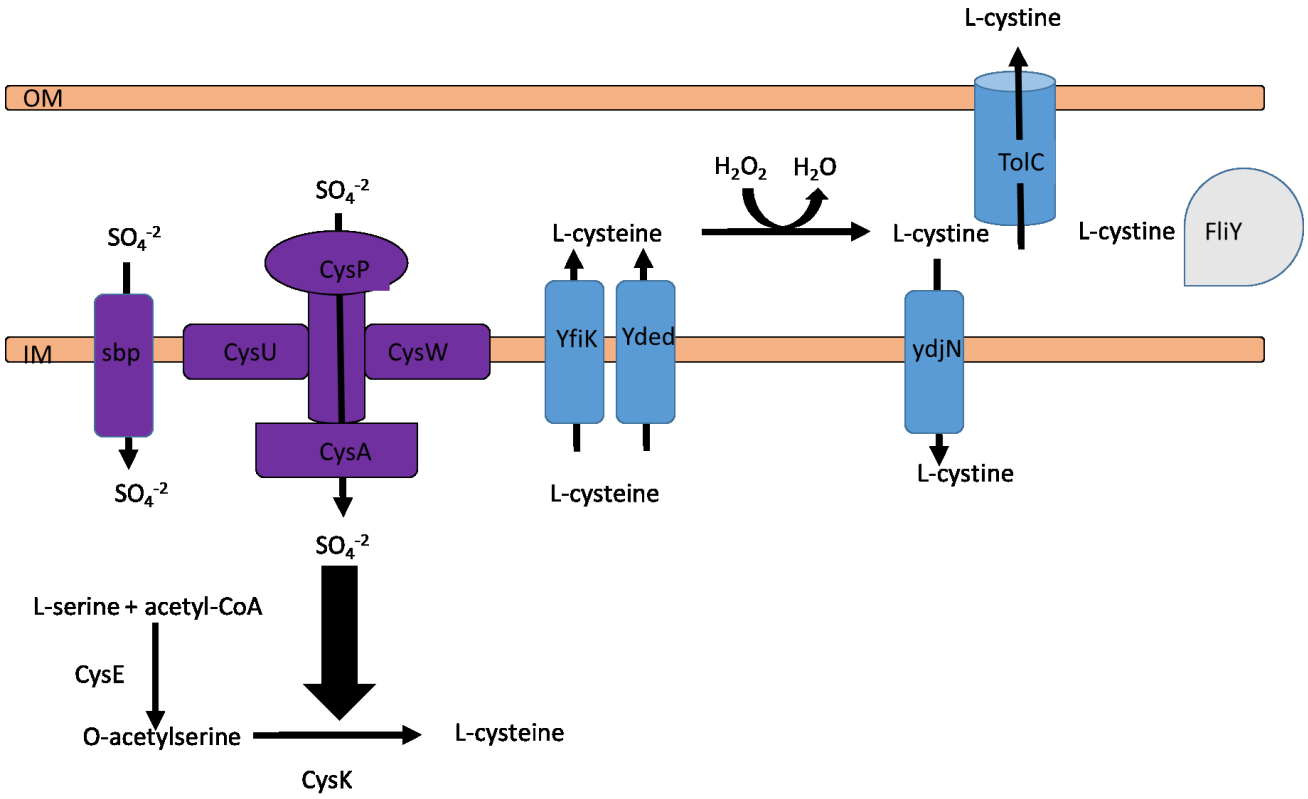

Figure 1.3: Cysteine and cystine generation and transport. Cysteine biosynthesis involves the incorporation of reduced sulphate into acetylated serine. Sulphate is imported through the CysAUWP and sbp importers, *cysE* encodes the serine transacetylase that converts L-serine and acetyl-CoA into O-acetylserine. Reduced sulphate is introduced into acetylated serine by the *cysK* encoded two O-acetyl-serine-sulphydrylase to form L-cysteine. Cytoplasmic concentrations of cysteine are strictly controlled due to its highly reactive nature. Cysteine can be exported to the periplasm, via YdeD or YfiK, where it is believed to act as an antioxidant by reducing hydrogen peroxide. The oxidized cysteine (cystine) can then either be exported out of the cell via TolC or imported into the cytoplasm via FliY or YdjN (Kredich, 2008a).

1.5 Role of zinc in metabolism

Metals play essential roles in cellular metabolism including maintaining protein structure and activity. In enteric bacteria, metals are involved in conserved processes such as central metabolism, respiration and transcription. As a defense mechanism, mammalian hosts often chelate metals as a response to infection and bacteria have developed mechanisms to maintain metal homeostasis in different environments that they may encounter.

After iron, zinc is the second most abundant heavy transitional element found in living organisms (Broadley *et al.*, 2007). In eukaryotes, zinc is known to have a vital role in growth, development and immune activity. It is essential for biomembrane formation and catalytic function in some metalloenzymes. It is also associated with transcription as evidenced from the abundance of zinc-finger transcription factors (Haase and Rink, 2014). Zinc deficiency has been associated with diarrheal diseases, including those caused by enteropathogenic *E. coli* (EPEC), though the mechanism by which it causes diarrhoea is not fully understood (Skrovanek *et al.*, 2014).

The role of zinc in bacteria is associated with providing catalytic and structural stability in peptidases and proteases. The NelC type III secretion system effector present in EPEC and enterohemorrhagic *E. coli* (EHEC) that destabilizes the host immune response during infection by cleaving p65 in the human NF-κB complex has been shown to require zinc for *in-vitro* activity (Li *et al.*, 2014). Cytosolic zinc concentrations are tightly controlled by unique zinc transporters that maintain zinc homeostasis (Blencowe and Morby, 2003). The chaperone CpxP was crystallised with zinc and there were several zinc ions found to be bound to the structure. Though the significance of zinc sites in the CpxP structure remain to be elucidated, the similarities in structure between CpxP and ZraP, another member of the CpxP family of chaperones that is a zinc-

dependent chaperone, hint at common periplasmic functions (Thede *et al.*, 2011). In addition the mRNA levels of this family of chaperones was found to be increased by, 11, 47 and 21 fold for *cpxP*, *spy* and *zraP* respectively in response to zinc (Lee, Barrett and Poole, 2005).

1.5.1 Zinc uptake

Zinc is an essential element required within the cell for various purposes. The concentration of zinc within the cell is kept at a constant in order to provide the cell with sufficient amounts of zinc, and yet prevent toxicity. The uptake of extracellular zinc into the periplasm is believed to be unspecific with most evidence in the literature supporting the idea that extracellular zinc enters the periplasm via TonB dependent transporters under low zinc conditions (Blindauer, 2015).

Intracellular zinc concentration in *E*. coli is sensed by the cytoplasmic transcriptional regulator Zur (Zinc uptake regulator) which has a high sensitivity for zinc in the femtomolar range. The concentration of intracellular zinc is estimated to be in the micromolar range with most of it being bound to proteins hence limiting the availability of free zinc. Under surplus zinc conditions, zinc bound Zur represses genes that are involved in zinc uptake. During conditions of zinc depletion, genes involved in zinc uptake and acquisition are induced and the uptake is mediated by many zinc transporters with different affinities for zinc (Patzer and Hantke, 1998; Grass *et al.*, 2002; Graham *et al.*, 2009).

ZnuABC is a high-affinity zinc uptake system belonging to the ATP-binding cassette (ABC) family of transporters. ZnuA being the periplasmic substrate binding protein with a high affinity for zinc. ZnuA has two binding sites: a high affinity site with a conserved histidine and aspartate residue that is extremely useful under zinc limiting conditions and a second site that is

believed to have a lower affinity for zinc (Hantke, 2005). The protein forms a complex with the inner membrane permease ZnuB which helps facilitate the transfer of zinc into the channel protein ZnuB (Figure 1.4). ZnuC is a cytoplasmic ATPase involved in the ATP hydrolysis driven uptake of zinc though the direct mechanism of this has not been fully explored (Blindauer, 2015). ZupT, a recently identified zinc transporter, is a member of the ZIP (zinc-regulated transporters and iron-regulated transporter-like proteins) family of proteins identified as either zinc or iron transporters. ZupT has a lower affinity for zinc than ZnuABC, as deletion of *zupT* shows less growth sensitivity in the presence of excess EDTA as compared to strains lacking *znuABC* (Grass *et al.*, 2002). ZnuA can acquire zinc from ZinT, a lower affinity zinc binding protein, whose physiological role is not fully understood because *zinT* mutants appear to be vital only in conditions of severe zinc depletion in in *E. coli* strains lacking *znuABC* (Graham *et al.*, 2009).

1.5.2 Zinc efflux

At high concentrations, zinc is known to be toxic to the cell. Intracellular zinc concentrations are tightly controlled by zinc transporters and under conditions of excess zinc, efflux is mediated by the high affinity zinc binding transporter ZntA (Figure 1.4). ZntA is a P-type ATPase that confers resistance to Zn(II), Pb(II) and Cd(II). Expression of *zntA* is mediated by ZntR and it is evidenced, by *in vitro* studies, that ZntR senses zinc in the fentomolar range. On the other hand, it has been shown that ZntR senses external zinc in the millimolar range suggesting that most intracellular zinc is present as protein complexes or zinc chelating compounds (Blindauer, 2015). ZntR belongs to the MerR-like prokaryotic transcriptional regulators. In wild type cells, the reported MIC for zinc is 2.25 mM whereas in mutants lacking either *zntA* or *zntR* the MIC drops to about 0.75 mM (Brocklehurst *et al.*, 1999). Zinc bound ZntR activates the transcription of *zntA*

by unwinding and widening the minor grove of DNA at the *zntA* promotor region thereby creating suitable conditions for RNA polymerase binding.

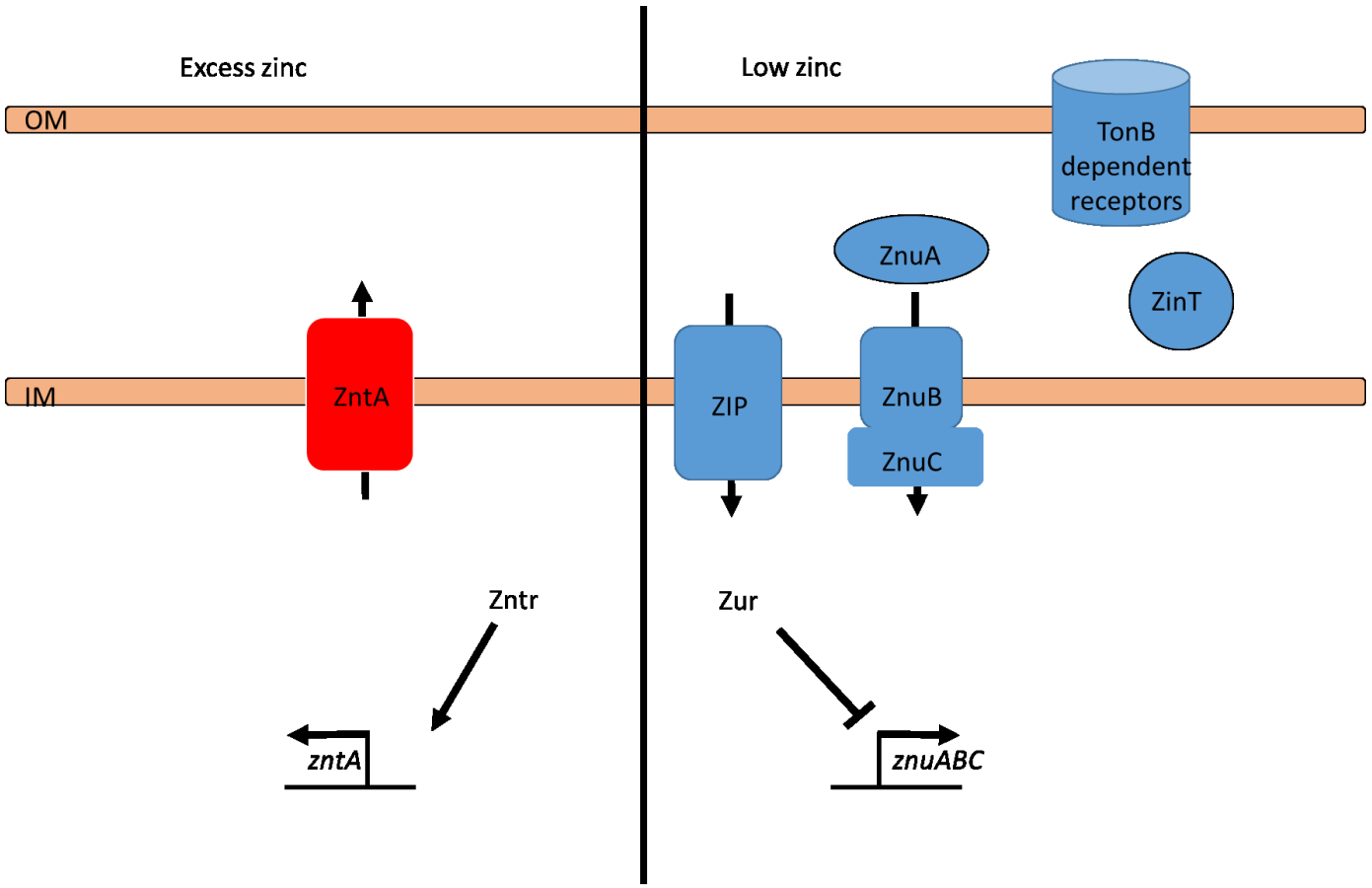

Figure 1.4: Zinc transport in *E. coli*. Extracellular zinc is believed to enter the periplasm through TonB dependent transporters in an unspecific form. Cytoplasmic concentrations of zinc are sensed by Zur which regulates *znuABC* transporter genes. Under low zinc conditions, *znuABC* transporter genes, ZIP proteins and *zinT* are highly induced. Zinc bound represses these genes under excess zinc conditions. *zntA* is highly expressed under excess conditions and is regulated by ZntR which senses cytoplasmic zinc concentrations in the femtomolar range (Blindauer, 2015).

1.6 Effects of excess zinc on *E. coli*

Zinc concentrations that exceed ambient levels, in nature, come mostly because of anthropogenic activity (Klimek, 2012). There is also evidence that suggests that the immune system may compartmentalise excess zinc as an antimicrobial response. Bursts of free zinc have been observed inside macrophages as well as intraphagosomal zinc accumulation in cells infected with *Mycobacterium tuberculosis*. Excess zinc is proposed to have several toxic effects on *E. coli* attributed to sulfhydryl groups in proteins, particularly those involved in respiration (Hughes and Poole, 1989). It has been demonstrated that zinc can attack iron-sulphur clusters through S_N1 substitution by binding to either cysteinyl or bridging sulfur atoms (Fang and Imlay, 2012). As a highly thiophilic metal, zinc has been shown to affect growth by causing damage to 6-phosphogluconate dehydratase. However, intracellular concentrations of zinc are tightly controlled by *zntA* to keep cytoplasmic concentrations in check. Mutants that lack a zinc export system are more sensitive to its growth inhibition effect (Fang and Imlay, 2012). In other studies, it has been demonstrated that zinc can arrest respiration by binding cytochrome *bd* or NADH (Kasahara and Anraku, 1974; Beard, Hughes and Poole, 1995). Therefore, zinc homeostasis is crucial to protecting respiratory targets that face potential inhibition by excess zinc.

Various genomic analyses have been undertaken to uncover the toxic effects that excess zinc has on *E. coli*. Excess copper and zinc have been shown to induce the Cpx and σ^E stress responses in *Salmonella* which regulate genes involved in detoxification, such as *tolC*, which is believed to restore metal balance (Pontel *et al.*, 2014). The BaeSR two component system has also been linked with zinc detoxification through its regulation of the *mdtABC* and *mdtD* transporters as well as the *spy* chaperon (Wang and Fierke, 2013). In one microarray study, it was demonstrated

that excess zinc results in the upregulation of cysteine biosynthetic genes suggesting that cysteine plays a role in the transient trapping of zinc to maintain zinc homeostasis. The same microarray also revealed that excess zinc led to an increase in expression of the gene encoding the protease *degP* (Yamamoto and Ishihama, 2005). In EPEC, excess zinc has been shown to result in the downregulation of *bfp* and genes encoded by the locus of enterocyte effacement (LEE) (Crane *et al.*, 2007). In vivo studies also demonstrated that excess zinc resulted in reduced adherence in rabbit models.

1.7 Thesis objectives

The demonstrated effect that zinc has on *E. coli* physiology and genetic expression suggest an involvement of the Cpx response in mediating its toxic effects. I hypothesized that excess zinc disrupts the envelope's integrity affecting the selectivity of molecules across the membrane. Consequently, the redox balance of the periplasm is affected - making it more reducing. The Cpx response is activated, as a result of these changes, in order to maintain the oxidative state of the periplasm. The aims of this thesis were to investigate the involvement of the Cpx response in mediating adaptation to zinc toxicity and, as such, the specific objectives laid out were:

1. Determine the mechanism of activation of the Cpx response by zinc and elucidate the role of the Cpx response in mediating zinc toxicity.

    a. Investigate whether zinc activation of the Cpx response occurs via an envelope signal.

    b. Examine whether the Cpx response contributes to resistance to excess zinc.

2. Examine the role of the cysteine in alleviating stress caused by excess zinc as well as the regulation of genes in the cysteine regulon during zinc stress.

a. Confirm induction of the *cys* regulon by *nlpE* overexpression and by excess zinc.

b. Determine whether the Cpx response is required for activation of the *cys* regulon under excess zinc conditions.

c. Determine the role of cysteine in mediating adaptation to zinc toxicity.

1.8 **References**

Acosta, N., Pukatzki, S. and Raivio, T. L. (2015) 'The Vibrio cholerae Cpx envelope stress response senses and mediates adaptation to low iron', Journal of Bacteriology, 197(2), pp. 262–276. doi: 10.1128/JB.01957-14.

Arechaga, I. (2013) 'Membrane invaginations in bacteria and mitochondria: common features and evolutionary scenarios.', Journal of molecular microbiology and biotechnology. Karger Publishers, 23(1–2), pp. 13–23. doi: 10.1159/000346515.

Berkmen, M. (2012) 'Production of disulfide-bonded proteins in Escherichia coli', Protein Expression and Purification, 82(1), pp. 240–251. doi: 10.1016/j.pep.2011.10.009.

Blencowe, D. K. and Morby, A. P. (2003) 'Zn(II) metabolism in prokaryotes.', FEMS microbiology reviews, 27(2–3), pp. 291–311. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12829272 (Accessed: 28 March 2018).

Blindauer, C. A. (2015) 'Advances in the molecular understanding of biological zinc transport', Chemical Communications. Royal Society of Chemistry, 51(22), pp. 4544–4563. doi: 10.1039/C4CC10174J.

Braun, V. (1975) 'Covalent lipoprotein from the outer membrane of Escherichia coli.', Biochimica et biophysica acta, 415(3), pp. 335–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/52377 (Accessed: 15 May 2018).

Breyton, C., Haase, W., Rapoport, T. A., Kühlbrandt, W. and Collinson, I. (2002) 'Three-dimensional structure of the bacterial protein-translocation complex SecYEG.', Nature, 418(6898), pp. 662–5. doi: 10.1038/nature00827.

Brocklehurst, K. R., Hobman, J. L., Lawley, B., Blank, L., Marshall, S. J., Brown, N. L. and Morby, A. P. (1999) 'ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of zntA in Escherichia coli', Molecular Microbiology. Wiley/Blackwell (10.1111), 31(3), pp. 893–902. doi: 10.1046/j.1365-2958.1999.01229.x.

Bryan, L. E. and Van den Elzen, H. M. (1976) 'Streptomycin accumulation in susceptible and resistant strains of Escherichia coli and Pseudomonas aeruginosa.', Antimicrobial agents and chemotherapy, 9(6), pp. 928–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/820248 (Accessed: 27 March 2018).

Capra, E. J. and Laub, M. T. (2012) 'Evolution of two-component signal transduction systems.', Annual review of microbiology. NIH Public Access, 66, pp. 325–47. doi: 10.1146/annurev-micro-092611-150039.

Chopra, I. (1988) 'Molecular mechanisms involved in the transport of antibiotics into bacteria', Parasitology. Cambridge University Press, 96(S1), pp. S25–S44. doi: 10.1017/S0031182000085966.

Clifton, L. A., Skoda, M. W. A., Le Brun, A. P., Ciesielski, F., Kuzmenko, I., Holt, S. A. and Lakey, J. H. (2015) 'Effect of Divalent Cation Removal on the Structure of Gram-

Negative Bacterial Outer Membrane Models', Langmuir, 31(1), pp. 404–412. doi: 10.1021/la504407v.

Conway, T. and Cohen, P. S. (2015) 'Commensal and Pathogenic Escherichia coli Metabolism in the Gut', Microbiology spectrum, 3(3), p. 10.1128/microbiolspec.MBP-0006-2014. doi: 10.1128/microbiolspec.MBP-0006-2014.

Crane, J. K., Naeher, T. M., Shulgina, I., Zhu, C. and Boedeker, E. C. (2007) 'Effect of zinc in enteropathogenic Escherichia coli infection', Infection and Immunity, 75(12), pp. 5974–5984. doi: 10.1128/IAI.00750-07.

Danese, P. N., Oliver, G. R., Barr, K., Bowman, G. D., Rick, P. D. and Silhavy, T. J. (1998) 'Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates degP transcription in Escherichia coli', Journal of Bacteriology, 180(22), pp. 5875–5884. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0031765192&partnerID=tZOtx3y1.

Danese, P. N. and Silhavy, T. J. (1997) 'The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in Escherichia coli.', Genes & development, 11(9), pp. 1183–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9159399 (Accessed: 27 March 2018).

Danese, P. N. and Silhavy, T. J. (1998) 'CpxP, a stress-combative member of the Cpx regulon', Journal of Bacteriology, 180(4), pp. 831–839. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9473036 (Accessed: 27 March 2018).

Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. B. and Silhavy, J. (1995) 'The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene specifying the stress-inducible periplasmic protease , DegP', pp. 387–398.

Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. and Silhavy, T. J. (1995) 'The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP.', Genes & development, 9(4), pp. 387–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7883164 (Accessed: 27 March 2018).

DiGiuseppe, P. A. and Silhavy, T. J. (2003) 'Signal Detection and Target Gene Induction by the CpxRA Two-Component System', Journal of Bacteriology. American Society for Microbiology, 185(8), pp. 2432–2440. doi: 10.1128/JB.185.8.2432-2440.2003.

Dong, J., Iuchi, S., Kwan, H. S., Lu, Z. and Lin, E. C. (1993) 'The deduced amino-acid sequence of the cloned cpxR gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of Escherichia coli.', Gene, 136(1–2), pp. 227–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8294007 (Accessed: 27 March 2018).

Dorel, C., Lejeune, P. and Rodrigue, A. (2006) 'The Cpx system of Escherichia coli, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities?', Research in Microbiology, 157(4), pp. 306–314. doi: 10.1016/j.resmic.2005.12.003.

Dufresne, K. and Paradis-Bleau, C. (2015) 'Biology and Assembly of the Bacterial Envelope', in. Springer, Cham, pp. 41–76. doi: 10.1007/978-3-319-23603-2_3.

Fairman, J. W., Noinaj, N. and Buchanan, S. K. (2011) 'The structural biology of β-barrel membrane proteins: a summary of recent reports', Current Opinion in Structural Biology. Elsevier Current Trends, 21(4), pp. 523–531. doi: 10.1016/J.SBI.2011.05.005.

Flores-Kim, J. and Darwin, A. J. (2016) 'The Phage Shock Protein Response', Annual Review of Microbiology, 70(1), pp. 83–101. doi: 10.1146/annurev-micro-102215-095359.

Friedrich, T., Dekovic, D. K. and Burschel, S. (2016) 'Assembly of the Escherichia coli NADH:ubiquinone oxidoreductase (respiratory complex I)', Biochimica et Biophysica Acta (BBA) - Bioenergetics. Elsevier, 1857(3), pp. 214–223. doi: 10.1016/J.BBABIO.2015.12.004.

Goemans, C., Denoncin, K. and Collet, J.-F. (2014) 'Folding mechanisms of periplasmic proteins', Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. Elsevier, 1843(8), pp. 1517–1528. doi: 10.1016/J.BBAMCR.2013.10.014.

Graham, A. I., Hunt, S., Stokes, S. L., Bramall, N., Bunch, J., Cox, A. G., McLeod, C. W. and Poole, R. K. (2009) 'Severe zinc depletion of Escherichia coli: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins.', The Journal of biological chemistry. American Society for Biochemistry and Molecular Biology, 284(27), pp. 18377–89. doi: 10.1074/jbc.M109.001503.

Grass, G., Wong, M. D., Rosen, B. P., Smith, R. L. and Rensing, C. (2002) 'ZupT is a Zn(II) uptake system in Escherichia coli.', Journal of bacteriology. American Society for Microbiology, 184(3), pp. 864–6. doi: 10.1128/JB.184.3.864-866.2002.

Groisman, E. A. (2001) 'The pleiotropic two-component regulatory system PhoP-PhoQ.', Journal of bacteriology. American Society for Microbiology, 183(6), pp. 1835–42. doi: 10.1128/JB.183.6.1835-1842.2001.

Guédon, E. and Martin-verstraete, I. (2006) 'Cysteine Metabolism and Its Regulation in Bacteria', (August 2015). doi: 10.1007/7171.

Guest, R. L. and Raivio, T. L. (2016) 'Role of the Gram-Negative Envelope Stress Response in the Presence of Antimicrobial Agents', Trends in Microbiology. Elsevier Ltd, 24(5), pp. 377–390. doi: 10.1016/j.tim.2016.03.001.

Guest, R. L., Wang, J., Wong, J. L. and Raivio, T. L. (2017) 'A Bacterial Stress Response Regulates Respiratory Protein Complexes To Control Envelope Stress Adaptation', Journal of Bacteriology. Edited by V. J. DiRita, 199(20), pp. e00153-17. doi: 10.1128/JB.00153-17.

Haase, H. and Rink, L. (2014) 'Multiple impacts of zinc on immune function', Metallomics, 6(7), p. 1175. doi: 10.1039/c3mt00353a.

Hancock, R. E. W. and McPhee, J. B. (2005) 'Salmonella's Sensor for Host Defense Molecules', Cell. Cell Press, 122(3), pp. 320–322. doi: 10.1016/J.CELL.2005.07.023.

Hantke, K. (2005) ‘Bacterial zinc uptake and regulators’, Current Opinion in Microbiology, 8(2), pp. 196–202. doi: 10.1016/j.mib.2005.02.001.

Hughes, M. N. (Martin N. and Poole, R. K. (1989) Metals and micro-organisms. Chapman and Hall. Available at: https://www.springer.com/gp/book/9780412244001 (Accessed: 3 May 2018).

Hung, D. L., Raivio, T. L., Jones, C. H., Silhavy, T. J. and Hultgren, S. J. (2001) ‘Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili.’, The EMBO journal, 20(7), pp. 1508–18. doi: 10.1093/emboj/20.7.1508.

Johnson, J. R., Gajewski, A., Lesse, A. J. and Russo, T. A. (2003) ‘Extraintestinal pathogenic Escherichia coli as a cause of invasive nonurinary infections.’, Journal of clinical microbiology. American Society for Microbiology (ASM), 41(12), pp. 5798–802. doi: 10.1128/JCM.41.12.5798-5802.2003.

Juncker, A. S., Willenbrock, H., von Heijne, G., Brunak, S., Nielsen, H. and Krogh, A. (2003) ‘Prediction of lipoprotein signal peptides in Gram-negative bacteria’, Protein Science. Wiley-Blackwell, 12(8), pp. 1652–1662. doi: 10.1110/ps.0303703.

Kamio, Y. and Nikaido, H. (1976) ‘Outer membrane of Salmonella typhimurium: accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium’, Biochemistry. American Chemical Society, 15(12), pp. 2561–2570. doi: 10.1021/bi00657a012.

Kertesz, M. A. (2001) ‘Bacterial transporters for sulfate and organosulfur compounds.’, Research in microbiology, 152(3–4), pp. 279–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11421275 (Accessed: 28 March 2018).

Klimek, B. (2012) ‘Effect of long-term zinc pollution on soil microbial community resistance to repeated contamination.’, Bulletin of environmental contamination and toxicology. Springer, 88(4), pp. 617–22. doi: 10.1007/s00128-012-0523-0.

Kredich, N. M. (2008) ‘Biosynthesis of Cysteine’, EcoSal Plus. asm Pub2Web, 3(1). doi: 10.1128/ecosalplus.3.6.1.11.

Kudva, R., Denks, K., Kuhn, P., Vogt, A., Müller, M. and Koch, H.-G. (2013) ‘Protein translocation across the inner membrane of Gram-negative bacteria: the Sec and Tat dependent protein transport pathways’, Research in Microbiology. Elsevier Masson, 164(6), pp. 505–534. doi: 10.1016/J.RESMIC.2013.03.016.

Kurabayashi, K., Hirakawa, Y., Tanimoto, K., Tomita, H. and Hirakawa, H. (2014) ‘Role of the CpxAR Two-Component Signal Transduction System in Control of Fosfomycin Resistance and Carbon Substrate Uptake’, Journal of Bacteriology, 196(2), pp. 248–256. doi: 10.1128/JB.01151-13.

Landeta, C., Boyd, D. and Beckwith, J. (2018) ‘Disulfide bond formation in prokaryotes’, Nature Microbiology, 3(3), pp. 270–280. doi: 10.1038/s41564-017-0106-2.

Lee, L. J., Barrett, J. A. and Poole, R. K. (2005) ‘Genome-wide transcriptional response of chemostat-cultured Escherichia coli to zinc.’, Journal of bacteriology.

American Society for Microbiology, 187(3), pp. 1124–34. doi: 10.1128/JB.187.3.1124-1134.2005.

Li, W., Liu, Y., Sheng, X., Yin, P., Hu, F., Liu, Y., Chen, C., Li, Q., Yan, C. and Wang, J. (2014) 'Structure and mechanism of a type III secretion protease, NleC.', Acta crystallographica. Section D, Biological crystallography, 70(Pt 1), pp. 40–7. doi: 10.1107/S1399004713024619.

Luirink, J., Yu, Z., Wagner, S. and de Gier, J.-W. (2012) 'Biogenesis of inner membrane proteins in Escherichia coli', Biochimica et Biophysica Acta (BBA) - Bioenergetics. Elsevier, 1817(6), pp. 965–976. doi: 10.1016/J.BBABIO.2011.12.006.

MacRitchie, D. M., Ward, J. D., Nevesinjac, A. Z. and Raivio, T. L. (2008) 'Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic Escherichia coli', Infection and Immunity, 76(4), pp. 1465–1475. doi: 10.1128/IAI.01265-07.

Magalon, A., Arias-Cartin, R. and Walburger, A. (2012) 'Supramolecular Organization in Prokaryotic Respiratory Systems', Advances in Microbial Physiology. Academic Press, 61, pp. 217–266. doi: 10.1016/B978-0-12-394423-8.00006-8.

McEwen, J. and Silverman, P. (1980a) 'Chromosomal mutations of Escherichia coli that alter expression of conjugative plasmid functions.', Proceedings of the National Academy of Sciences of the United States of America, 77(1), pp. 513–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6102380 (Accessed: 27 March 2018).

McEwen, J. and Silverman, P. (1980b) ‘Mutations in genes cpxA and cpxB of Escherichia coli K-12 cause a defect in isoleucine and valine syntheses.’, Journal of bacteriology, 144(1), pp. 68–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6998970 (Accessed: 27 March 2018).

McEwen, J. and Silverman, P. M. (1982) ‘Mutations in genes cpxA and cpxB alter the protein composition of Escherichia coli inner and outer membranes.’, Journal of bacteriology, 151(3), pp. 1553–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7050092 (Accessed: 27 March 2018).

Messens, J. and Collet, J.-F. (2006) ‘Pathways of disulfide bond formation in Escherichia coli.’, The international journal of biochemistry & cell biology, 38(7), pp. 1050–62. doi: 10.1016/j.biocel.2005.12.011.

Mileykovskaya, E. and Dowhan, W. (1997) ‘The Cpx two-component signal transduction pathway is activated in Escherichia coli mutant strains lacking phosphatidylethanolamine’, Journal of Bacteriology, 179(4), pp. 1029–1034. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0031039158&partnerID=tZOtx3y1.

Miyadai, H., Tanaka-Masuda, K., Matsuyama, S. and Tokuda, H. (2004) ‘Effects of lipoprotein overproduction on the induction of DegP (HtrA) involved in quality control in the Escherichia coli periplasm.’, The Journal of biological chemistry. American Society for Biochemistry and Molecular Biology, 279(38), pp. 39807–13. doi: 10.1074/jbc.M406390200.

Narita, S. (2011) ‘ABC Transporters Involved in the Biogenesis of the Outer Membrane in Gram-Negative Bacteria’, Bioscience, Biotechnology, and Biochemistry. Japan Society for Bioscience, Biotechnology, and Agrochemistry, 75(6), pp. 1044–1054. doi: 10.1271/bbb.110115.

Nevesinjac, A. Z. and Raivio, T. L. (2005) ‘The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic Escherichia coli.’, Journal of bacteriology. American Society for Microbiology, 187(2), pp. 672–86. doi: 10.1128/JB.187.2.672-686.2005.

Otto, K. and Silhavy, T. J. (2002) ‘Surface sensing and adhesion of Escherichia coli controlled by the Cpx-signaling pathway.’, Proceedings of the National Academy of Sciences of the United States of America, 99(4), pp. 2287–92. doi: 10.1073/pnas.042521699.

Palmela, C., Chevarin, C., Xu, Z., Torres, J., Sevrin, G., Hirten, R., Barnich, N., Ng, S. C. and Colombel, J.-F. (2018) ‘Adherent-invasive Escherichia coli in inflammatory bowel disease’, Gut, 67(3), pp. 574–587. doi: 10.1136/gutjnl-2017-314903.

Patzer, S. and Hantke, K. (1998) ‘The ZnuABC high-affinity zinc uptake system and its regulator Zur in Escherichia coli’, Mol Microbiol, 28.

Pogliano, J., Lynch, A. S., Belin, D., Lin, E. C. and Beckwith, J. (1997) ‘Regulation of Escherichia coli cell envelope proteins involved in protein folding and degradation by

the Cpx two-component system.', Genes & development, 11(9), pp. 1169–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9159398 (Accessed: 27 March 2018).

Pontel, L. B., Scampoli, N. L., Porwollik, S., Checa, S. K., McClelland, M. and Soncini, F. C. (2014) 'Identification of a Salmonella ancillary copper detoxification mechanism by a comparative analysis of the genome-wide transcriptional response to copper and zinc excess', Microbiology, 160(8), pp. 1659–1669. doi: 10.1099/mic.0.080473-0.

Price, N. L. and Raivio, T. L. (2009) 'Characterization of the Cpx regulon in Escherichia coli strain MC4100', Journal of Bacteriology, 191(6), pp. 1798–1815. doi: 10.1128/JB.00798-08.

Pucci, M. J. and Bush, K. (2013) 'Investigational Antimicrobial Agents of 2013', Clinical Microbiology Reviews, 26(4), pp. 792–821. doi: 10.1128/CMR.00033-13.

Raetz, C. R. H. and Whitfield, C. (2002) 'Lipopolysaccharide Endotoxins', Annual Review of Biochemistry. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 71(1), pp. 635–700. doi: 10.1146/annurev.biochem.71.110601.135414.

Rainwater, S. and Silverman, P. M. (1990) 'The Cpx proteins of Escherichia coli K-12: evidence that cpxA, ecfB, ssd, and eup mutations all identify the same gene.', Journal of bacteriology, 172(5), pp. 2456–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2185221 (Accessed: 27 March 2018).

Raivio, T. L. (2005) 'MicroReview: Envelope stress responses and Gram-negative bacterial pathogenesis', Molecular Microbiology. Wiley/Blackwell (10.1111), 56(5), pp. 1119–1128. doi: 10.1111/j.1365-2958.2005.04625.x.

Raivio, T. L., Leblanc, S. K. D. and Price, N. L. (2013) 'The Escherichia coli Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity', Journal of Bacteriology, 195(12), pp. 2755–2767. doi: 10.1128/JB.00105-13.

Raivio, T. L., Popkin, D. L. and Silhavy, T. J. (1999) 'The Cpx envelope stress response is controlled by amplification and feedback inhibition.', Journal of bacteriology. American Society for Microbiology, 181(17), pp. 5263–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10464196 (Accessed: 27 March 2018).

Raivio, T. L. and Silhavy, T. J. (1997) 'Transduction of envelope stress in Escherichia coli by the Cpx two-component system . Transduction of Envelope Stress in Escherichia coli by the Cpx Two-Component System', American Society for Microbiology, 179(24), pp. 7724–7733. doi: 10.1128/JB.179.24.7724-7733.1997.

Raivio, T. L. and Silhavy, T. J. (2001) 'Periplasmic stress and ECF sigma factors.', Annual review of microbiology, 55, pp. 591–624. doi: 10.1146/annurev.micro.55.1.591.

Rollauer, S. E., Sooreshjani, M. A., Noinaj, N. and Buchanan, S. K. (2015) 'Outer membrane protein biogenesis in Gram-negative bacteria.', Philosophical transactions of

the Royal Society of London. Series B, Biological sciences. The Royal Society, 370(1679), p. 20150023. doi: 10.1098/rstb.2015.0023.

Russo, T. A. and Johnson, J. R. (2000) 'Proposal for a New Inclusive Designation for Extraintestinal Pathogenic Isolates of Escherichia coli: ExPEC', The Journal of Infectious Diseases, 181(5), pp. 1753–1754. doi: 10.1086/315418.

Sankaran, K. and Wu, H. C. (1994) 'Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol.', The Journal of biological chemistry, 269(31), pp. 19701–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8051048 (Accessed: 26 March 2018).

Silhavy, T. J., Kahne, D. and Walker, S. (2010) 'The bacterial cell envelope.', Cold Spring Harbor perspectives in biology. Cold Spring Harbor Laboratory Press, 2(5), p. a000414. doi: 10.1101/cshperspect.a000414.

Singh, T., Das, S., Ramachandran, V. G., Shah, D., Saha, R., Dar, S. A. and Rai, A. (2017) 'Typical & atypical enteropathogenic escherichia coli in diarrhoea & their role as carrier in children under five', Indian Journal of Medical Research, 145(April), pp. 551–557. doi: 10.4103/ijmr.IJMR_25_15.

Skrovanek, S., Diguilio, K., Bailey, R., Huntington, W., Urbas, R., Mayilvaganan, B., Mercogliano, G., Mullin, J. M., Skrovanek, S., Diguilio, K. and Mullin, J. M. (2014) 'Zinc and gastrointestinal disease', 5(4), pp. 496–513. doi: 10.4291/wjgp.v5.i4.496.

Snyder, W. B., Davis, L. J., Danese, P. N., Cosma, C. L. and Silhavy, T. J. (1995) 'Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway.', Journal of bacteriology, 177(15), pp. 4216–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7635808 (Accessed: 27 March 2018).

van Stelten, J., Silva, F., Belin, D. and Silhavy, T. J. (2009) 'Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY.', Science (New York, N.Y.). American Association for the Advancement of Science, 325(5941), pp. 753–6. doi: 10.1126/science.1172221.

Takagi, H. (2010) 'The L -Cysteine / L -Cystine Shuttle System Provides Reducing Equivalents to the Periplasm in Escherichia coli * □', 285(23), pp. 17479–17487. doi: 10.1074/jbc.M109.081356.

Thede, G. L., Arthur, D. C., Edwards, R. A., Buelow, D. R., Wong, J. L., Raivio, T. L. and Glover, J. N. M. (2011) 'Structure of the periplasmic stress response protein CpxP.', Journal of bacteriology. American Society for Microbiology, 193(9), pp. 2149–57. doi: 10.1128/JB.01296-10.

Urdaneta, V. and Casadesús, J. (2017) 'Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts', Frontiers in Medicine. Frontiers Media SA, 4, p. 163. doi: 10.3389/FMED.2017.00163.

Vogt, S. L., Nevesinjac, A. Z., Humphries, R. M., Donnenberg, M. S., Armstrong, G. D. and Raivio, T. L. (2010) 'The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic Escherichia coli bundle-forming pilus', Molecular Microbiology, 76(5), pp. 1095–1110. doi: 10.1111/j.1365-2958.2010.07145.x.

Vogt, S. L. and Raivio, T. L. (2012) 'Just scratching the surface: An expanding view of the Cpx envelope stress response', FEMS Microbiology Letters, 326(1), pp. 2–11. doi: 10.1111/j.1574-6968.2011.02406.x.

Vollmer, W., Blanot, D. and De Pedro, M. A. (2008) 'Peptidoglycan structure and architecture', FEMS Microbiology Reviews, 32(2), pp. 149–167. doi: 10.1111/j.1574-6976.2007.00094.x.

Wang, D. and Fierke, C. A. (2013) 'The BaeSR regulon is involved in defense against zinc toxicity in E. coli', Metallomics. The Royal Society of Chemistry, 5(4), p. 372. doi: 10.1039/c3mt20217h.

Weatherspoon-Griffin, N., Yang, D., Kong, W., Hua, Z. and Shi, Y. (2014) 'The CpxR/CpxA two-component regulatory system up-regulates the multidrug resistance cascade to facilitate Escherichia coli resistance to a model antimicrobial peptide.', The Journal of biological chemistry, 289(47), pp. 32571–82. doi: 10.1074/jbc.M114.565762.

Whitfield, C., Szymanski, C. M. and Aebi, M. (2015) Eubacteria, Essentials of Glycobiology. Cold Spring Harbor Laboratory Press. doi: 10.1101/GLYCOBIOLOGY.3E.021.

Van Wielink, J. E. and Duine, J. A. (1990) ‘How big is the periplasmic space?’, Trends in Biochemical Sciences. Elsevier Current Trends, 15(4), pp. 136–137. doi: 10.1016/0968-0004(90)90208-S.

Wong, J. L. (2015) ‘Cellular role of the inhibitor-chaperone CpxP in’.

Yamamoto, K. and Ishihama, A. (2005) ‘Transcriptional Response of Escherichia coli to External Zinc’, 187(18), pp. 6333–6340. doi: 10.1128/JB.187.18.6333.

Zhang, G., Meredith, T. C. and Kahne, D. (2013) ‘On the essentiality of lipopolysaccharide to Gram-negative bacteria’, Current Opinion in Microbiology. Elsevier Current Trends, 16(6), pp. 779–785. doi: 10.1016/J.MIB.2013.09.007.

Zhao, B. and Houry, W. A. (2010) ‘Acid stress response in enteropathogenic gammaproteobacteria: an aptitude for survival.’, Biochemistry and cell biology = Biochimie et biologie cellulaire, 88(2), pp. 301–14. doi: 10.1139/o09-182.

Chapter 2: Materials and Methods

2.1 Bacterial strains and plasmids used in this study.

All the mutants and plasmids used in this study were constructed using standard genetic techniques. The strains and plasmids are listed in Table (1).

2.2 Media and growth conditions.

Strains were grown in Luria-Bertani (LB) broth containing 5 g NaCl, 10 g Tryptone and 5 g yeast extract per litre (BD Lifesciences). Antibiotics used were added in the following concentrations: 100 µg/mL ampicillin, 50 µg/mL kanamycin, 50 µg/mL chloramphenicol (all Sigma). 1 M stock solution of zinc chloride was used for zinc assays.

2.3 Luminescence assays.

Single isolated colonies of each strain were inoculated into 5 mL of LB broth with the appropriate antibiotics and grown overnight with shaking at 225 rpm in a test tube rack at 37 ºC. Cultures were then diluted 1:50 into 5 mL fresh LB broth plus antibiotics and grown for 2 hrs under the same conditions. Zinc chloride, DTT or mQH_2O was then added to the cultures all at the same volume to indicated final concentrations. The cultures were then transferred to 96 well plates incubated at 37 ºC for 4 hours luminescence and OD_{600} values being taken every hour.

2.4 Preparation of $P1_{vir}$ lysates.

Mutations in the MC4100 strain were introduced using $P1_{vir}$ transduction. Lysates were prepared from the KEIO collection (Baba *et al.*, 2006) by inoculating the desired mutant in 5 mL of LB overnight with shaking at 225 rpm in a test tube rack at 37 ºC. The culture was then transferred into 5 mL fresh LB containing 5 mM $CaCl_2$ and 0.2 % glucose at a 1:50 dilution the following day and incubated for 30 min under the same conditions. 100 µL of $P1_{vir}$ lysate (≈ 10^9 pfu/mL) was then added to the culture and incubated further until the cells were completely lysed.

100 µL of chloroform was added to the lysate, vortexed briefly and then centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a sterile vile and then mixed with 100 µL of chloroform before storing at 4 ºC (Thomason, Costantino and Court, 2007).

2.5 Transduction mutagenesis.

Single isolated colonies of the recipient strain were inoculated in 5 mL of LB overnight with shaking at 225 rpm in a test tube rack at 37 ºC. The cells were then pelleted and resuspended in 2.5 mL of mQH_2O water containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$. 100 µL of the desired lysate was then added to 100 µL of the resuspended cells and incubated at 30 ºC for 30 min without shaking. 1 mL of LB containing 10 mM of citrate was then added and the cells were incubated at 37 ºC for another 30 min without shaking. The cells were then pelleted, resuspended in 100 µL of 1 M citrate and then streaked on LB plates containing 50 µg/mL of kanamycin (Thomason, Costantino and Court, 2007).

2.6 RNA isolation.

RNA was isolated using the MasterPure Complete DNA and RNA Purification Kit from Epicentre. Briefly, overnight cultures were diluted 1:50 in 5 mL fresh LB and grown for 2 hours with shaking at 225 rpm in a test tube rack at 37 ºC. $ZnCl_2$ was then added to the media to a final concentration of 0.5 mM to half of the culture and a similar volume of MilliQ water was added to the rest and left to grow for another 5 min under the same conditions. 1 mL of the culture was then pelleted by centrifugation and the supernatant discarded leaving approximately 25 µL of liquid. The tube was vortexed to resuspend the cells before adding 300 µL of Tissue and Cell lysis solution containing proteinase K. The mixture was then incubated at 65 ºC for 15 min with vortexing every 5 min. The samples were cooled on ice for 5 min before precipitating the nucleic acid. 175 µL of MPC Protein Precipitation Reagent was then added to 300 µL of the lysed sample and vortexed

for 10 sec. The debris was pelleted by centrifuging the sample at 4 ºC for 10 min at 13000 rpm. The supernatant was transferred to a new microcentrifuge tube to precipitate the RNA. 500 µL of cold isopropanol was added to the recovered supernatant and the tube was inverted 40 times before pelleting the total nucleic acid by centrifugation at 4 ºC for 10 min at 13000 rpm.

2.7 RNA purification.

Two separate kits were used to remove contaminating DNA from the total nucleic acid (Ambion DNA-*free* kit). In brief, the isopropanol was carefully poured off and residual liquid pipetted out before adding 200 µL of DNAse I solution (5 µL of RNase-Free DNase I added to 195 µL with 1X DNase Buffer). The solution was then incubated at 37 ºC for 30 min to remove the contaminating DNA. 200 µL of 2X T and C Lysis Solution was then added to the mixture and vortexed for 5 sec. The 200 µL of MPC Protein Precipitation Reagent was then added to the mixture and vortexed for 10 sec before incubating on ice for 5 min. The debris was pelleted by centrifuging the sample at 4 ºC for 10 min at 13000 rpm and the supernatant containing the RNA was transferred to a new microcentrifuge tube. 500 µL of cold isopropanol was added to the recovered supernatant and the tube was inverted 40 times to precipitate the RNA. The RNA was pelleted by centrifugation at 4 ºC for 10 min at 13,000 rpm. The isopropanol was poured off and the RNA pellet was rinsed twice with ethanol before resuspending the RNA in 35 µL of TE buffer. For the second DNA degradation, using the Ambion DNA-*free* kit, the total nucleic acid was diluted to 10 µg nucleic acid/50 µL. 0.1 volume 10X DNase I Buffer was then added to solution followed by 0.5 µL of DNase I and incubated at 37 ºC for 30 min. 0.5 µL of DNase I was added again to the mixture and allowed to incubate for another 30 min at 37 ºC. 0.1 volume DNase Inactivation Reagent was added to the mixture and allowed to incubate at room temperature for 2

min. The mixture was centrifuged at 10,000 × g for 1.5 min and finally the RNA was transferred to a fresh tube.

2.8 Reverse transcription.

The RT-PCR master mix was prepared using the following: 1 µL of 250 ng/µL random primers or 0.83 µL of 300 ng/µL random primers, 1 µL dNTP mix (10 mM each = 10 µL each + 60 µL H_2O), 0.17 µL sterile mQH_2O, 4 µL 5x First-Strand buffer, 2 µL 0.1 M DTT, 1 µL RNAse OUT, 2 µL of RNA and either 1 µL of Superscript II RT or 1 µL of mQH_2O. The RNA was then reverse transcribed in a thermocycler at Incubate at:

25 ºC – 10 min

42 ºC – 50 min

70 ºC – 15 min

4 ºC – hold.

2.9 Tetrazolium violet assay.

To determine the reducing power of the periplasm under excess zinc conditions, overnight cultures were diluted 1:50 in 4 mL fresh LB and grown for 2 hours with shaking at 225 rpm in a test tube rack at 37 ºC. $ZnCl_2$ was then added to the media to a final concentration of 1 mM to half of the culture and a similar volume of mQH_2O water was added to the rest and left to grow for another 2 hours. 0.01 % [w/v] 2,5-Diphenyl-3-(1-naphthyl)tetrazolium chloride was then added to the culture which was transferred to a 96 well plate and incubated further for another 15 min with shaking at 225 rpm at 37 ºC. The OD_{495} and OD_{600} measurements were then taken and recorded.

2.10 Thiol quantifications.

To determine intracellular thiol concentrations, I utilized the Measure-It thiol quantification kit from Invitrogen. In brief, cells were grown overnight and sub-cultured in 4 mL fresh LB the next day for 2 hours before adding $ZnCl_2$ to a final concentration of 0.5 mM to half of the culture and a similar volume of mQH_2O water was added to the rest. The cells were then grown for another 2 hours washed and resuspended in 2 mL of phosphate buffer saline (PBS) before lysing in a ultrasonic water-bath sonicator at maximum exposure for 5 min. 10 µL of the lysate was then added to 100 µL of the Measure-iT thiol quantitation reagent and incubated at room temperature for 5 min. Fluorescent measurements were then taken at 435/540 nm to quantify the thiol concentration against a prepared standard.

2.11 β-galactosidase assays.

Single isolated colonies of each strain were inoculated into 5 mL of LB broth with the appropriate antibiotics and grown overnight with shaking at 225 rpm in a test tube rack at 37 ºC. Cultures were then diluted 1:50 into 4 mL fresh LB broth plus antibiotics and grown for 2 hrs under the same conditions. Zinc chloride or mQH_2O was then added to half the cultures all at the same volume to the indicated final concentrations and grown for another 2 hrs. Cells were then centrifuged and resuspended in 2 mL of buffer Z (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001M $MgSO_4$ and 0.05 M β-mercaptoethanol at pH 7.1) before being lysed using EDTA and chloroform. β-galactosidase activity was then measured in a microtiter plate as previously described (Slauch and Silhavy, 1991).

2.12 Growth curves.

Single isolated colonies of the specified bacterial strains were inoculated into 5 mL of LB broth with the appropriate antibiotics and grown overnight with shaking at 225 rpm in a test tube

rack at 37 ºC. Cultures were then diluted 1:50 into 4 mL fresh LB broth plus antibiotics and grown for 2 hours, under the same conditions. $ZnCl_2$ was then added to the media to a final concentration of 1 mM to half of the culture and a similar volume of mQH_2O water was added to the rest and left to grow for another 6 hours in a 96 well microtiter plate. OD_{600} values were recorded every 2 hrs.

2.13 Bacterial transformations.

Single isolated colonies of the specified bacterial strains were inoculated into 5 mL of LB broth with the appropriate antibiotics and grown overnight with shaking at 225 rpm in a test tube rack at 37 ºC. Cultures were then diluted 1:50 into 2 mL of fresh LB broth plus the necessary antibiotics and grown for 2 hrs at the same conditions before pelleting the cells and resuspending them in 2 mL of solution containing 0.1 M $CaCl_2$, 0.1 M MOPS buffer at pH 6.5. The cells were incubated on ice for 30 min then pelleted and recovered in 200 µL of magic formula. 3 µL of the plasmid were added and incubated on ice for another 10 min before heat-shock at 42.5 ºC for 30 sec. 1 mL of LB was added and incubated at 30 ºC with shaking at 225 rpm for 1 hour before plating on LB plates containing appropriate antibiotics.

2.14 Plasmid isolation.

Plasmids were isolated using the GenElute Plasmid Miniprep kit from Sigma using the provided protocol. In brief, single isolated colonies were inoculated into 5 mL of LB broth overnight at 37 ºC with shaking at 225 rpm in a test tube rack. Cells where then centrifuged and plasmids isolated using the manufactures guidelines. Isolated plasmids were eluted into 50 µL of mQH_2O and stored at -20 ºC.

2.15 Construction of lux reporter plasmids.

The plasmid pNLP10 was used to construct the luminescent reporter. The promotor region of the gene of interest was amplified using the primers containing the BamHI and EcoRI restriction sites as overhangs, as shown listed (Table 2). The pNLP10 plasmid and amplified DNA were then restriction digested with appropriate enzymes and heat inactivated, at temperatures suggested by the manufacturer, before ligation to introduce the promoter of the gene of interest upstream of the *luxABCDE* genes. The ligated product was then transformed into *E. coli* Top10 cells to amplify the plasmid before isolation.

2.16 Sequencing.

Sequencing of plasmids to confirm correct insertions was done using Sanger sequencing provided by the Molecular Biology Service Unit (MBSU) at the University of Alberta.

2.17 FRT recombination.

To transform mutants created using the KEIO library (Baba *et al.*, 2006) with luminescent reporters the kanamycin cassette had to be flipped out using FRT recombination. In brief, mutants were transformed with the pFLP2 plasmid carrying the FRT recombinase gene as described earlier. Cells were recovered at 30 ºC for 1 hour, with shaking at 225 rpm, before plating on LB plates containing 100 µg/mL ampicillin and incubated overnight at 37 ºC to activate the recombinase. Transformants were then purified and streaked on both LB plates and LB plates containing 50 µg/mL kanamycin to check for mutants that had lost the kanamycin cassette. Colonies that did not grow on kanamycin plates were then grown in LB for 6 hours at 30 ºC with shaking at 225 rpm before plating on LB plates containing 5 % sucrose and lacking NaCl. Cells were grown for 2 days at room temperature and purified before screening for loss of the pFLP2 plasmid.

Table 1: Strains and plasmids used in this study

Strain	Description	Source
C6706	*Vibrio cholerae E1 Tor* C6706 strain; (Sm^R) streptomycin-resistant spontaneous	(Chin *et al.*, 2011)
MC4100	F- *araD139* Δ*(argF-lac)U169 rpsL150(Str^r) relA1 flbB301 decC1 ptsF25 rbsR*	(Casadaban and Cohen, 1979)
JZ101	MC4100 Δ*cpxR*	This study
JZ102	MC4100 Δ*cpxA*	This study
JZ103	MC4100 Δ*cpxP*	This study
JZ104	MC4100 Δ*degP*	This study
JZ105	MC4100 Δ*nlpE*	This study
JZ106	MC4100 *pNLP15*	This study
JZ107	MC4100 Δ*cpxR pNLP15*	This study
JZ108	MC4100 Δ*cpxA pNLP15*	This study
JZ109	MC4100 Δ*cpxP pNLP15*	This study
JZ110	MC4100 Δ*degP pNLP15*	This study
JZ111	MC4100 Δ*nlpE pNLP15*	This study
JZ112	Top10 *pJZ1*	This study
JZ113	MC4100 *pJZ1*	This study
JZ114	MC4100 Δ*cpxA pJZ1*	This study
JZ115	MC4100 Δ*cpxP pJZ1*	This study
JZ116	MC4100 Δ*nlpE pJZ1*	This study
JZ117	MC4100 Δ*degP pJZ1*	This study

JZ118	MC4100 *pCA-24N*	This study
JZ119	MC4100 *cysK::kan*	This study
JZ120	MC4100 *pCA-nlpE pJZ1*	This study
JZ121	MC4100 *pCA-24N pJZ1*	This study
JZ122	MC4100 Δ*cysK pNLP15 pCA-nlpE*	This study
JZ123	MC4100 Δ*cysK pNLP15 pCA-24N*	This study
JZ124	MC4100 *pCA-nlpE pNLP15*	This study
JZ125	MC4100 *pCA-24N pNLP15*	This study
TR50	MC4100 *cpxP'-'lacZ* at λRS88	(T L Raivio and Silhavy, 1997)
JZ126	TR50 *dsbA::kan*	This study
JZ127	TR50 *yfiK::kan*	This study
JZ128	TR50 *ydjN::kan*	This study
JZ129	TR50 *ydeD::kan*	This study
JZ130	TR50 *fliy::kan*	This study
JZ131	TR50 *cysK::kan*	This study
JZ132	TR50 *zntA::kan*	This study

Plasmid	Description	Source
pNLP10	Vector bearing promotorless *luxCDABE* fusion	(Price and Raivio, 2009)
pNLP15	Vector bearing *cpxP* promotor	(Price and Raivio, 2009)
pJZ1	Vector bearing *cysK* promotor	This study
pCA-nlpE	High-copy IPTG-inducible over-expression of *nlpE*	(Kitagawa *et al.*, 2006)
pCA-24N	High-copy IPTG-inducible vector, Cam^R	(Kitagawa *et al.*, 2006)

Table 2: Primers used in this study

qPCR primers		
Gene	**Forward**	**Reverse**
ydjN	GCGTTCCTGAATCCATCG	CCGTTCTGACCAATGGTTG
fliy	CTCCCGTCAGGAGTCTGG	TCCGCAATTGCATCATTC
yded	GCACGACCGAAAGTACCACT	CTGCGCAAAACTGATGGTTA
yfiK	GATTGGGACGTTTGGCAAT	AACAATCGCTGAAACAGATGC
zntA	ATCCGGGTGAAGAACTTACG	GTTAAACTTATGCCGTCGAA
cpxP	CACCACGCTGTTAGGGATG	CTTAACGCATTCGCTGTCAC
znuC	ATTAACGCACCGATGCAAA	CAATGCTCGCGCTAATAGTACA
cysK	CGATCTCAAGCTGGTCGATAA	CGCGGTAGAAATCGCTTC
degP	GCCGCGTAATTTCCAGCAGTT	GCCCCAGCGCAGAGACAATC
spy	AGCGTGACCAGATGAAACG	GTATCGCTGGCAATGATGTC
rpoD	GCCGAAGCAGTTTGACTACC	CTTCACCGATGGACATACGA
Cloning primers		
Name	**Sequence (restriction site underlined)**	**Source**
cysK	TTCAAAAATCGGATCCATGGCCTGTCCTTA	(Yamamoto *et al.*, 2011)
cysK	ACGCGATGTGGAATTCACAATCTACCGG	(Yamamoto *et al.*, 2011)

qPCR primers were designed using ROCHE primer design tool

(https://lifescience.roche.com/en_ca/brands/universal-probe-library.html)

2.18 References

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L. and Mori, H. (2006) 'Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection.', Molecular systems biology, 2, p. 2006.0008. doi: 10.1038/msb4100050.

Casadaban, M. J. and Cohen, S. N. (1979) 'Lactose genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: in vivo probe for transcriptional control sequences.', Proceedings of the National Academy of Sciences of the United States of America, 76(9), pp. 4530–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/159458 (Accessed: 7 May 2018).

Chin, C.-S., Sorenson, J., Harris, J. B., Robins, W. P., Charles, R. C., Jean-Charles, R. R., Bullard, J., Webster, D. R., Kasarskis, A., Peluso, P., Paxinos, E. E., Yamaichi, Y., Calderwood, S. B., Mekalanos, J. J., Schadt, E. E. and Waldor, M. K. (2011) 'The Origin of the Haitian Cholera Outbreak Strain', New England Journal of Medicine. Massachusetts Medical Society , 364(1), pp. 33–42. doi: 10.1056/NEJMoa1012928.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H. and Mori, H. (2006) 'Complete set of ORF clones of Escherichia coli ASKA library (A Complete Set of E. coli K-12 ORF Archive): Unique Resources for Biological Research', DNA Research, 12(5), pp. 291–299. doi: 10.1093/dnares/dsi012.

Price, N. L. and Raivio, T. L. (2009) 'Characterization of the Cpx regulon in Escherichia coli strain MC4100', Journal of Bacteriology, 191(6), pp. 1798–1815. doi: 10.1128/JB.00798-08.

Raivio, T. L. and Silhavy, T. J. (1997) ‘Transduction of envelope stress in Escherichia coli by the Cpx two-component system.’, Journal of bacteriology, 179(24), pp. 7724–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9401031 (Accessed: 7 May 2018).

Slauch, J. M. and Silhavy, T. J. (1991) ‘Genetic fusions as experimental tools.’, Methods in enzymology, 204, pp. 213–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1658562 (Accessed: 30 April 2018).

Thomason, L. C., Costantino, N. and Court, D. L. (2007) ‘E. coli Genome Manipulation by P1 Transduction’, in Current Protocols in Molecular Biology. Hoboken, NJ, USA: John Wiley & Sons, Inc., p. 1.17.1-1.17.8. doi: 10.1002/0471142727.mb0117s79.

Yamamoto, K., Oshima, T., Nonaka, G., Ito, H. and Ishihama, A. (2011) ‘Induction of the Escherichia coli cysK gene by genetic and environmental factors’, (1987). doi: 10.1111/j.1574-6968.2011.02364.x.

Chapter 3: Results

3.1 Ojective 1: Determination of the mechanism of activation of the Cpx response by zinc

3.1.1 Zinc generates an envelope stress signal that activates the Cpx response

The envelope is crucial to the cells as it protects bacteria from hostile environments and is the site where most virulence determinants reside in. Envelope stress response systems such as the Cpx 2CSTS, and others discussed earlier in this thesis, sense damage to the envelope and regulate genes to maintain its integrity. To begin with, I wanted to investigate whether zinc causes damage to the envelope. To test this, I conducted a lysis assay by exposing the cells to lysozyme and taking OD_{600} measurements over time to determine whether zinc destabilised the outer membrane. Gram-negative bacteria such as *E. coli* are generally resistant to lysozyme because of their outer membrane. I tested to see whether excess zinc would cause the outer membrane to become more permeable to lysozyme thereby indicating an envelope signal that Cpx response could sense. As a control, I used EDTA to chelate magnesium and calcium that stabilize the negatively charged LPS on the outer membrane, thereby making the cells more susceptible to lysozyme (Wooley and Blue, 1975). I did not observe any changes in the cells that had zinc and lysozyme perhaps owing to the concentration of zinc added and the short time frame used (Figure 3.1A). I then tried spotting *E. coli* onto LB plates containing either 100 μg/L vancomycin, 1 mM zinc or both. Unlike gram-positive bacteria, gram-negative bacteria are very resistant to vancomycin (up to concentrations of about 400 μg/mL) owing to their more selective outer membrane (Zhou *et al.*, 2015). I again expected that if excess zinc made the outer membrane more permeable, the cells would become susceptible to vancomycin. The results from this experiment showed that the cells did indeed became more susceptible to vancomycin in the presence of excess zinc giving evidence to suggest that zinc affects outer membrane permeability (Figure 3.1B).

I then sought to confirm zinc activation of the Cpx response and investigate whether it was involved in alleviating zinc induced stress. I grew wild-type MC4100 and mutants containing deletions in genes encoding Cpx signaling proteins or regulon members including: *nlpE* encoding the lipoprotein involved in surface sensing and whose overproduction induces the Cpx pathway; *cpxR* the response regulator of the Cpx pathway; *cpxA* the sensor kinase of the Cpx response - deletion of *cpxA* results in the constitutive activation of the response; *cpxP* a periplasmic chaperon that inhibits the Cpx response through interaction with CpxA; and *degP* the periplasmic protease that degrades misfolded proteins and CpxP. I then compared growth in each strain with or without zinc. The Δ*degP* mutant was unable to grow in the presence of excess zinc (Figures 3.2A and 3.2B) suggesting that *degP* could be involved in alleviating zinc toxicity. DegP is a periplasmic serine endoprotease that is involved in the cleavage of misfolded proteins including CpxP (Paul N Danese *et al.*, 1995). CpxP is known to inhibit activation of the Cpx response via interactions with the sensor kinase CpxA and activation of the response occurs via degradation of CpxP by the DegP protease. The Δ*cpxR* mutant grew very poorly as compared to the Δ*cpxA* mutant and wild-type when grown in excess zinc (Figures 3.2A and 3.2B) suggesting that Cpx activity plays a role in mediating zinc resistance in *E. coli*. Deleting *cpxA* does not remove the Cpx response but instead removes phosphatase activity that is required to cleave phosphate from CpxR that is auto-phosphorylated with acetyl-phosphate heterologous kinases. Taken together, this data provided further evidence to support the idea that under excess zinc conditions, the Cpx response could be sensing changes in periplasmic homeostasis and thereby elevating the expression of protein folding and degrading factors to alleviate zinc toxicity. However, as *degP* is also regulated by the σ^E response, that data also suggests that this response could be playing a critical role in survival under excess zinc conditions as the Δ*degP* mutant was the most susceptible to excess zinc.

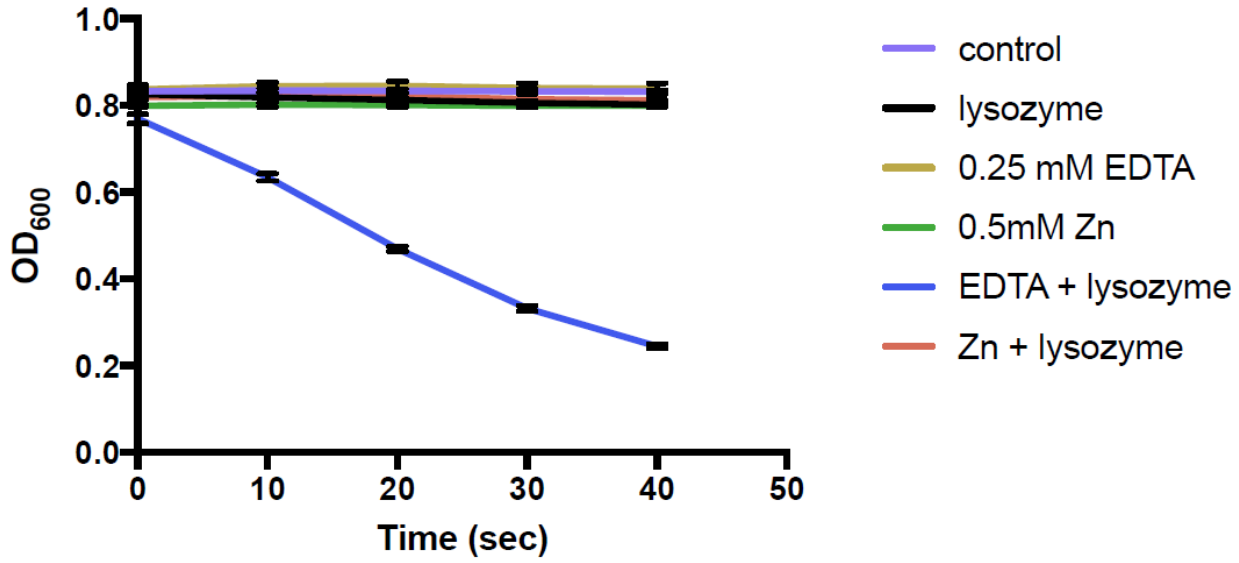

Figure 3.1A: *E. coli* susceptibility to lysozyme in excess zinc. Wildtype MC4100 was grown overnight in 5 mL LB at 37 ºC with aeration. 1:50 subcultures were made in 2 mL LB for each experimental group and control indicated and grown for 2 hours. Cells were washed twice in PBS before introducing the indicated experimental conditions. The OD_{600} was then measured every 10 seconds for 40 seconds using a Perkin Elmer Victor 1420 plate reader. The OD_{600} value is plotted against time. Data for lysis assay represents the mean and standard deviation of each triplicate set of samples. A representative result of two independent experiments is shown here.

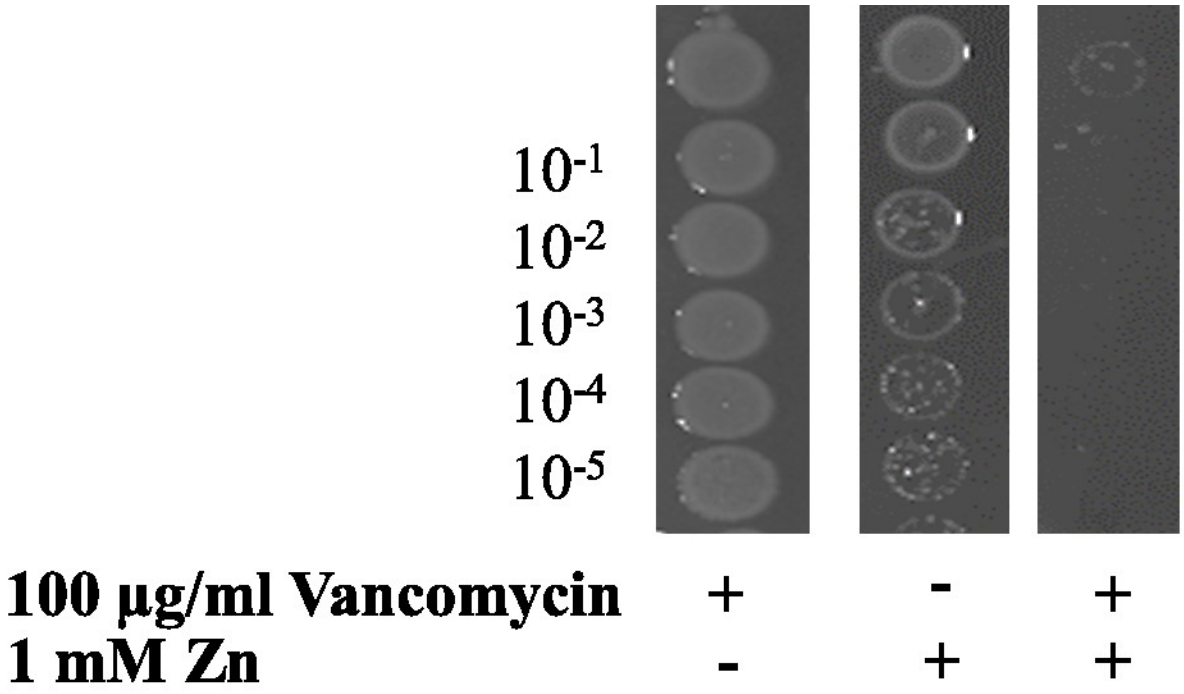

Figure 3.1B: Excess zinc increases *E. coli* susceptibility to vancomycin. Wildtype MC4100 was grown overnight in 5 mL LB at 37 ºC with aeration. Microdilutions of the cells were then made, at the indicated dilution factors, on plates containing 100 µg/mL vancomycin, or 1 mM $ZnCl_2$, or 100 µg/mL vancomycin and 1 mM $ZnCl_2$. Cells were then incubated at 37 ºC overnight. Data are representative of at least 2 independent experiments.

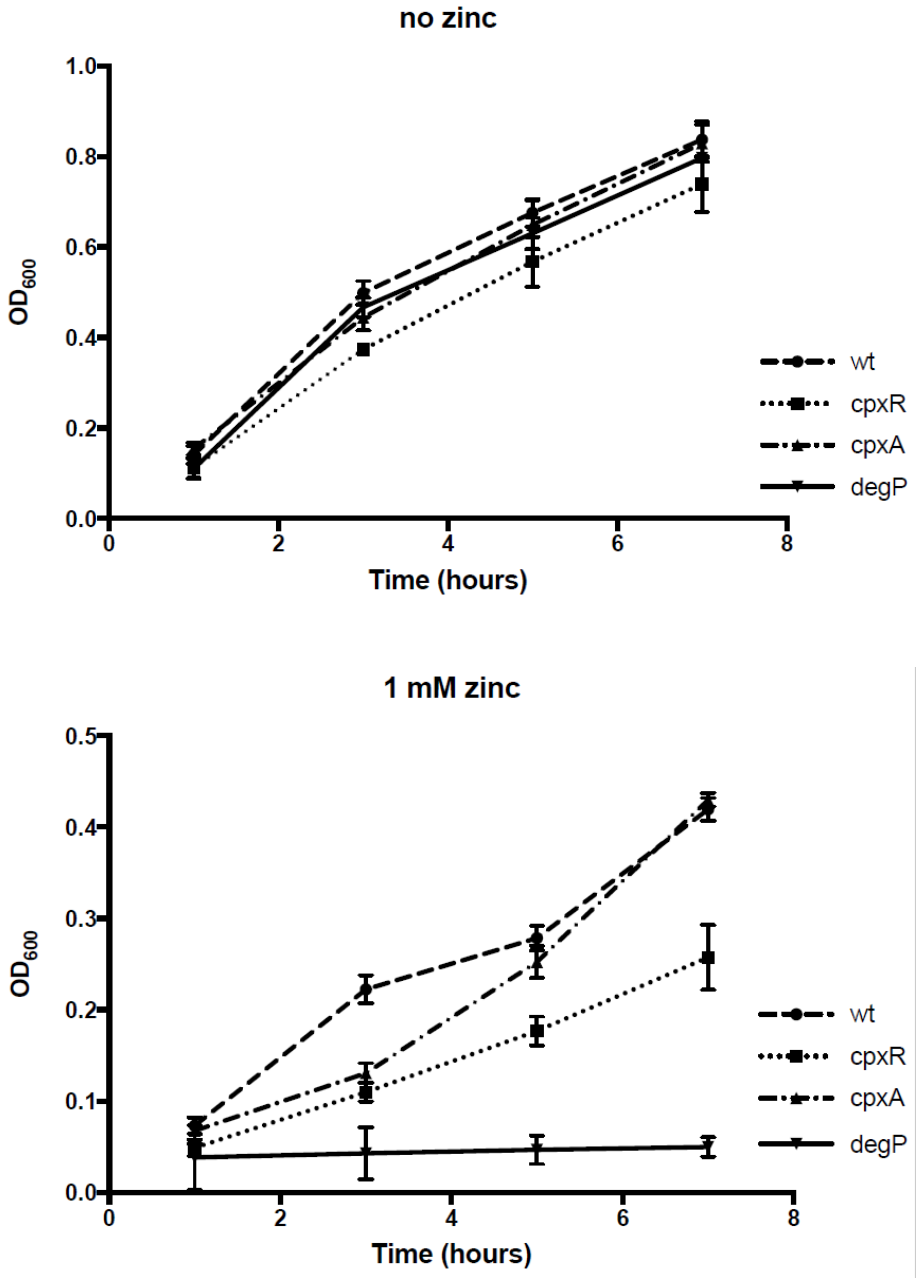

Figure 3.2: Deletion of *degP* leads to no growth in 1 mM Zn and deletion of *cpxR* leads to slow growth. A, growth profile of wild type MC4100, *cpxR*, *cpxA* and *degP* mutants in LB. **B,** growth profile of wild type MC4100, *cpxR*, *cpxA* and *degP* mutants in LB with added 1 mM zinc. Indicated strains were grown overnight in 5 mL LB at 37 ºC with aeration. 1:50 subcultures were made in 4 mL LB for each strain and grown for 2 hours before adding either zinc to a final

concentration of 1 mM to half the culture and an equal volume mQH_2O water was added to the rest and left to grow for another 6 hours in a 96 well microtiter plate under the same conditions. OD_{600} values were recorded every 2 hrs using Perkin Elmer Victor 1420 plate reader. The average OD_{600} and standard deviation of each triplicate set of samples is plotted against time. A representative of result at least two independent experiments is shown here.

3.1.2 Excess zinc activates the Cpx response in *E. coli* and *Vibrio cholerae*

As previous results showed that the Δ*cpxR* mutant had a growth defect in the presence of excess zinc I hypothesized that activation of the Cpx pathways was necessary for growth under these conditions. To confirm zinc induced activation of the Cpx response, I followed the activity of a *cpxP-lux* transcriptional reporter encoded on the plasmid pNLP15, under conditions of excess zinc and in untreated LB. The plasmid pNLP15 encodes for bacterial luciferase, with the *cpxP* promoter sequence cloned upstream of the *luxCDABE* genes, to report Cpx activity via luminescence. *cpxP* expression in *E. coli* is known to be strictly under the control of *cpxR* and as such is often used to determine the level of Cpx pathway activity (Danese and Silhavy, 1998; Price and Raivio, 2009). I spotted *E. coli* and *V. cholerae* carrying the *cpxP-lux* reporter gene on LB plates containing zinc and observed luminescence activity in the absence and presence of zinc. Results showed that zinc activated the Cpx response in both *E. coli* and *V. cholerae* suggesting that this response could be conserved among enteric Gram-negative bacteria (Figure 3.3).

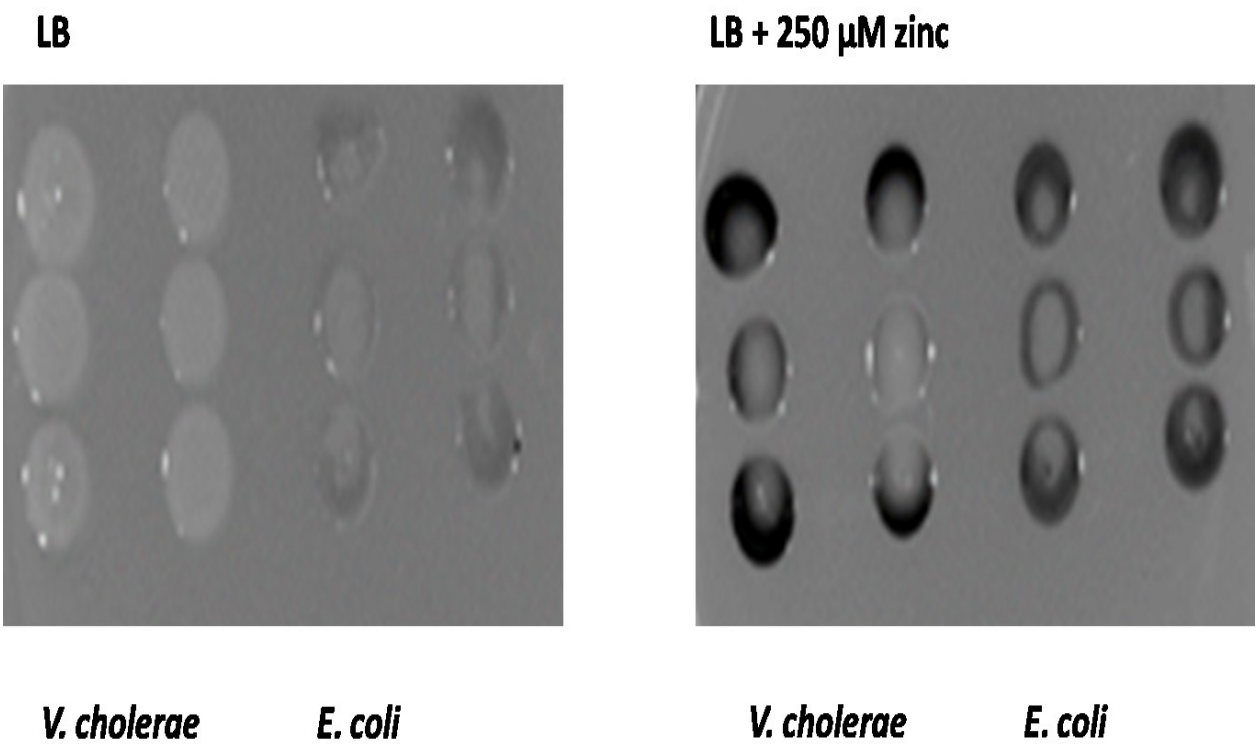

Figure 3.3: Zn activation of the Cpx response is conserved in *Vibrio cholerae* and *E. coli*. *E. coli* MC4100 *pNLP15* and *V. cholera* C6706 *pN3* containing the *pcpxP-lux* plasmids were grown overnight in 5 mL LB at 37 ºC with aeration. Cells were then spotted on LB and LB supplemented with 250 µM Zn plates containing and left to grow overnight at 37 ºC. Luminescence was then measured under an imager. Dark spots indicate luminescence. Data are representative result of two independent experiments.

3.1.3 Zinc activation of the Cpx response in *E. coli* is dependent on *cpxR* and *cpxA*

To investigate whether zinc activation of the Cpx response occurs via an envelope signal – whether it is dependent on the envelope-localized signaling proteins NlpE, CpxP, CpxA, and/or the cytoplasmic response regulator CpxR, I followed the activity of a *cpxP-lux* transcriptional reporter in Δ*cpxR*, Δ*cpxA*, Δ*cpxP*, Δ*nlpE* and Δ*degP* mutants under excess zinc conditions and compared them to those of the wild type. I observed an increase in Cpx pathway activity in the presence of zinc in wild type, Δ*nlpE* and Δ*cpxP* mutants but not in the Δ*cpxR* or Δ*cpxA* mutants suggesting that activation of the response was due to an extracytoplasmic signal that was sensed by CpxA, but did not require the surface sensing lipoprotein NlpE, the cytoplasmic chaperone/inhibitor CpxP, or the periplasmic protease DegP (Figure 3.4). This data also provided further evidence to support the idea that under excess zinc conditions, the Cpx pathway could be sensing changes in periplasmic homeostasis and thereby elevating the expression of protein folding and degrading factors, or other Cpx-regulated genes, to alleviate zinc toxicity.

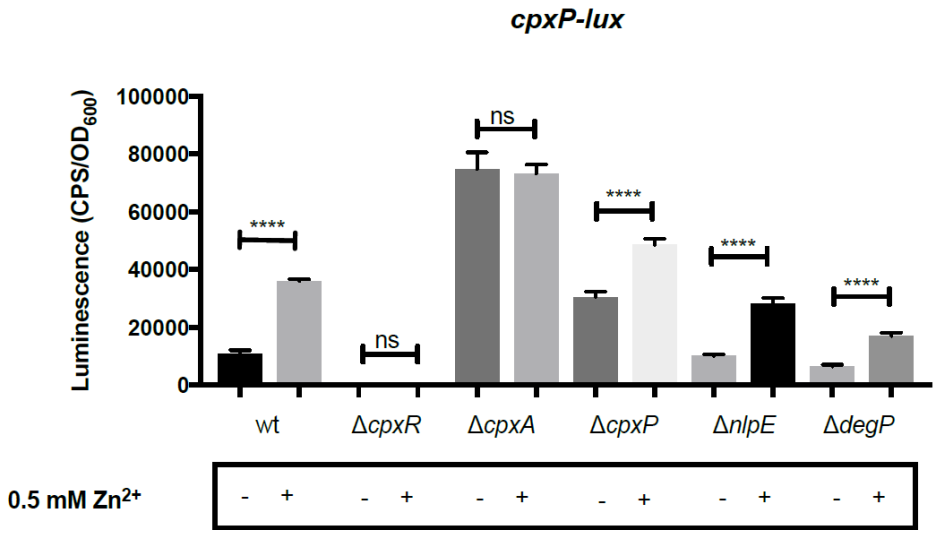

Figure 3.4: Zn activation of the Cpx response is dependent on CpxA and CpxR. *cpxP* expression in wild-type, Δ*cpxR*, Δ*cpxA*, Δ*cpxP*, Δ*nlpE* and Δ*degP* mutants grown in LB compared to conditions of excess zinc. Indicated strains were grown overnight in 5 mL LB with appropriate antibiotics at 37 ºC with aeration. 1:50 subcultures were made in 4 mL LB for each strain and grown for 2 hours before adding either zinc to a final concentration of 1 mM to half the culture and an equal volume mQH_2O water was added to the rest and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted. A representative result of at least two independent experiments is shown here. **** indicates a statistically significant difference with P value of ≤ 0.0001, ns denotes a statistically insignificant difference with a P value of > 0.05 as compared to the relevant LB control (one-way ANOVA multiple comparison test).

3.2 Objective 2: Analysis of Cpx regulated *cysK* and cysteine transporter expression

A microarray carried out by Yamamato showed that 9 out of the 26 genes that were induced more than two-fold, by addition of excess external zinc, were organised in the regulon for cysteine biosynthesis in *E. coli* (Yamamoto and Ishihama, 2005). There are about 70 genes that are involved in cysteine biosynthesis, each of these being located within one of the 9 operons: *cysS*p, *cysZ*p, *cysK*p, *cysP*p, *cysD*p, *cysJ*p, *nirB*p, *cysE*p, or *metB*p promoter regions. These operons include genes that are involved in sulphate uptake and enzymes involved in cysteine production, to name but a few (Kredich, 2008b). The authors hypothesised that the increase in the intracellular level of cysteine, may play a role in transient trapping of excess free zinc prior to export of excess zinc. The microarray data also showed more than four-fold induction by zinc of the *htrA* (*degP*) gene encoding a periplasmic protease, which is involved in degradation of damaged proteins including CpxP (Yamamoto and Ishihama, 2005). In another microarray used to characterise genes under the control of the Cpx regulon, it was observed that the genes involved in cysteine biosynthesis and *degP* are as well highly expressed upon activation of the Cpx response via *nlpE* overexpression (Price and Raivio, 2009; Raivio, Leblanc and Price, 2013). This combined evidence suggested to us that zinc could be inducing the expression of cysteine biosynthetic genes by activating the Cpx response.

I sought to determine whether the Cys regulon was indeed induced by the Cpx response under excess zinc conditions. For this purpose, I cloned the promoter region of *cysK* upstream of the *luxCDABE* genes on the pNLP10 plasmid to use as a reporter for *cysK* transcriptional activity. *cysK* encodes for cysteine synthase A, the enzyme that is responsible for the catalytic conversion of O-acetyl-L-serine and hydrogen sulphide into L-cysteine and acetate (Kredich, 2008a). I

followed both *cysK* and *cpxP* expression under different concentrations of zinc and observed a direct correlation between the concentration of zinc and expression levels of both *cysK* and *cpxP* (Figure 3.5). I also affirmed findings that activation of the Cpx response via *nlpE* overexpression induces expression of *cysK* using the same *cysK-lux* plasmid (Figure 3.6). To determine whether activation of the Cys regulon was dependent on the Cpx response under excess zinc conditions I followed *cysK* promoter activity in the Δ*cpxA*, Δ*cpxR*, Δ*cpxP*, Δ*degP* and Δ*nlpE* mutants under excess zinc conditions and found that *cysK* expression was induced independently of the Cpx response. However, I did observe that the fold change of induction of *cysK* in the Δ*cpxA* and Δ*cpxR* mutants was considerably less than the wild type and Δ*nlpE* mutant suggesting that the Cpx response could have an additive effect on induction of cysteine biosynthetic genes upon exposure to excess zinc (Figure 3.7). After 2 hours of exposure to 0.5 mM of zinc, I observed over 20-fold induction of reported *cysK* expression in wild type and the Δ*nlpE* mutant whereas only about 10-fold induction was observed in in the Δ*cpxA* and Δ*cpxR* mutants. In addition the basal levels of *cysK* were much higher in the Δ*cpxA* and Δ*cpxR* mutants compared to the wild-type and Δ*nlpE* mutant suggesting to us that the Cpx response could be repressing *cysK* activity under normal conditions.

To confirm our findings, I conducted a qPCR and evaluated the fold change of *cpxP* and *cysK* in a Δ*cpxR* mutant and compared it to the wild type under excess zinc conditions. The results indeed showed that *cysK* was upregulated about 2-fold in the Δ*cpxR* mutant under normal growth conditions in untreated LB and that there was about twice as much induction of *cysK* in the wild type than in the Δ*cpxR* mutant upon exposure to excess zinc (Table 3.1). These results supported that idea that the CpxR response represses *cysK* under normal conditions. I hypothesized that under normal conditions, the Cpx response represses *cysK* to reduce cysteine levels that would otherwise

be exported to the periplasm and make it more reducing - a condition that could have a negative impact on protein folding.

To get a better understanding of the possible role cysteine plays in alleviating zinc induced stress and how the Cpx response is implicated, I investigated changes in intracellular thiols under conditions of excess zinc in wild type, Δ*cpxA*, Δ*cpxR* and a cysteine auxotroph Δ*cysK* (Kredich, 2008a). I observed an increase in intracellular thiol concentrations in the wild type upon exposure to excess zinc. I also observed an overall reduced intracellular thiol concentration which was not impacted by zinc in the Δ*cysK* mutant, as was anticipated for a cysteine auxotroph. Interestingly, the thiol concentration in the Δ*cpxA* and Δ*cpxR* mutants was also not impacted by zinc albeit the basal level of thiols was much higher compared to wild type (Figure 3.8). This result being consistent with the observed *cysK* activity already shown (Figure 3.7)

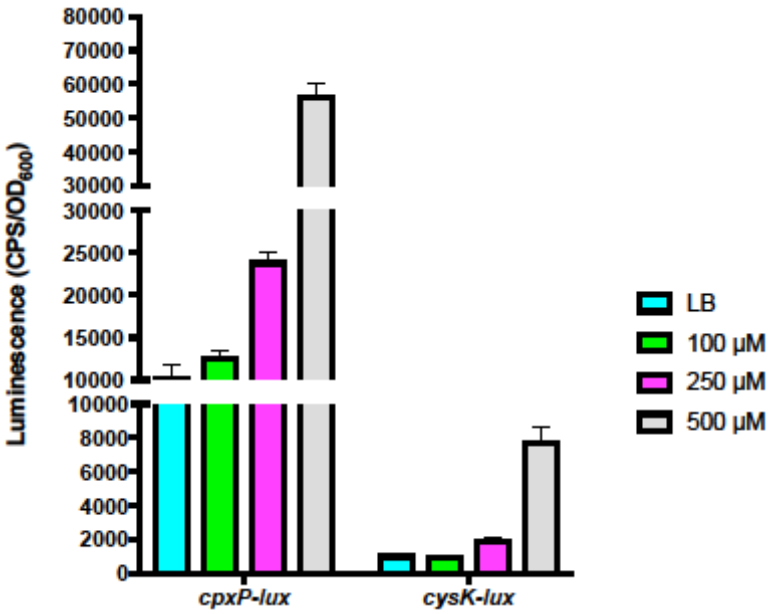

Figure 3.6: Zn activation of the Cpx response and *cys* regulon correlates with concentration of excess zinc added to media. *E. coli* MC4100 carrying either the *pNLP15 cpxP-lux* reporter or *pJZ1 cysK-lux* reporter plasmids were grown overnight in 5 mL LB with appropriate antibiotics at 37 ºC with aeration. 1:50 subcultures were made in 2 mL LB for each strain and grown for 2 hours before adding either zinc to final indicated concentrations and an equal volume mQH_2O water to the control and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted. A representative result of at least two independent experiments is shown here.

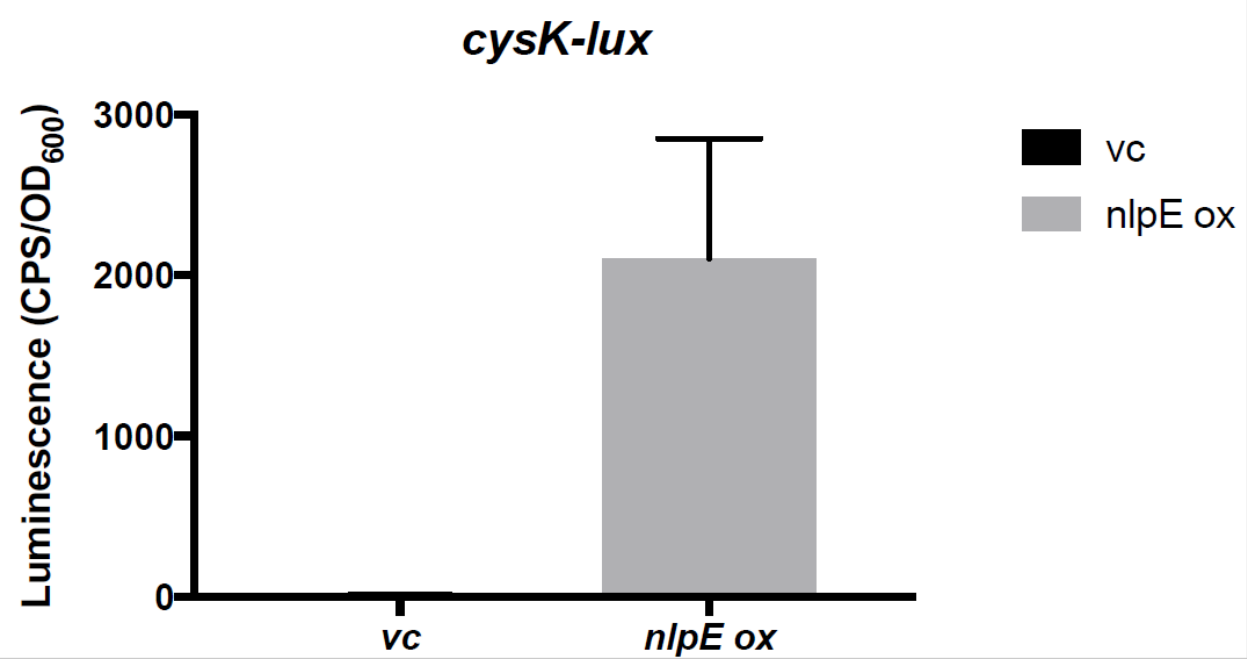

Figure 3.7: *nlpE* overexpression upregulates *cysK* expression. *E. coli* MC4100 carrying *pJZ1 cysK-lux* reporter and either *pCA24-N* vector or *pCA-nlpE* overexpression plasmids were grown overnight in 5 mL LB with appropriate antibiotics at 37 °C with aeration. 1:50 subcultures were made in 2 mL LB for each strain and grown for 2 hours before adding IPTG to final concentration of 0.05 mM and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted against time. A representative result of two independent experiments is shown here.

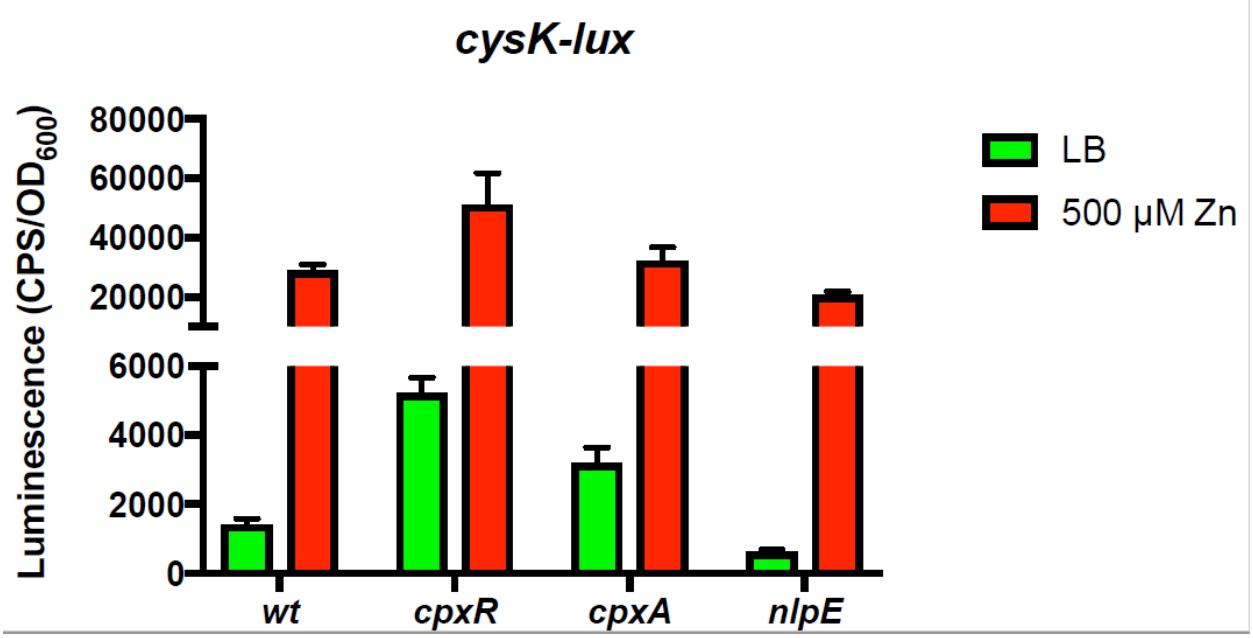

Figure 3.8: Zn activation of the *cys* regulon is independent of the Cpx response. *E. coli* MC4100 wild-type and indicated mutants carrying the *pJZ1 cysK-lux* reporter plasmids were grown overnight in 5 mL LB with appropriate antibiotics at 37 ºC with aeration. 1:50 subcultures were made in 4 mL LB for each strain and grown for 2 hours before adding either zinc to final indicated concentration to half the culture and an equal volume of mQH_2O water to the rest and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted. A representative result of at least two independent experiments is shown here.

Table 3.1: qPCR results showing relative fold change of indicated genes in wt upon exposure to excess zinc and Δ*cpxR* mutant with and without zinc

		no Zinc		0.5 mM Zinc			
		Δ*cpxR*		*wt*		Δ*cpxR*	
Gene	Description	X Change (compared to WT)	Stdev	X Change (compared to no zinc)	Stdev	X Change (compared to WT no zinc)	Stdev
cpxP	chaperone, auxiliary inhibitor of CpxA	0.100	0.006	19.299	3.248	0.072	0.002
cysK	cysteine biosynthesis	2.216	0.247	24.721	0.620	5.550	0.136
spy	periplasmic chaperone	0.621	0.078	56.599	2.263	0.799	0.202
degP	periplasmic serine endoprotease DegP	0.060	0.109	35.990	8.703	0.103	0.374
zntA	Zn exporter	2.064	0.257	179.079	28.284	55.398	7.279
znuC	Zn importer	0.743	0.103	0.565	0.023	0.254	0.017
yfiK	transporter involved in the efflux of O-acetylserine and cysteine	0.041	0.001	0.645	0.186	0.339	0.457
ydeD	secretion of O-acetylserine and cysteine	0.092	0.040	0.830	0.101	0.860	0.087
ydjN	periplasmic binding protein of a cystine/cysteine ABC transport system	0.065	0.007	6.950	3.137	14.613	0.179
fliy	L-cystine uptake	0.002	0.000	0.527	0.461	0.284	0.715

Table 3.2 qPCR results showing cycle times of indicated genes in wt upon exposure to excess zinc and Δ*cpxR* mutant with and without zinc

		wt		Δ*cpxR*		wt + 0.5 mM Zn		Δ*cpxR* + 0.5 mM Zn	
Gene	Description	Avg	Stdev	Avg	Stdev	Avg	Stdev	Avg	Stdev
rpoD	RNA polymerase sigma factor	19.79	2.10	18.60	0.10	20.03	0.36	18.07	0.18
cpxP	chaperone, auxiliary inhibitor of CpxA	20.97	0.08	23.11	0.09	16.94	0.25	23.06	0.04
cysK	cysteine biosynthesis	20.33	0.07	18.00	0.16	15.94	0.04	16.14	0.04
spy	periplasmic chaperone	23.69	0.11	23.19	0.18	18.10	0.06	22.29	0.38
degP	periplasmic serine endoprotease DegP	21.25	0.47	24.13	2.20	16.32	0.35	22.81	2.96
zntA	Zn exporter	25.86	0.26	23.63	0.18	18.62	0.23	18.35	0.19
znuC	Zn importer	18.76	1.30	18.00	0.21	19.82	0.06	19.02	0.10
yfiK	transporter involved in the efflux of O-acetylserine and cysteine	27.43	0.29	30.83	0.04	28.30	0.45	27.27	1.64
ydeD	secretion of O-acetylserine and cysteine	24.73	0.42	26.98	0.55	25.24	0.18	23.23	0.14
ydjN	periplasmic binding protein of a cystine/cysteine ABC transport system	24.26	0.90	27.01	0.16	21.70	0.73	18.67	0.02
fliy	L-cystine uptake	24.71	0.06	32.88	0.16	25.88	1.13	24.81	2.47

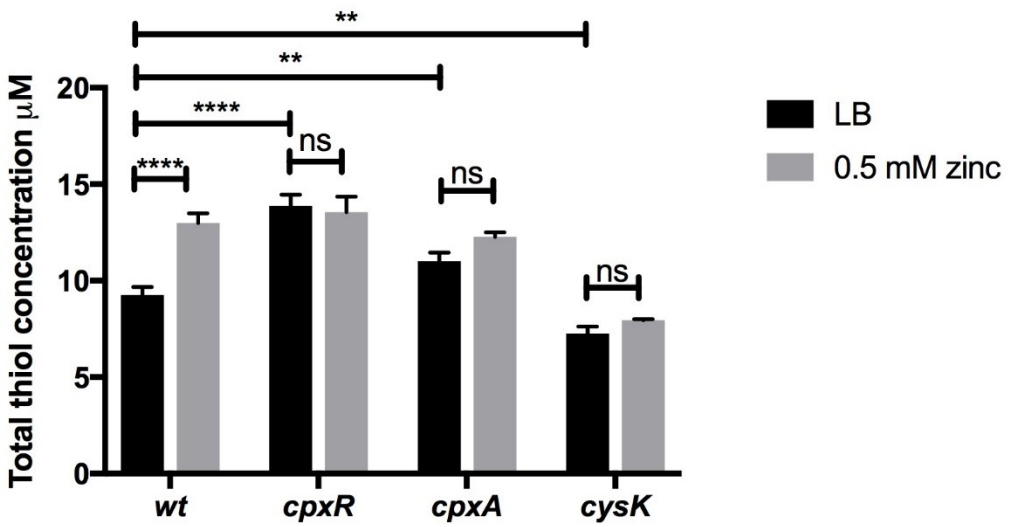

Figure 3.9: Intracellular thiol concentrations are affected by zinc in a Cpx and *cysK* dependent manner. Thiol concentrations were measured using the Measure-It thiol quantification kit from Invitrogen. *E. coli* MC4100 wild-type and indicated mutants were grown overnight and sub-cultured in 4 mL fresh LB the next day for 2 hours before adding $ZnCl_2$ to a final concentration of 0.5 mM to half of the culture and a similar volume of mQH_2O water was added to the rest. The cells were then grown for another 2 hours washed and resuspended in 2 mL of phosphate buffer saline (PBS) before lysing in a ultrasonic water-bath sonicator at maximum exposure for 5 min. 10 µL of the lysate was then added to 100 µL of the Measure-iT thiol quantitation reagent and incubated at room temperature for 5 min. Fluorescent measurements were then taken at 435/540 nm to quantify the thiol concentration against a prepared standard. Average thiol concentrations and standard deviations of sextuplet samples is plotted for each strain. A representative result of two independent experiments is shown here. **** indicates a statistically significant difference with P value of ≤ 0.0001, ** indicates a statistically significant difference with P value of ≤ 0.01, ns denotes a statistically insignificant difference with a P value of > 0.05 (one-way ANOVA multiple comparison test).

3.2.2 DTT activates the Cpx response

As previous results showed that the Cys regulon was up-regulated independent of the Cpx response under excess zinc conditions, I postulated that the Cpx response could be sensing reducing equivalents, such as cysteine, that are exported into the periplasm upon exposure to excess zinc. For this purpose, I investigated whether adding a known reducing agent such as DTT (Sinha, Langford and Kroll, 2004), that can cross the outer membrane, would activate the Cpx response. I exposed the cells to DTT and measured luminescence activity of both *cpxP-lux* and *cysK-lux* reporter genes. Consistent with our hypothesis, DTT activated the Cpx response in a *cpxA* dependent manner and resulted in a downregulation of *cysK* (Figures 3.10A and 3.10B).

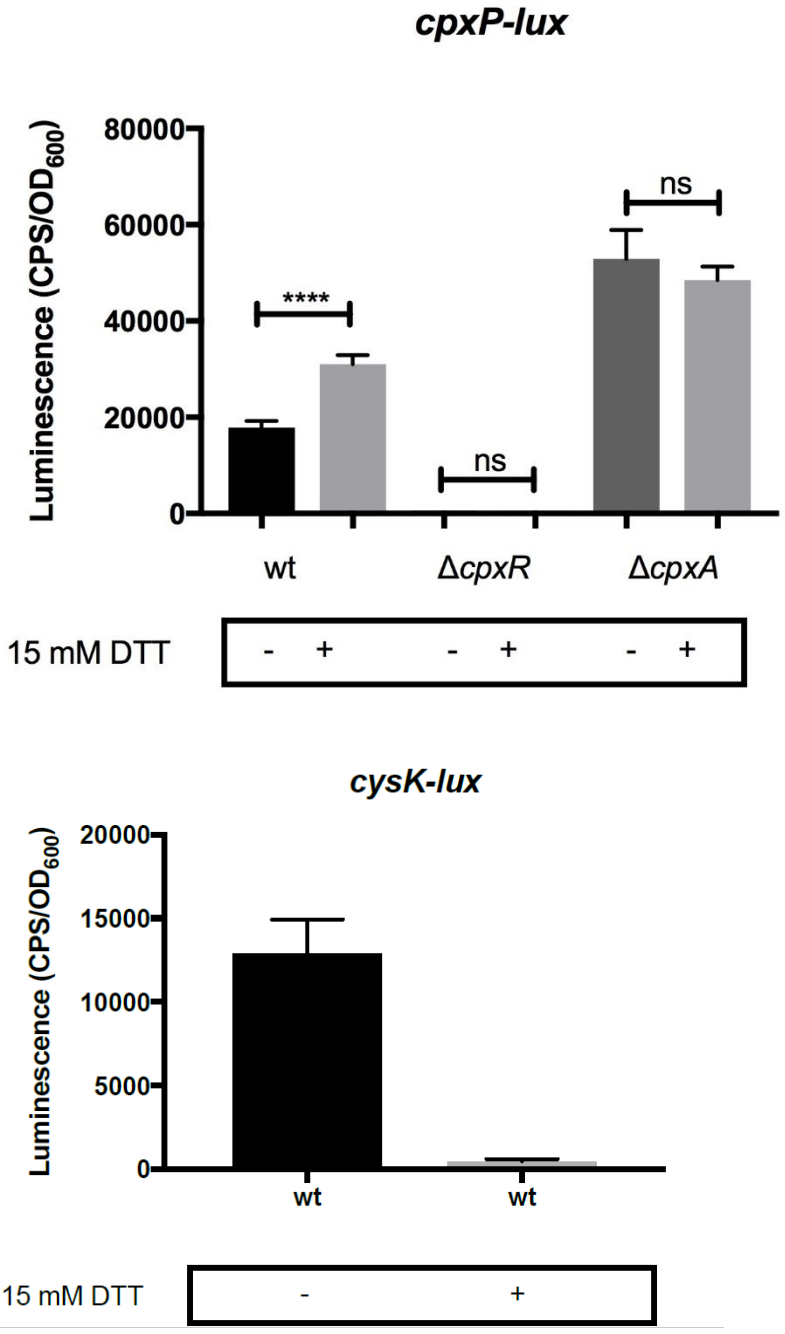

Figure 3.10: DTT effect on the Cpx response and *cysK* activity. A, DTT activates the Cpx response in a *cpxA* and *cpxR* dependent manner and **B,** downregulates *cysK*. *E. coli* MC4100 wild-type and indicated mutants carrying either the *pNLP15 cpxP-lux* reporter or the *pJZ1 cysK-lux* reporter plasmids were grown overnight in 5 mL LB with appropriate antibiotics at 37 ºC with

aeration. 1:50 subcultures were made in 4 mL LB for each strain and grown for 2 hours before adding either DTT to final indicated concentration to half the culture and an equal volume of mQH_2O water to the rest and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted. A representative result of at least two independent experiments is shown here. **** indicates a statistically significant difference with P value of ≤ 0.0001, ns denotes a statistically insignificant difference with a P value of > 0.05 as compared to the relevant LB control (one-way ANOVA multiple comparison test).

The DTT results suggested to me that the Cpx response could be sensitive to changes in periplasmic reducing potential. To complement the DTT results I looked to determine whether deleting genes that alter periplasmic redox would affect zinc induced activation of the Cpx response. For this purpose, I constructed a cysteine auxotroph by deleting *cysK* so as to decrease the number of reducing equivalents that could be transported to the periplasm. I also constructed a Δ*dsbA* mutant that was meant to make the periplasm more reducing. *dsbA* encodes for the bacterial thiol disulphide oxidoreductase that is responsible for introducing disulphide bonds in extracytoplasmic proteins as they enter the periplasm. In its absence, the periplasm becomes more reducing due to the accumulation of extracellular proteins containing reduced sulphur (Ping *et al.*, 2012). I then examined the effect that zinc had on the Cpx response in these mutants. As expected for the Δ*cysK* mutant there was a down-regulation of the Cpx response with luminescence of the Cpx-regulated *cpxP-lux* reporter gene significantly below that of the basal wild type levels (Figure 3.11). I also saw elevated levels of Cpx activity in the *dsbA* mutant as expected (Figure 3.12).

I further investigated whether adding exogenous cysteine would activate the Cpx response or affect zinc induced activation of the response. My tests showed no significant difference between samples supplemented with cysteine as compared to LB even in the presence of excess zinc (Figure 3.13). The results suggested that the exogenously added cysteine could either be degraded quickly, not imported into the cell or that the concentration I used was too low to affect Cpx activity. The levels of cysteine within the cells are tightly controlled due to the high reactivity of cysteine that makes it toxic at high concentrations (Harris, 1981). There are at least 5 known cysteine desulfhydrases in *E. coli* and it is thought this high number of cysteine degrading enzymes helps to keep cysteine concentrations at a low level (Loddeke *et al.*, 2017).

As the data collected thus far showed that *cysK* upregulation under excess zinc conditions was independent of the Cpx response I wondered whether the PhoPQ system was regulating *cysK* under these conditions instead. The PhoPQ response is a two-component system that responds to limiting extracellular Mg^{2+} and Ca^{2+} as well as the presence of cationic antimicrobial peptides. In Salmonella, the PhoPQ system regulates the expression of genes involved in Mg^{2+} transport and it is believed that the PhoPQ response is activated to recruit Mg^{2+} required to maintain stability of LPS in the outer membrane (Groisman, 2001). It is also believed to sense periplasmic redox as it is also activated by DTT and by deletion of *dsbA* (Lippa and Goulian, 2012). Furthermore, there is a predicted PhoP binding site on the *cysB* promoter - the major regulator of cysteine biosynthetic genes. To this end, I tested whether deletions in *cpxR, cpxA, phoQ, cysK* or *dsbA* would have an effect on sensitivity to vancomycin in the presence of excess zinc. I did not see a significant effect in the Δ*cpxR,* Δ*cpxA* or Δ*phoQ* mutants compared to the wild-type upon exposure to vancomycin and excess zinc. I did however, notice that the Δ*cysK* mutant seemed more resistant at 10 µg/mL vancomycin in excess zinc but not at 50 µg/mL. On the other hand, the Δ*dsbA* mutant seemed the most sensitive to vancomycin at either concentration (Figure 3.14).

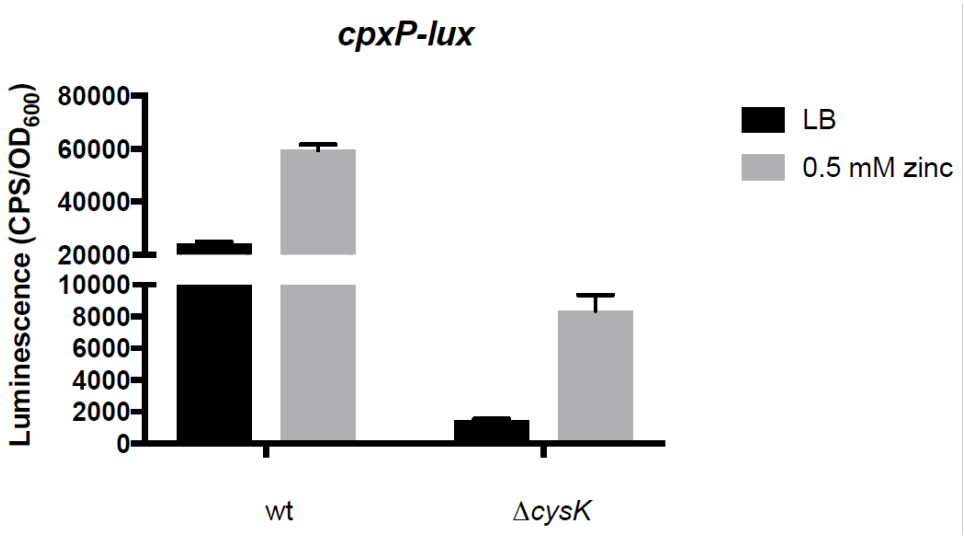

Figure 3.11: basal Cpx pathway activity is lowered in a Δ*cysK* mutant. *E. coli* MC4100 wild-type and indicated mutants carrying the *pNLP15 cpxP-lux* reporter plasmid were grown overnight in 5 mL LB with appropriate antibiotics at 37 ºC with aeration. 1:50 subcultures were made in 4 mL LB for each strain and grown for 2 hours before adding either zinc to a final concentration of 0.5 mM to half the culture and an equal volume mQH_2O water was added to the rest and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted. A representative result of at least two independent experiments is shown here.

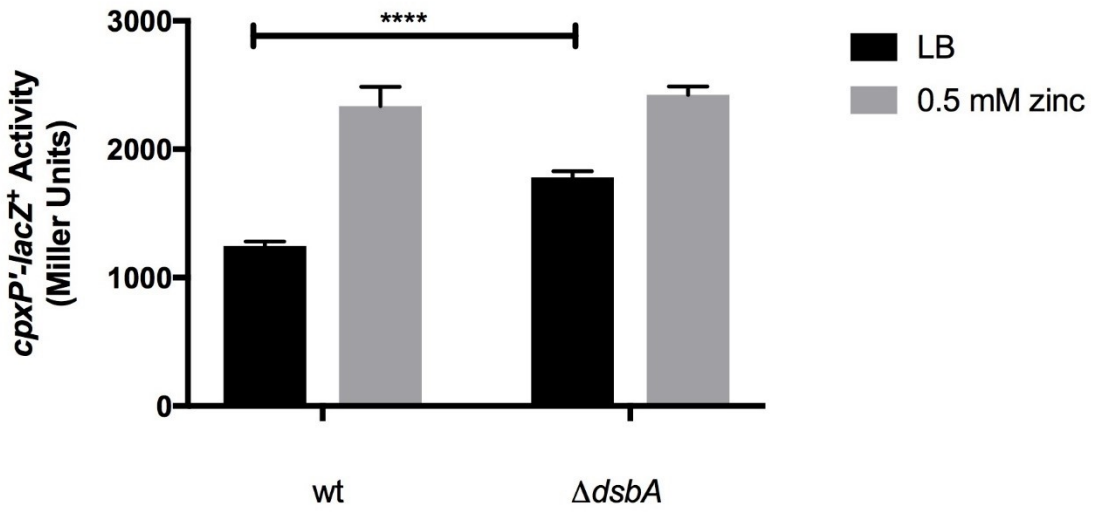

Figure 3.12: the Cpx response is activated in a Δ*dsbA* mutant. Single isolated colonies of *E. coli* TR50 and indicated mutants were inoculated into 5 mL of LB broth with the appropriate antibiotics and grown overnight with aeration at 37 ºC. Cultures were then diluted 1:50 into 4 mL fresh LB broth plus antibiotics and grown for 2 hrs under the same conditions. Zinc chloride or mQH_2O was then added to half the cultures all at the same volume to the indicated final concentrations and grown for another 2 hrs. Cells were then centrifuged and resuspended in 2 mL of buffer Z before being lysed using EDTA and chloroform. β-galactosidase activity was then measured. The average Miller Units (indicative of β-galactosidase activity) and standard deviation of each sextuplet set of samples is plotted for each strain. A representative result of two independent experiments is shown here. A**** indicates a statistically significant difference with P value of ≤ 0.0001 as compared to the relevant LB control (one-way ANOVA multiple comparison test).

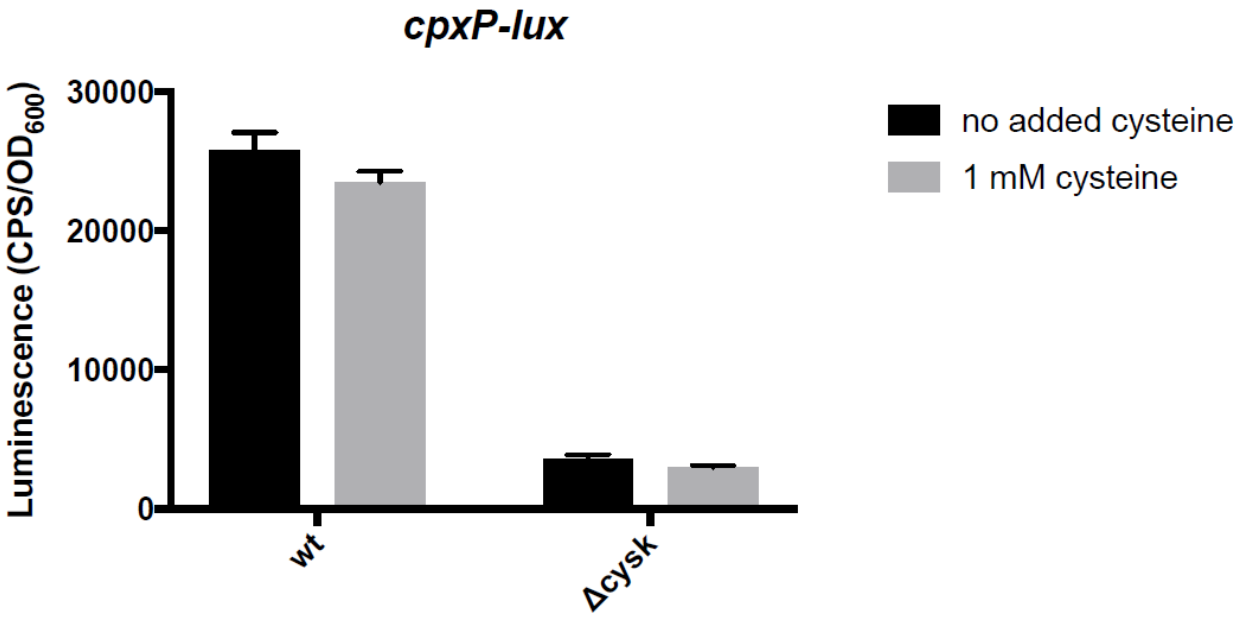

Figure 3.13: exogenous cysteine does not restore Cpx activity in a Δ*cysK* mutant. *E. coli* MC4100 wild-type and indicated mutants carrying the *pNLP15 cpxP-lux* reporter plasmid were grown overnight in 5 mL LB with appropriate antibiotics at 37 °C with aeration. 1:50 subcultures were made in 4 mL LB for each strain and grown for 2 hours before adding either cysteine to a final concentration of 1 mM to half the culture and an equal volume mQH_2O water was added to the rest and left to grow for another 2 hours in a 96 well microtiter plate. The OD_{600} and luminescence value, measured as counts per second (CPS), was then recorded using a Perkin Elmer Victor 1420 plate reader. The average CPS/OD_{600} and standard deviation of each sextuplet set of samples is plotted. A representative result of at least two independent experiments is shown here.

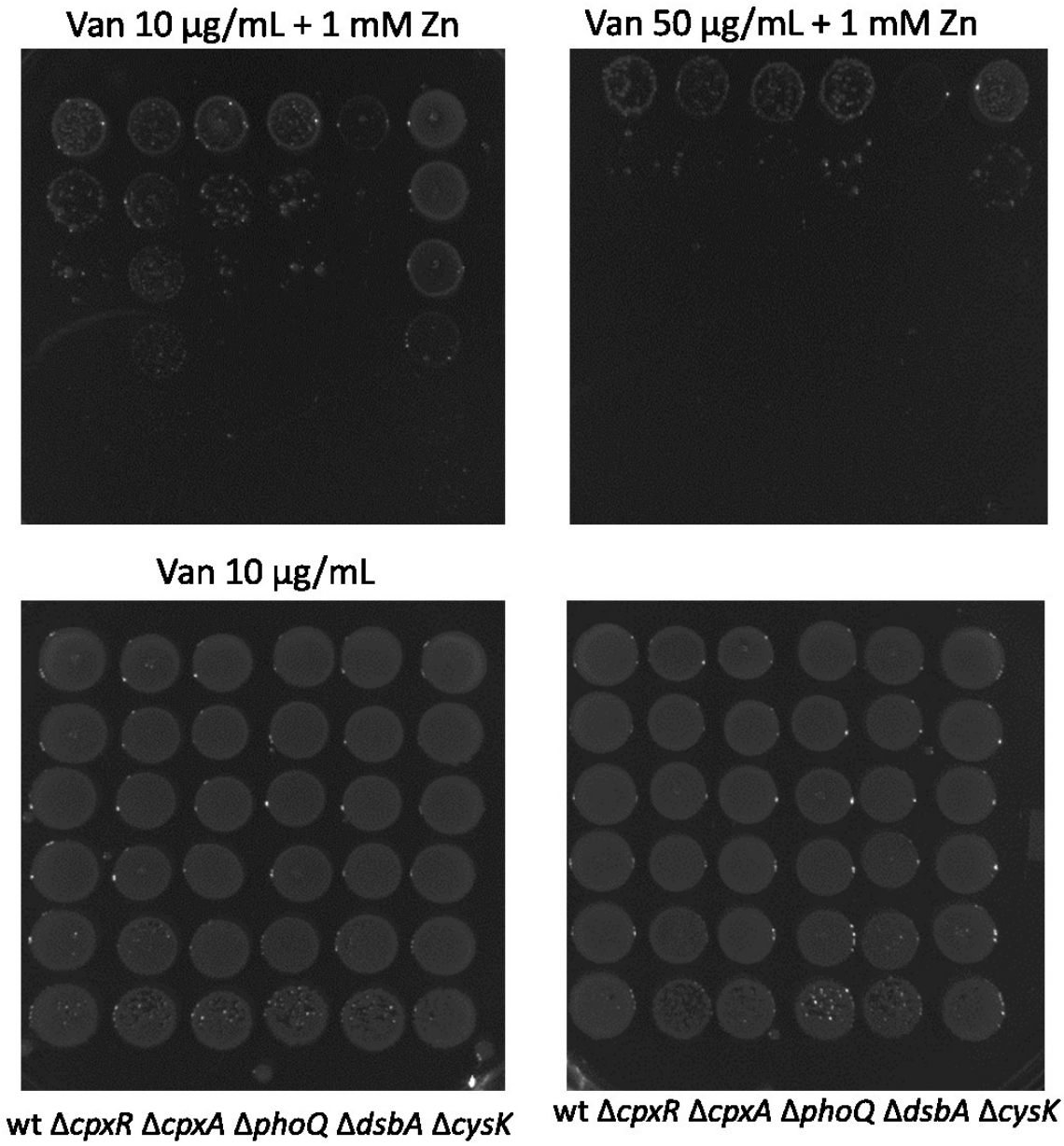

Figure 3.14: Deletions in a *phoQ, dsbA* and *cysK* have no effect on vancomycin susceptibility in the presence of excess zinc. Wildtype MC4100 and indicated mutants were grown overnight in 5 mL LB at 37 ºC with aeration. Microdilutions of the cells were then made on plates containing 10 µg/mL vancomycin, or 1 mM $ZnCl_2$, or 50 µg/mL vancomycin and 1 mM $ZnCl_2$. Cells were then incubated at 37 ºC overnight. Data are representative of at least 2 independent experiments.

3.2.3 Effect of deleting zinc or cysteine transporters on zinc induced activation of the Cpx response

In another study conducted to show whether the Cpx response altered the expression of zinc exporters, wild-type, Δ*cpxR*, Δ*cpxA* and Δ*cpxP* mutants were transformed with a *zntA-lux* reporter and the luminescence of the transformants was examined on LB agar containing a gradient of zinc. In the wild-type strain and Δ*cpxP*, *zntA-lux* activity increased with increasing amounts of zinc until cell death whereas in the Δ*cpxA* and Δ*cpxR* mutants, luminescence remained low at all concentrations where growth was observed, even at lethal concentrations of zinc (Wong, 2015). Our qPCR experiments showed similar results in that *zntA* was upregulated about 3 times more in wild-type with zinc compared to the Δ*cpxR* mutant (Table 3.1). I also examined expression of cysteine transporters from the qPCR experiments and noticed a reduction in expression of the transporters *ydjN*, *yfiK*, *ydeD* and *fliy* in the Δ*cpxR* mutant. Cysteine can be exported to the periplasm, via YdeD or YfiK, where it is believed to act as an antioxidant by reducing hydrogen peroxide. The oxidized cysteine (cystine) can then either be exported out of the cell via TolC or imported into the cytoplasm via FliY or YdjN (Kredich, 2008a). Under conditions of excess zinc, I noticed that the cystine importer *ydjN*, was upregulated twice as much in the Δ*cpxR* mutant as compared to the wild type (Table 3.1)

Collectively, the data suggested that the Cpx response regulated metal and cysteine transporters to alleviate envelope stress that affects periplasmic redox balance. To test this, we investigated Cpx activity in strains lacking cysteine or zinc transporters in the presence of excess zinc. Our results showed that the zinc induced activation of the Cpx response was dependent on *zntA*. I did not see any differences in Cpx activation in mutants lacking certain cysteine transporters

which could mean that single deletions of these transporters may not have an effect on cysteine trafficking. As previously observed (Fig. 3.11), I did, however, notice reduced basal activity of the Cpx response in a *cysK* cysteine auxotroph, thereby supporting the idea that altering thiol concentrations impacts the Cpx response (Figure 3.15).

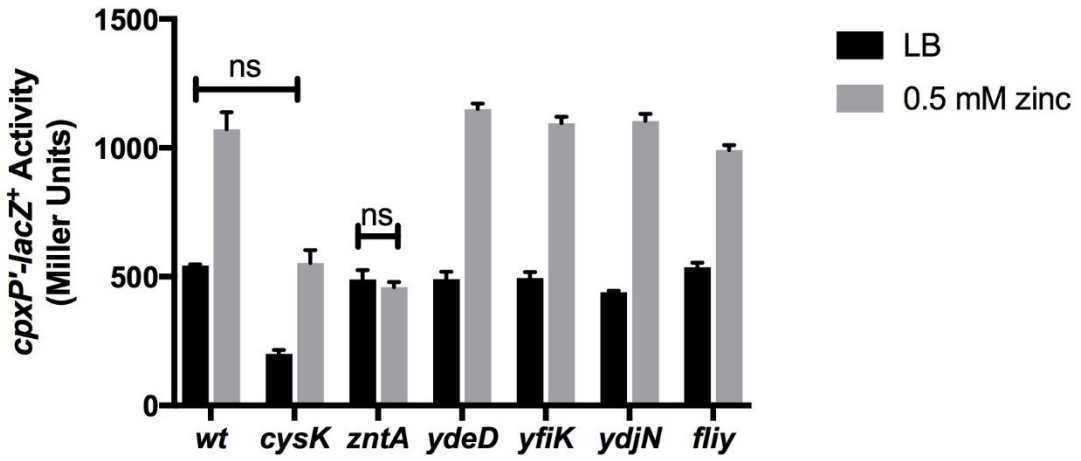

Figure 3.15: Zinc induced activation of the Cpx response is dependent on *zntA*. Single isolated colonies of *E. coli* TR50 and indicated mutants were inoculated into 5 mL of LB broth with the appropriate antibiotics and grown overnight with aeration at 37 ºC. Cultures were then diluted 1:50 into 4 mL fresh LB broth plus antibiotics and grown for 2 hrs under the same conditions. Zinc chloride or mQH_2O was then added to half the cultures all at the same volume to the indicated final concentrations and grown for another 2 hrs. Cells were then centrifuged and resuspended in 2 mL of buffer Z before being lysed using EDTA and chloroform. β-galactosidase activity was then measured. The average Miller Units (indicative of β-galactosidase activity) and standard deviation of each sextuplet set of samples is plotted for each strain. A representative result of two independent experiments is shown here. ns denotes a statistically insignificant difference as compared to the relevant LB control or wild-type LB control (one-way ANOVA multiple comparison test).

3.2.4 Analysis of periplasmic reducing potential under excess zinc conditions

Finally, I wanted to confirm that zinc does indeed affect the periplasmic reducing potential. For this purpose, I utilised tetrazolium violet to determine periplasmic redox balance. Tetrazolium salts are widely used to determine bacterial metabolic activity. Tetrazolium violet in its reduced form appears red whereas as its oxidised form appears yellow. It has been shown that tetrazolium is oxidised in the periplasm and as such can be used to determine periplasmic reducing potential. In the presence of excess zinc, I observed a reduction in periplasmic reducing power (Figure 3.16). I performed the same assay using DTT and observed similar results (Figure 3.17). These results seemed to contradict the evidence that we had so far, however, it is important to note that TTC is reduced by NADPH in the periplasm via respiration (Ping *et al.*, 2012). I believe that these results may be an indication of respiratory activity rather than a quantification of reducing equivalents. In this case, the results could be suggesting that the Cpx response reduces respiratory activity in the presence of either zinc or DTT to restore periplasmic redox.

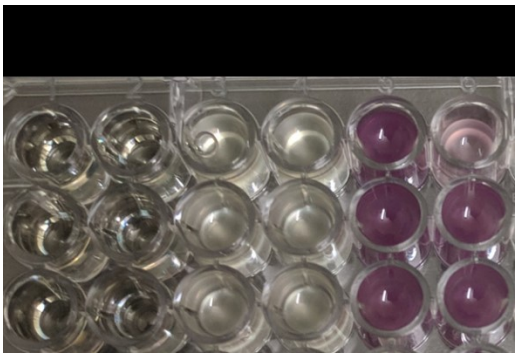

Figure 3.16: Zinc affects periplasmic reducing potential. *E. coli* MC4100 was grown overnight in 5 mL LB at 37 ºC with aeration. 1:50 subcultures were made in 4 mL LB and grown for 2 hours before adding either zinc to a final concentration of 1 mM to half the culture and an equal volume mQH_2O water was added to the rest and left to grow for another 2 hours under the same conditions. 0.01 % [w/v] 2,5-Diphenyl-3-(1-naphthyl)tetrazolium chloride was then added to the culture which was transferred to a 96 well plate and incubated further for another 15 min. A representative result of 6 isolated colonies from two independent experiments is shown here.

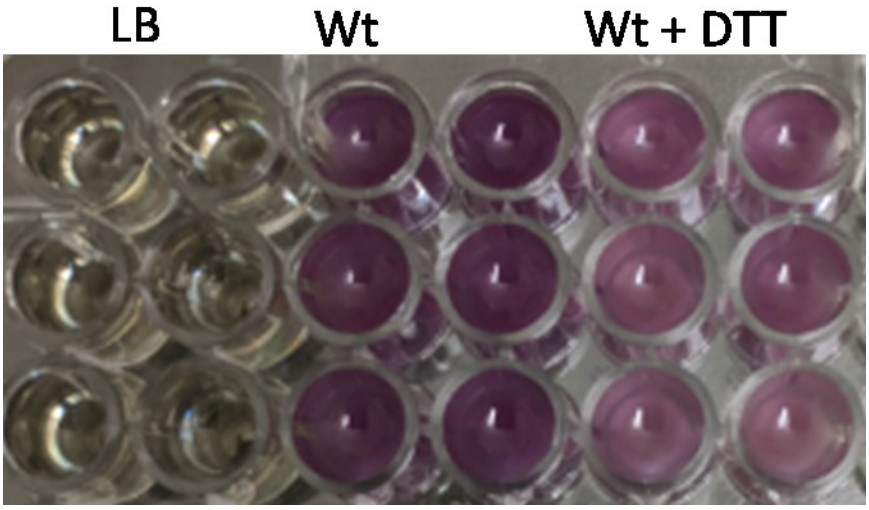

Figure 3.17: DTT affects periplasmic reducing potential. *E. coli* MC4100 was grown overnight in 5 mL LB at 37 ºC with aeration. 1:50 subcultures were made in 4 mL LB and grown for 2 hours before adding either DTT to a final concentration of 15 mM to half the culture and an equal volume mQH_2O water was added to the rest and left to grow for another 2 hours under the same conditions. 0.01 % [w/v] 2,5-Diphenyl-3-(1-naphthyl)tetrazolium chloride was then added to the culture which was transferred to a 96 well plate and incubated further for another 15 min. A representative result of 6 isolated colonies from two independent experiments is shown here.

3.3 References

Danese, P. N. and Silhavy, T. J. (1998) 'CpxP, a stress-combative member of the Cpx regulon', Journal of Bacteriology, 180(4), pp. 831–839. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9473036 (Accessed: 27 March 2018).

Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. B. and Silhavy, J. (1995) 'The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene specifying the stress-inducible periplasmic protease , DegP', pp. 387–398.

Harris, C. L. (1981) 'Cysteine and growth inhibition of Escherichia coli: threonine deaminase as the target enzyme.', Journal of bacteriology, 145(2), pp. 1031–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7007336 (Accessed: 5 June 2018).

Kredich, N. M. (2008) 'Biosynthesis of Cysteine', EcoSal Plus. asm Pub2Web, 3(1). doi: 10.1128/ecosalplus.3.6.1.11.

Lippa, A. M. and Goulian, M. (2012) 'Perturbation of the Oxidizing Environment of the Periplasm Stimulates the PhoQ/PhoP System in Escherichia coli', Journal of Bacteriology, 194(6), pp. 1457–1463. doi: 10.1128/JB.06055-11.

Loddeke, M., Schneider, B., Oguri, T., Mehta, I., Xuan, Z. and Reitzer, L. (2017) 'Anaerobic Cysteine Degradation and Potential Metabolic Coordination in Salmonella enterica and Escherichia coli.', Journal of bacteriology. American Society for Microbiology, 199(16), pp. e00117-17. doi: 10.1128/JB.00117-17.

Ping, L., Mavridou, D. A. I., Emberly, E., Westermann, M. and Ferguson, S. J. (2012) 'Vital Dye Reaction and Granule Localization in Periplasm of Escherichia coli', PLoS ONE. Edited by B. Lei, 7(6), p. e38427. doi: 10.1371/journal.pone.0038427.

Price, N. L. and Raivio, T. L. (2009) 'Characterization of the Cpx regulon in Escherichia coli strain MC4100', Journal of Bacteriology, 191(6), pp. 1798–1815. doi: 10.1128/JB.00798-08.

Raivio, T. L., Leblanc, S. K. D. and Price, N. L. (2013) 'The Escherichia coli Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity', Journal of Bacteriology, 195(12), pp. 2755–2767. doi: 10.1128/JB.00105-13.

Sinha, S., Langford, P. R. and Kroll, J. S. (2004) 'Functional diversity of three different DsbA proteins from Neisseria meningitidis', Microbiology. Microbiology Society, 150(9), pp. 2993–3000. doi: 10.1099/mic.0.27216-0.

Wong, J. L. (2015) 'Cellular role of the inhibitor-chaperone CpxP in'.

Wooley, R. E. and Blue, J. L. (1975) 'In-vitro effect of edta-tris-lysozyme solutions on selected pathogenic bacteria.', Journal of medical microbiology, 8(1), pp. 189–94. doi: 10.1099/00222615-8-1-189.

Yamamoto, K. and Ishihama, A. (2005) 'Transcriptional Response of Escherichia coli to External Zinc', 187(18), pp. 6333–6340. doi: 10.1128/JB.187.18.6333.

Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., Nguyen, M. Q., Yeh, P. and Miller, J. H. (2015) ‘Synergistic interactions of vancomycin with different antibiotics against Escherichia coli: trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type E. coli.’, Antimicrobial agents and chemotherapy. American Society for Microbiology, 59(1), pp. 276–81. doi: 10.1128/AAC.03502-14.

Chapter 4: Discussion

Zinc is an essential metal that shares characteristics with magnesium. It is the 25th most abundant element on the earth's crust and the second most abundant heavy metal. Concentrations of zinc that exceed ambient levels come mostly as a result of anthropogenic activity (Klimek, 2012). In nature however, zinc and most other metals are scarce due to sequestration by organic materials. It has been well documented that this sequestration in animals helps prevent potential pathogens from colonizing and infecting the host. As some of these metals play essential roles in functioning of the host cells, complete deficiency is not a solution to preventing bacterial infection. Zinc deficiency, for example, has been well documented to lead to infectious diseases including diarrhoea caused by EPEC. Supplementation of zinc, in these cases, has been shown to prevent such infections (Skrovanek *et al.*, 2014).

Bacteria have to adapt to changes in metal concentrations that might occur as environmental conditions change. Bacteria have an exceptional ability to sequester zinc and up until recently it has been almost impossible to attain complete zinc deficiency in bacteria, due to this ability. Low concentrations of zinc affect growth in *E. coli* and recently it has been shown that metal depletion activates the Cpx response (Wong, 2015). Higher concentrations of zinc, on the other hand, are toxic to *E. coli*, inducing the transcription of cysteine biosynthetic genes and the *degP* encoding periplasmic protease, causing a reduction in *bfp* expression and type III secretion, and affecting respiratory complexes (Hughes and Poole, 1989; Yamamoto and Ishihama, 2005; Crane *et al.*, 2007; Mellies *et al.*, 2012). These results implicate the Cpx response in multiple ways because activation of the Cpx response by *nlpE* overexpression has been shown to induce the expression of cysteine biosynthetic genes and *degP*, reduce the expression of *bfp* and type III secretion and also affect regulation of respiratory complexes (MacRitchie *et al.*, 2008; MacRitchie, Acosta and Raivio, 2012; Raivio, Leblanc and Price, 2013; Guest *et al.*, 2017). This study sought

to elucidate the effects that excess zinc has on *E. coli* and investigate the possible role that the Cpx response has in alleviating toxic effects of excess zinc.

4.1 Excess zinc increases outer membrane permeability

Initially, I sought to investigate whether excess zinc elicits an envelope signal that could induce the Cpx response. The envelope is crucial to the cells - it protects bacteria from hostile environments and is the site where most virulence determinants reside. The Cpx response is among the many envelope stress response systems that is involved in maintaining envelope integrity. Damages to the cell envelope could be an inducing signal for the Cpx response. I started by testing whether excess zinc would make the cells more susceptible to lysozyme. Chelating divalent cations such as Mg^{2+} and Ca^{2+}, that stabilize the otherwise repulsive forces of the strongly negatively charged LPS on the envelope, makes the cells more susceptible to the action of lysozyme (Wooley and Blue, 1975). I wanted to determine whether zinc disrupts membrane stability when in excess. The results showed no significant difference between treatment with/without zinc perhaps due to limited exposure time to zinc. I took a different approach using vancomycin to test whether the cells became more susceptible to it in the presence of excess zinc. Gram-negative bacteria are particularly resistant to vancomycin, on its own, owing to their selectively permeable outer membrane (Zhou *et al.*, 2015). In the presence of zinc however, I noticed an increased susceptibility to vancomycin up to concentrations of about 50 mg/μL (Figure 3.1). These results suggested to me that excess zinc causes the outer membrane to become more permeable and thus makes the cells more susceptible to vancomycin. This gave us more evidence to believe that zinc-induced stress causes an envelope signal that could be sensed by the Cpx response.

To determine whether the Cpx response is involved in resistance to zinc I tested whether deletion of certain components of the Cpx response would alter growth in the presence of excess zinc. I also included a Δ*degP* mutant as *degP* is a member of the Cpx regulon and has been previously shown to be strongly induced in the presence of excess zinc. The results showed that deletion of *cpxR* made the cells more susceptible to growth retardation caused by zinc as compared to the wild type, Δ*cpxA,* Δ*cpxP* and Δ*nlpE* mutants. It was observed that the Δ*degP* mutant was unable to grow in the presence of excess zinc (Figures 3.2A and 3.2B) suggesting that *degP* could be involved in alleviating zinc toxicity. DegP is a periplasmic serine endoprotease that is involved in the cleavage of misfolded proteins that could potentially prove to be toxic to the cell if left to accumulate (Danese *et al.*, 1995). This action is thought to facilitate survival of the bacterial cell in environments that induce oxidative damage of proteins. Taken together, this data provided further evidence to support the idea that under excess zinc conditions, the Cpx response could be sensing changes in periplasmic homeostasis and thereby elevating the expression of protein folding and degrading factors to alleviate zinc toxicity.

4.2 Zinc induced activation of the Cpx response is conserved in gram-negative bacteria and occurs via an extracytoplasmic signal

I sought to confirm activation of the Cpx response by zinc in *E. coli* and *V. cholerae* to determine if this was conserved among gram-negative bacteria. I did, in fact, observe activation of the Cpx response in both strains and this happened in a concentration dependent manner. To confirm whether this was due to an extracytoplasmic signal, I tested whether deleting components of the Cpx response would alter induction by zinc. I noticed from the results that deleting *cpxA* and *cpxR* resulted in no change in Cpx activity suggesting that zinc activation of the Cpx response

occurred through an extracytoplasmic signal that was sensed by CpxA. Taken together with the vancomycin results, this suggested that excess zinc causes an envelope signal that the Cpx response could be responding to. The fact that the Δ*degP* mutant was completely susceptible to excess zinc also gave us reason to believe that zinc could be causing damage to envelope proteins perhaps through oxidative stress and that the Cpx response could be responding to this by increasing the expression of protein folding and degrading factors such as *degP*.

4.3 Role of cysteine in alleviating zinc induced stress

Cysteine serves as a precursor to glutathione as well as for iron sulphur clusters. It is also known for its ability to bind metals, an attribute of its thiol group. Glutathione is an important antioxidant in plants, animals, fungi, and some bacteria and archaea and can prevent damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals (Guédon and Martin-verstraete, 2006). A microarray carried out by Yamamato showed that addition of excess external zinc induced the expression of many genes that are organised in the regulon for cysteine biosynthesis in *E. coli*. With the evidence provided thus far - showing that zinc could be causing oxidative damage I wondered if the Cpx response could be inducing the expression of cysteine biosynthetic genes in the presence of excess zinc to alleviate oxidative stress. I began by confirming Cpx induction of *cysK* by overexpression of *nlpE*. I did in fact observe an induction of *cysK* by *nlpE* overexpression. I then determined whether the Cpx response was involved in activating *cysK* in the presence of excess zinc. Our results showed that the *cysK* was induced independently of the Cpx response.

To get a better understanding of the possible role cysteine plays in alleviating zinc induced stress and how the Cpx response is implicated, I investigated changes in intracellular thiols under

conditions of excess zinc in wild-type, Δ*cpxA*, Δ*cpxR* and a cysteine auxotroph Δ*cysK*. I observed an increase in intracellular thiol concentrations in the wild-type upon exposure to excess zinc. I also observed an overall reduced intracellular thiol concentration which was not impacted by zinc in the Δ*cysK* mutant, as was anticipated for a cysteine auxotroph. Interestingly, the thiol concentration in the Δ*cpxA* and Δ*cpxR* mutants was also not impacted by zinc albeit the basal levels of thiol concentration were much higher than wild-type levels (Figure 3.5). These results suggested to us that the Cpx effect on cysteine trafficking could help alleviate oxidative stress that is induced by zinc.

4.4 Alterations in periplasmic redox potential affect the Cpx response

As previous results showed that the Cys regulon was upregulated independently of the Cpx response under excess zinc conditions, I wondered if the Cpx response sensed reducing equivalents, such as cysteine, that are exported into the periplasm upon exposure to excess zinc. Our tests with DTT, a known reducing agent that is permeable to the outer membrane, gave evidence to support the idea that the Cpx response indeed sensed changes in periplasmic homeostasis. The periplasm is a site for protein folding. Proteins that pass through the periplasm are slowly oxidised by DsbA to introduce disulphide bridges. Optimum conditions for DsbA include acidic pH and an oxidising environment. The Cpx response is known to sense conditions that affect protein folding and as such it would make sense for the Cpx response to sense conditions that are not optimal for DsbA such as alkaline pH and in this case the presence of excess reducing equivalents in the periplasm.

An independent study from our lab tested whether deleting components of thioredoxins that affected periplasmic homeostasis would lead to alterations in zinc induced activation of the

Cpx response. This work showed a higher basal activity of the Cpx response in Δ*dsbA* and Δ*dsbB* mutants but lower in the Δ*dsbC* and Δ*dsbD* mutants (Ravio and Zhu, unpublished findings). Deleting *dsbA* or *dsbB* has been shown to increase the reducing potential of the periplasm whereas DsbC and DsbD are believed to be the source of reducing power to the periplasm and so it was hypothesised that deleting *dsbC* and *dsbD* should lower the reducing potential of the periplasm thereby reducing the basal activity of the Cpx response whereas deleting *dsbA* and *dsbB* would have the opposite effect.

4.6 Zinc activation of the Cpx response is dependent of *zntA*

In an effort to determine whether the Cpx response affected the expression of the zinc transporter *zntA*, it was found that deleting either *cpxA* or *cpxR* affected the expression of *zntA* even under lethal concentrations of zinc (Wong, 2015). The qPCR data confirmed these results and also showed differences in the expression of cysteine transporters. Combined with this observation, the data up to this stage suggested that the Cpx response regulated metal and cysteine transporters to alleviate envelope stress that affects periplasmic redox balance. To test this, I investigated Cpx activity in strains lacking cysteine or zinc transporters in the presence of excess zinc. Our results showed that the zinc induced activation of the Cpx response was dependent on *zntA*. I did not see any differences in Cpx activation in mutants lacking certain cysteine transporters which could mean that single deletions of these transporters may not influence cysteine trafficking. I did, however, notice reduced basal activity of the Cpx response in a cysteine auxotroph thereby supporting the idea that altering thiol concentrations impacts the Cpx response.

4.7 Zinc affects periplasmic redox homeostasis

Finally, I wanted to confirm that zinc does indeed affect the periplasmic reducing potential. For this purpose, I utilised tetrazolium violet to determine periplasmic redox balance. It has been shown that tetrazolium is oxidised in the periplasm and as such can be used to determine periplasmic reducing potential. In the presence of excess zinc, I indeed observed less periplasmic reducing power. Tetrazolium salts are believed to be reduced by NADPH via respiration (Ping *et al.*, 2012). For this reason, tetrazolium salts can be used as an estimation for respiratory activity and viability. The results obtained from this experiment could indicate damage to respiratory complexes by excess zinc as viability tests did show that the concentration used was not completely lethal. Iron sulphur clusters could be the target of excess zinc as their sulfhydryl groups have a high affinity for metals such as zinc.

Model

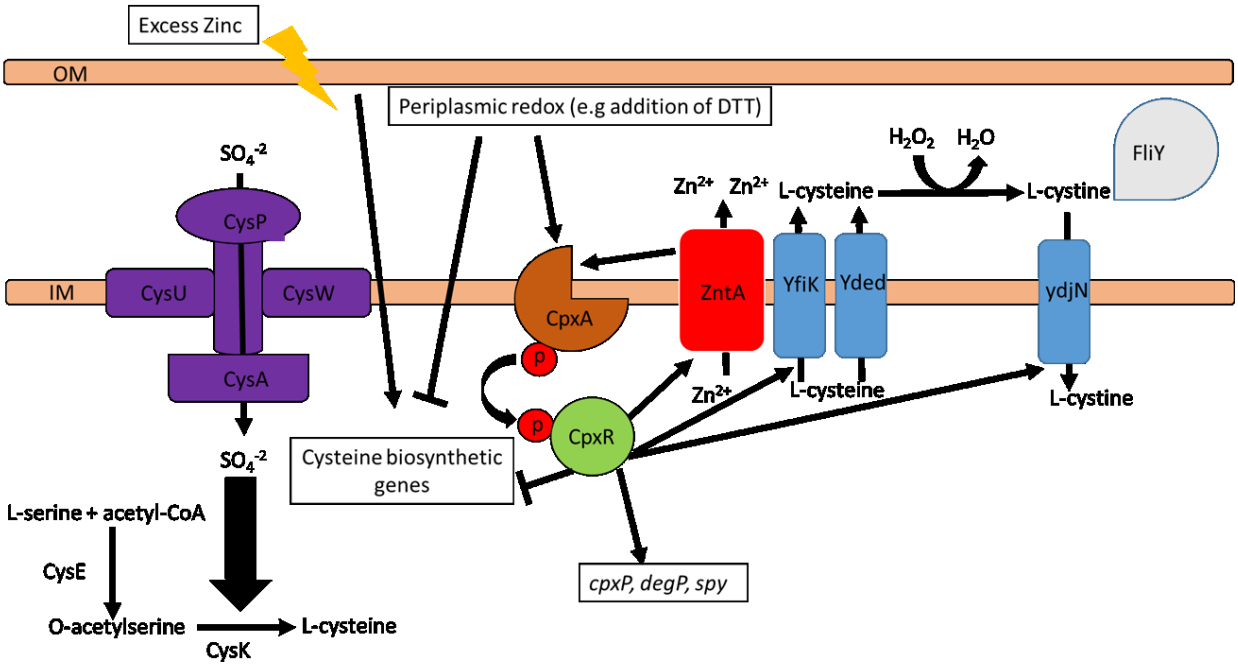

Figure 4.1: Proposed model describing how the Cpx response senses accumulation of cysteine in the periplasm brought about by excess zinc and how the Cpx response could be maintaining homeostasis by controlling the regulation of L-cysteine/L-cystine transporters.

The model above summarises the current findings of this thesis. Zinc likely causes instability in the cellular envelope that makes it more permeable to toxic metabolites. Through an unknown mechanism, the cys regulon is upregulated under excess zinc conditions and cysteine is exported into the periplasmic space. CpxA then senses these changes in the periplasm and upregulates transporters to funnel out excess reducing equivalents that could disrupt protein folding in the periplasm and thereby help maintain the oxidative state of the periplasm. For the proper functioning of DsbA, the periplasm needs to be oxidising. By controlling the redox state of the periplasm, the Cpx response could help maintain envelope integrity under excess zinc conditions.

4.8 Concluding remarks

This study aimed at investigating the effects of excess zinc on *E. coli* and the role that the Cpx response has on alleviating the toxic effects of by excess zinc. Overall my findings showed that zinc elicits an extracytoplasmic signal that is sensed by CpxA. I also show that zinc induced activation of the Cpx response is dependent on the zinc transporter ZntA. The data I collected provided evidence to suggest that the Cpx response affects metal and cysteine transporters in the presence of excess zinc. I believe that the Cpx response regulates these transporter genes in order to maintain periplasmic homeostasis. Evidence from the literature point out several mechanisms by which the Cpx response could alleviate toxic zinc effects, including regulation of respiratory complexes and synthesis of envelope structures such as the bundle forming pilus. Taken together I believe that, in the presence of excess zinc, the Cpx response maintains periplasmic homeostasis to provide optimal conditions for the proper folding of envelope proteins to maintain envelope integrity.

4.9 References

Crane, J. K., Naeher, T. M., Shulgina, I., Zhu, C. and Boedeker, E. C. (2007) 'Effect of zinc in enteropathogenic Escherichia coli infection', Infection and Immunity, 75(12), pp. 5974–5984. doi: 10.1128/IAI.00750-07.

Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. B. and Silhavy, J. (1995) 'The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene specifying the stress-inducible periplasmic protease , DegP', pp. 387–398.

Guédon, E. and Martin-verstraete, I. (2006) 'Cysteine Metabolism and Its Regulation in Bacteria', (August 2015). doi: 10.1007/7171.

Guest, R. L., Wang, J., Wong, J. L. and Raivio, T. L. (2017) 'A Bacterial Stress Response Regulates Respiratory Protein Complexes To Control Envelope Stress Adaptation', Journal of Bacteriology. Edited by V. J. DiRita, 199(20), pp. e00153-17. doi: 10.1128/JB.00153-17.

Hughes, M. N. (Martin N. and Poole, R. K. (1989) Metals and micro-organisms. Chapman and Hall. Available at: https://www.springer.com/gp/book/9780412244001 (Accessed: 3 May 2018).

Klimek, B. (2012) 'Effect of long-term zinc pollution on soil microbial community resistance to repeated contamination.', Bulletin of environmental contamination and toxicology. Springer, 88(4), pp. 617–22. doi: 10.1007/s00128-012-0523-0.

MacRitchie, D. M., Acosta, N. and Raivio, T. L. (2012) 'DegP is involved in Cpx-mediated posttranscriptional regulation of the type III secretion apparatus in enteropathogenic Escherichia coli.', Infection and immunity. American Society for Microbiology (ASM), 80(5), pp. 1766–72. doi: 10.1128/IAI.05679-11.

MacRitchie, D. M., Ward, J. D., Nevesinjac, A. Z. and Raivio, T. L. (2008) 'Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic Escherichia coli', Infection and Immunity, 76(4), pp. 1465–1475. doi: 10.1128/IAI.01265-07.

Mellies, J. L., Thomas, K., Turvey, M., Evans, N. R., Crane, J., Boedeker, E. and Benison, G. C. (2012) 'Zinc-induced envelope stress diminishes type III secretion in enteropathogenic Escherichia coli', BMC Microbiology, 12, pp. 1–10. doi: 10.1186/1471-2180-12-123.

Ping, L., Mavridou, D. A. I., Emberly, E., Westermann, M. and Ferguson, S. J. (2012) 'Vital Dye Reaction and Granule Localization in Periplasm of Escherichia coli', PLoS ONE. Edited by B. Lei, 7(6), p. e38427. doi: 10.1371/journal.pone.0038427.

Raivio, T. L., Leblanc, S. K. D. and Price, N. L. (2013) 'The Escherichia coli Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity', Journal of Bacteriology, 195(12), pp. 2755–2767. doi: 10.1128/JB.00105-13.

Skrovanek, S., Diguilio, K., Bailey, R., Huntington, W., Urbas, R., Mayilvaganan, B., Mercogliano, G., Mullin, J. M., Skrovanek, S., Diguilio, K. and Mullin, J. M. (2014) 'Zinc and gastrointestinal disease', 5(4), pp. 496–513. doi: 10.4291/wjgp.v5.i4.496.

Wong, J. L. (2015) ‘Cellular role of the inhibitor-chaperone CpxP in’.

Wooley, R. E. and Blue, J. L. (1975) ‘In-vitro effect of edta-tris-lysozyme solutions on selected pathogenic bacteria.’, Journal of medical microbiology, 8(1), pp. 189–94. doi: 10.1099/00222615-8-1-189.

Yamamoto, K. and Ishihama, A. (2005) ‘Transcriptional Response of Escherichia coli to External Zinc’, 187(18), pp. 6333–6340. doi: 10.1128/JB.187.18.6333.

Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., Nguyen, M. Q., Yeh, P. and Miller, J. H. (2015) ‘Synergistic interactions of vancomycin with different antibiotics against Escherichia coli: trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type E. coli.’, Antimicrobial agents and chemotherapy. American Society for Microbiology, 59(1), pp. 276–81. doi: 10.1128/AAC.03502-14.

Zhu, T. (no date) Unpublished findings.

Bibliography

Acosta Amador, P. N. (2015) ‘Characterization of the Cpx Response in Vibrio cholerae’. doi: 10.7939/R3JH3DF3C.

Acosta, N., Pukatzki, S. and Raivio, T. L. (2015) ‘The Vibrio cholerae Cpx envelope stress response senses and mediates adaptation to low iron’, *Journal of Bacteriology*, 197(2), pp. 262–276. doi: 10.1128/JB.01957-14.

Arechaga, I. (2013) ‘Membrane invaginations in bacteria and mitochondria: common features and evolutionary scenarios.’, *Journal of molecular microbiology and biotechnology*. Karger Publishers, 23(1–2), pp. 13–23. doi: 10.1159/000346515.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L. and Mori, H. (2006) ‘Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection.’, *Molecular systems biology*, 2, p. 2006.0008. doi: 10.1038/msb4100050.

Beard, S. J., Hughes, M. N. and Poole, R. K. (1995) ‘Inhibition of the cytochrome bd-terminated NADH oxidase system in Escherichia coli K-12 by divalent metal cations.’, *FEMS microbiology letters*, 131(2), pp. 205–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7557331 (Accessed: 26 July 2018).

Berkmen, M. (2012) ‘Production of disulfide-bonded proteins in Escherichia coli’, *Protein Expression and Purification*, 82(1), pp. 240–251. doi: 10.1016/j.pep.2011.10.009.

Blencowe, D. K. and Morby, A. P. (2003) ‘Zn(II) metabolism in prokaryotes.’, *FEMS*

microbiology reviews, 27(2–3), pp. 291–311. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12829272 (Accessed: 28 March 2018).

Blindauer, C. A. (2015) 'Advances in the molecular understanding of biological zinc transport', *Chemical Communications*. Royal Society of Chemistry, 51(22), pp. 4544–4563. doi: 10.1039/C4CC10174J.

Braun, V. (1975) 'Covalent lipoprotein from the outer membrane of Escherichia coli.', *Biochimica et biophysica acta*, 415(3), pp. 335–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/52377 (Accessed: 15 May 2018).

Breyton, C., Haase, W., Rapoport, T. A., Kühlbrandt, W. and Collinson, I. (2002) 'Three-dimensional structure of the bacterial protein-translocation complex SecYEG.', *Nature*, 418(6898), pp. 662–5. doi: 10.1038/nature00827.

Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I. and Lux, A. (2007) 'Zinc in plants', *New Phytologist*. Wiley/Blackwell (10.1111), 173(4), pp. 677–702. doi: 10.1111/j.1469-8137.2007.01996.x.

Brocklehurst, K. R., Hobman, J. L., Lawley, B., Blank, L., Marshall, S. J., Brown, N. L. and Morby, A. P. (1999) 'ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of zntA in Escherichia coli', *Molecular Microbiology*. Wiley/Blackwell (10.1111), 31(3), pp. 893–902. doi: 10.1046/j.1365-2958.1999.01229.x.

Bryan, L. E. and Van den Elzen, H. M. (1976) 'Streptomycin accumulation in susceptible and resistant strains of Escherichia coli and Pseudomonas aeruginosa.', *Antimicrobial agents and*

chemotherapy, 9(6), pp. 928–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/820248 (Accessed: 27 March 2018).

Capra, E. J. and Laub, M. T. (2012) 'Evolution of two-component signal transduction systems.', *Annual review of microbiology*. NIH Public Access, 66, pp. 325–47. doi: 10.1146/annurev-micro-092611-150039.

Casadaban, M. J. and Cohen, S. N. (1979) 'Lactose genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: in vivo probe for transcriptional control sequences.', *Proceedings of the National Academy of Sciences of the United States of America*, 76(9), pp. 4530–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/159458 (Accessed: 7 May 2018).

Chin, C.-S., Sorenson, J., Harris, J. B., Robins, W. P., Charles, R. C., Jean-Charles, R. R., Bullard, J., Webster, D. R., Kasarskis, A., Peluso, P., Paxinos, E. E., Yamaichi, Y., Calderwood, S. B., Mekalanos, J. J., Schadt, E. E. and Waldor, M. K. (2011) 'The Origin of the Haitian Cholera Outbreak Strain', *New England Journal of Medicine*. Massachusetts Medical Society , 364(1), pp. 33–42. doi: 10.1056/NEJMoa1012928.

Chopra, I. (1988) 'Molecular mechanisms involved in the transport of antibiotics into bacteria', *Parasitology*. Cambridge University Press, 96(S1), pp. S25–S44. doi: 10.1017/S0031182000085966.

Clifton, L. A., Skoda, M. W. A., Le Brun, A. P., Ciesielski, F., Kuzmenko, I., Holt, S. A. and Lakey, J. H. (2015) 'Effect of Divalent Cation Removal on the Structure of Gram-Negative Bacterial Outer Membrane Models', *Langmuir*, 31(1), pp. 404–412. doi: 10.1021/la504407v.

Conway, T. and Cohen, P. S. (2015) 'Commensal and Pathogenic Escherichia coli Metabolism in the Gut', *Microbiology spectrum*, 3(3), p. 10.1128/microbiolspec.MBP-0006-2014. doi: 10.1128/microbiolspec.MBP-0006-2014.

Crane, J. K., Naeher, T. M., Shulgina, I., Zhu, C. and Boedeker, E. C. (2007) 'Effect of zinc in enteropathogenic Escherichia coli infection', *Infection and Immunity*, 75(12), pp. 5974–5984. doi: 10.1128/IAI.00750-07.

Danese, P. N., Oliver, G. R., Barr, K., Bowman, G. D., Rick, P. D. and Silhavy, T. J. (1998) 'Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates degP transcription in Escherichia coli', *Journal of Bacteriology*, 180(22), pp. 5875–5884. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0031765192&partnerID=tZOtx3y1.

Danese, P. N. and Silhavy, T. J. (1997) 'The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in Escherichia coli.', *Genes & development*, 11(9), pp. 1183–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9159399 (Accessed: 27 March 2018).

Danese, P. N. and Silhavy, T. J. (1998) 'CpxP, a stress-combative member of the Cpx regulon', *Journal of Bacteriology*, 180(4), pp. 831–839. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9473036 (Accessed: 27 March 2018).

Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. B. and Silhavy, J. (1995) 'The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene

specifying the stress-inducible periplasmic protease , DegP', pp. 387–398.

Danese, P. N., Snyder, W. B., Cosma, C. L., Davis, L. J. and Silhavy, T. J. (1995) 'The Cpx two-component signal transduction pathway of Escherichia coli regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP.', *Genes & development*, 9(4), pp. 387–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7883164 (Accessed: 27 March 2018).

DiGiuseppe, P. A. and Silhavy, T. J. (2003) 'Signal Detection and Target Gene Induction by the CpxRA Two-Component System', *Journal of Bacteriology*. American Society for Microbiology, 185(8), pp. 2432–2440. doi: 10.1128/JB.185.8.2432-2440.2003.

Dong, J., Iuchi, S., Kwan, H. S., Lu, Z. and Lin, E. C. (1993) 'The deduced amino-acid sequence of the cloned cpxR gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of Escherichia coli.', *Gene*, 136(1–2), pp. 227–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8294007 (Accessed: 27 March 2018).

Dorel, C., Lejeune, P. and Rodrigue, A. (2006) 'The Cpx system of Escherichia coli, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities?', *Research in Microbiology*, 157(4), pp. 306–314. doi: 10.1016/j.resmic.2005.12.003.

Dufresne, K. and Paradis-Bleau, C. (2015) 'Biology and Assembly of the Bacterial Envelope', in. Springer, Cham, pp. 41–76. doi: 10.1007/978-3-319-23603-2_3.

Fairman, J. W., Noinaj, N. and Buchanan, S. K. (2011) 'The structural biology of β-barrel

membrane proteins: a summary of recent reports', *Current Opinion in Structural Biology*. Elsevier Current Trends, 21(4), pp. 523–531. doi: 10.1016/J.SBI.2011.05.005.

Fang, F. and Imlay, J. A. (2012) 'Silver (I), Mercury (II), Cadmium (II), and Zinc (II) Target Exposed Enzymic Iron-Sulfur Clusters when They Toxify Escherichia coli', (I), pp. 3614–3621. doi: 10.1128/AEM.07368-11.

Flores-Kim, J. and Darwin, A. J. (2016) 'The Phage Shock Protein Response', *Annual Review of Microbiology*, 70(1), pp. 83–101. doi: 10.1146/annurev-micro-102215-095359.

Friedrich, T., Dekovic, D. K. and Burschel, S. (2016) 'Assembly of the Escherichia coli NADH:ubiquinone oxidoreductase (respiratory complex I)', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. Elsevier, 1857(3), pp. 214–223. doi: 10.1016/J.BBABIO.2015.12.004.

Goemans, C., Denoncin, K. and Collet, J.-F. (2014) 'Folding mechanisms of periplasmic proteins', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. Elsevier, 1843(8), pp. 1517–1528. doi: 10.1016/J.BBAMCR.2013.10.014.

Graham, A. I., Hunt, S., Stokes, S. L., Bramall, N., Bunch, J., Cox, A. G., McLeod, C. W. and Poole, R. K. (2009) 'Severe zinc depletion of Escherichia coli: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 284(27), pp. 18377–89. doi: 10.1074/jbc.M109.001503.

Grass, G., Wong, M. D., Rosen, B. P., Smith, R. L. and Rensing, C. (2002) 'ZupT is a Zn(II) uptake system in Escherichia coli.', *Journal of bacteriology*. American Society for

Microbiology, 184(3), pp. 864–6. doi: 10.1128/JB.184.3.864-866.2002.

Groisman, E. A. (2001) 'The pleiotropic two-component regulatory system PhoP-PhoQ.', *Journal of bacteriology*. American Society for Microbiology, 183(6), pp. 1835–42. doi: 10.1128/JB.183.6.1835-1842.2001.

Guédon, E. and Martin-verstraete, I. (2006) 'Cysteine Metabolism and Its Regulation in Bacteria', (August 2015). doi: 10.1007/7171.

Guest, R. L. and Raivio, T. L. (2016) 'Role of the Gram-Negative Envelope Stress Response in the Presence of Antimicrobial Agents', *Trends in Microbiology*. Elsevier Ltd, 24(5), pp. 377–390. doi: 10.1016/j.tim.2016.03.001.

Guest, R. L., Wang, J., Wong, J. L. and Raivio, T. L. (2017) 'A Bacterial Stress Response Regulates Respiratory Protein Complexes To Control Envelope Stress Adaptation', *Journal of Bacteriology*. Edited by V. J. DiRita, 199(20), pp. e00153-17. doi: 10.1128/JB.00153-17.

Haase, H. and Rink, L. (2014) 'Multiple impacts of zinc on immune function', *Metallomics*, 6(7), p. 1175. doi: 10.1039/c3mt00353a.

Hancock, R. E. W. and McPhee, J. B. (2005) 'Salmonella's Sensor for Host Defense Molecules', *Cell*. Cell Press, 122(3), pp. 320–322. doi: 10.1016/J.CELL.2005.07.023.

Hantke, K. (2005) 'Bacterial zinc uptake and regulators', *Current Opinion in Microbiology*, 8(2), pp. 196–202. doi: 10.1016/j.mib.2005.02.001.

Harris, C. L. (1981) ‘Cysteine and growth inhibition of Escherichia coli: threonine deaminase as the target enzyme.’, *Journal of bacteriology*, 145(2), pp. 1031–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7007336 (Accessed: 5 June 2018).

Hughes, M. N. (Martin N. and Poole, R. K. (1989) *Metals and micro-organisms*. Chapman and Hall. Available at: https://www.springer.com/gp/book/9780412244001 (Accessed: 3 May 2018).

Hung, D. L., Raivio, T. L., Jones, C. H., Silhavy, T. J. and Hultgren, S. J. (2001) ‘Cpx signaling pathway monitors biogenesis and affects assembly and expression of P pili.’, *The EMBO journal*, 20(7), pp. 1508–18. doi: 10.1093/emboj/20.7.1508.

Johnson, J. R., Gajewski, A., Lesse, A. J. and Russo, T. A. (2003) ‘Extraintestinal pathogenic Escherichia coli as a cause of invasive nonurinary infections.’, *Journal of clinical microbiology*. American Society for Microbiology (ASM), 41(12), pp. 5798–802. doi: 10.1128/JCM.41.12.5798-5802.2003.

Juncker, A. S., Willenbrock, H., von Heijne, G., Brunak, S., Nielsen, H. and Krogh, A. (2003) ‘Prediction of lipoprotein signal peptides in Gram-negative bacteria’, *Protein Science*. Wiley-Blackwell, 12(8), pp. 1652–1662. doi: 10.1110/ps.0303703.

Kamio, Y. and Nikaido, H. (1976) ‘Outer membrane of Salmonella typhimurium: accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium’, *Biochemistry*. American Chemical Society, 15(12), pp. 2561–2570. doi: 10.1021/bi00657a012.

Kasahara, M. and Anraku, Y. (1974) ‘Succinate- and NADH oxidase systems of Escherichia coli

membrane vesicles. Mechanism of selective inhibition of the systems by zinc ions.', *Journal of biochemistry*, 76(5), pp. 967–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4156035 (Accessed: 26 July 2018).

Kertesz, M. A. (2001) 'Bacterial transporters for sulfate and organosulfur compounds.', *Research in microbiology*, 152(3–4), pp. 279–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11421275 (Accessed: 28 March 2018).

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H. and Mori, H. (2006) 'Complete set of ORF clones of Escherichia coli ASKA library (A Complete Set of E. coli K-12 ORF Archive): Unique Resources for Biological Research', *DNA Research*, 12(5), pp. 291–299. doi: 10.1093/dnares/dsi012.

Klimek, B. (2012) 'Effect of long-term zinc pollution on soil microbial community resistance to repeated contamination.', *Bulletin of environmental contamination and toxicology*. Springer, 88(4), pp. 617–22. doi: 10.1007/s00128-012-0523-0.

Kredich, N. M. (2008a) 'Biosynthesis of Cysteine', *EcoSal Plus*. asm Pub2Web, 3(1). doi: 10.1128/ecosalplus.3.6.1.11.

Kredich, N. M. (2008b) 'Biosynthesis of Cysteine', *EcoSal Plus*, 3(1). doi: 10.1128/ecosalplus.3.6.1.11.

Kudva, R., Denks, K., Kuhn, P., Vogt, A., Müller, M. and Koch, H.-G. (2013) 'Protein translocation across the inner membrane of Gram-negative bacteria: the Sec and Tat dependent protein transport pathways', *Research in Microbiology*. Elsevier Masson, 164(6), pp. 505–534.

doi: 10.1016/J.RESMIC.2013.03.016.

Kurabayashi, K., Hirakawa, Y., Tanimoto, K., Tomita, H. and Hirakawa, H. (2014) 'Role of the CpxAR Two-Component Signal Transduction System in Control of Fosfomycin Resistance and Carbon Substrate Uptake', *Journal of Bacteriology*, 196(2), pp. 248–256. doi: 10.1128/JB.01151-13.

Landeta, C., Boyd, D. and Beckwith, J. (2018) 'Disulfide bond formation in prokaryotes', *Nature Microbiology*, 3(3), pp. 270–280. doi: 10.1038/s41564-017-0106-2.

Lee, L. J., Barrett, J. A. and Poole, R. K. (2005) 'Genome-wide transcriptional response of chemostat-cultured Escherichia coli to zinc.', *Journal of bacteriology*. American Society for Microbiology, 187(3), pp. 1124–34. doi: 10.1128/JB.187.3.1124-1134.2005.

Li, W., Liu, Y., Sheng, X., Yin, P., Hu, F., Liu, Y., Chen, C., Li, Q., Yan, C. and Wang, J. (2014) 'Structure and mechanism of a type III secretion protease, NleC.', *Acta crystallographica. Section D, Biological crystallography*, 70(Pt 1), pp. 40–7. doi: 10.1107/S1399004713024619.

Lippa, A. M. and Goulian, M. (2012) 'Perturbation of the Oxidizing Environment of the Periplasm Stimulates the PhoQ/PhoP System in Escherichia coli', *Journal of Bacteriology*, 194(6), pp. 1457–1463. doi: 10.1128/JB.06055-11.

Loddeke, M., Schneider, B., Oguri, T., Mehta, I., Xuan, Z. and Reitzer, L. (2017) 'Anaerobic Cysteine Degradation and Potential Metabolic Coordination in Salmonella enterica and Escherichia coli.', *Journal of bacteriology*. American Society for Microbiology, 199(16), pp. e00117-17. doi: 10.1128/JB.00117-17.

Luirink, J., Yu, Z., Wagner, S. and de Gier, J.-W. (2012) 'Biogenesis of inner membrane proteins in Escherichia coli', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. Elsevier, 1817(6), pp. 965–976. doi: 10.1016/J.BBABIO.2011.12.006.

MacRitchie, D. M., Acosta, N. and Raivio, T. L. (2012) 'DegP is involved in Cpx-mediated posttranscriptional regulation of the type III secretion apparatus in enteropathogenic Escherichia coli.', *Infection and immunity*. American Society for Microbiology (ASM), 80(5), pp. 1766–72. doi: 10.1128/IAI.05679-11.

MacRitchie, D. M., Ward, J. D., Nevesinjac, A. Z. and Raivio, T. L. (2008) 'Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic Escherichia coli', *Infection and Immunity*, 76(4), pp. 1465–1475. doi: 10.1128/IAI.01265-07.

Magalon, A., Arias-Cartin, R. and Walburger, A. (2012) 'Supramolecular Organization in Prokaryotic Respiratory Systems', *Advances in Microbial Physiology*. Academic Press, 61, pp. 217–266. doi: 10.1016/B978-0-12-394423-8.00006-8.

McEwen, J. and Silverman, P. (1980a) 'Chromosomal mutations of Escherichia coli that alter expression of conjugative plasmid functions.', *Proceedings of the National Academy of Sciences of the United States of America*, 77(1), pp. 513–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6102380 (Accessed: 27 March 2018).

McEwen, J. and Silverman, P. (1980b) 'Mutations in genes cpxA and cpxB of Escherichia coli K-12 cause a defect in isoleucine and valine syntheses.', *Journal of bacteriology*, 144(1), pp. 68–

73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6998970 (Accessed: 27 March 2018).

McEwen, J. and Silverman, P. M. (1982) ‘Mutations in genes cpxA and cpxB alter the protein composition of Escherichia coli inner and outer membranes.’, *Journal of bacteriology*, 151(3), pp. 1553–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7050092 (Accessed: 27 March 2018).

Mellies, J. L., Thomas, K., Turvey, M., Evans, N. R., Crane, J., Boedeker, E. and Benison, G. C. (2012) ‘Zinc-induced envelope stress diminishes type III secretion in enteropathogenic Escherichia coli’, *BMC Microbiology*, 12, pp. 1–10. doi: 10.1186/1471-2180-12-123.

Messens, J. and Collet, J.-F. (2006) ‘Pathways of disulfide bond formation in Escherichia coli.’, *The international journal of biochemistry & cell biology*, 38(7), pp. 1050–62. doi: 10.1016/j.biocel.2005.12.011.

Mileykovskaya, E. and Dowhan, W. (1997) ‘The Cpx two-component signal transduction pathway is activated in Escherichia coli mutant strains lacking phosphatidylethanolamine’, *Journal of Bacteriology*, 179(4), pp. 1029–1034. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0031039158&partnerID=tZOtx3y1.

Miyadai, H., Tanaka-Masuda, K., Matsuyama, S. and Tokuda, H. (2004) ‘Effects of lipoprotein overproduction on the induction of DegP (HtrA) involved in quality control in the Escherichia coli periplasm.’, *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(38), pp. 39807–13. doi: 10.1074/jbc.M406390200.

Narita, S. (2011) ‘ABC Transporters Involved in the Biogenesis of the Outer Membrane in

Gram-Negative Bacteria', *Bioscience, Biotechnology, and Biochemistry*. Japan Society for Bioscience, Biotechnology, and Agrochemistry, 75(6), pp. 1044–1054. doi: 10.1271/bbb.110115.

Nevesinjac, A. Z. and Raivio, T. L. (2005) 'The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic Escherichia coli.', *Journal of bacteriology*. American Society for Microbiology, 187(2), pp. 672–86. doi: 10.1128/JB.187.2.672-686.2005.

Otto, K. and Silhavy, T. J. (2002) 'Surface sensing and adhesion of Escherichia coli controlled by the Cpx-signaling pathway.', *Proceedings of the National Academy of Sciences of the United States of America*, 99(4), pp. 2287–92. doi: 10.1073/pnas.042521699.

Palmela, C., Chevarin, C., Xu, Z., Torres, J., Sevrin, G., Hirten, R., Barnich, N., Ng, S. C. and Colombel, J.-F. (2018) 'Adherent-invasive *Escherichia coli* in inflammatory bowel disease', *Gut*, 67(3), pp. 574–587. doi: 10.1136/gutjnl-2017-314903.

Patzer, S. and Hantke, K. (1998) 'The ZnuABC high-affinity zinc uptake system and its regulator Zur in Escherichia coli', *Mol Microbiol*, 28.

Ping, L., Mavridou, D. A. I., Emberly, E., Westermann, M. and Ferguson, S. J. (2012) 'Vital Dye Reaction and Granule Localization in Periplasm of Escherichia coli', *PLoS ONE*. Edited by B. Lei, 7(6), p. e38427. doi: 10.1371/journal.pone.0038427.

Pogliano, J., Lynch, A. S., Belin, D., Lin, E. C. and Beckwith, J. (1997) 'Regulation of Escherichia coli cell envelope proteins involved in protein folding and degradation by the Cpx two-component system.', *Genes & development*, 11(9), pp. 1169–82. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/9159398 (Accessed: 27 March 2018).

Pontel, L. B., Scampoli, N. L., Porwollik, S., Checa, S. K., McClelland, M. and Soncini, F. C. (2014) 'Identification of a Salmonella ancillary copper detoxification mechanism by a comparative analysis of the genome-wide transcriptional response to copper and zinc excess', *Microbiology*, 160(8), pp. 1659–1669. doi: 10.1099/mic.0.080473-0.

Price, N. L. and Raivio, T. L. (2009) 'Characterization of the Cpx regulon in Escherichia coli strain MC4100', *Journal of Bacteriology*, 191(6), pp. 1798–1815. doi: 10.1128/JB.00798-08.

Pucci, M. J. and Bush, K. (2013) 'Investigational Antimicrobial Agents of 2013', *Clinical Microbiology Reviews*, 26(4), pp. 792–821. doi: 10.1128/CMR.00033-13.

Raetz, C. R. H. and Whitfield, C. (2002) 'Lipopolysaccharide Endotoxins', *Annual Review of Biochemistry*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 71(1), pp. 635–700. doi: 10.1146/annurev.biochem.71.110601.135414.

Rainwater, S. and Silverman, P. M. (1990) 'The Cpx proteins of Escherichia coli K-12: evidence that cpxA, ecfB, ssd, and eup mutations all identify the same gene.', *Journal of bacteriology*, 172(5), pp. 2456–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2185221 (Accessed: 27 March 2018).

Raivio, T. L. (2005) 'MicroReview: Envelope stress responses and Gram-negative bacterial pathogenesis', *Molecular Microbiology*. Wiley/Blackwell (10.1111), 56(5), pp. 1119–1128. doi: 10.1111/j.1365-2958.2005.04625.x.

Raivio, T. L., Leblanc, S. K. D. and Price, N. L. (2013) 'The Escherichia coli Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity', *Journal of Bacteriology*, 195(12), pp. 2755–2767. doi: 10.1128/JB.00105-13.

Raivio, T. L., Popkin, D. L. and Silhavy, T. J. (1999) 'The Cpx envelope stress response is controlled by amplification and feedback inhibition.', *Journal of bacteriology*. American Society for Microbiology, 181(17), pp. 5263–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10464196 (Accessed: 27 March 2018).

Raivio, T. L. and Silhavy, T. J. (1997) 'Transduction of envelope stress in Escherichia coli by the Cpx two-component system.', *Journal of bacteriology*, 179(24), pp. 7724–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9401031 (Accessed: 7 May 2018).

Raivio, T. L. and Silhavy, T. J. (1997) 'Transduction of envelope stress in Escherichia coli by the Cpx two-component system . Transduction of Envelope Stress in Escherichia coli by the Cpx Two-Component System', *American Society for Microbiology*, 179(24), pp. 7724–7733. doi: 10.1128/JB.179.24.7724-7733.1997.

Raivio, T. L. and Silhavy, T. J. (2001) 'Periplasmic stress and ECF sigma factors.', *Annual review of microbiology*, 55, pp. 591–624. doi: 10.1146/annurev.micro.55.1.591.

Rollauer, S. E., Sooreshjani, M. A., Noinaj, N. and Buchanan, S. K. (2015) 'Outer membrane protein biogenesis in Gram-negative bacteria.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. The Royal Society, 370(1679), p. 20150023. doi:

10.1098/rstb.2015.0023.

Russo, T. A. and Johnson, J. R. (2000) ‘Proposal for a New Inclusive Designation for Extraintestinal Pathogenic Isolates of *Escherichia coli:* ExPEC’, *The Journal of Infectious Diseases*, 181(5), pp. 1753–1754. doi: 10.1086/315418.

Sankaran, K. and Wu, H. C. (1994) ‘Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol.’, *The Journal of biological chemistry*, 269(31), pp. 19701–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8051048 (Accessed: 26 March 2018).

Silhavy, T. J., Kahne, D. and Walker, S. (2010) ‘The bacterial cell envelope.’, *Cold Spring Harbor perspectives in biology*. Cold Spring Harbor Laboratory Press, 2(5), p. a000414. doi: 10.1101/cshperspect.a000414.

Singh, T., Das, S., Ramachandran, V. G., Shah, D., Saha, R., Dar, S. A. and Rai, A. (2017) ‘Typical & atypical enteropathogenic escherichia coli in diarrhoea & their role as carrier in children under five’, *Indian Journal of Medical Research*, 145(April), pp. 551–557. doi: 10.4103/ijmr.IJMR_25_15.

Sinha, S., Langford, P. R. and Kroll, J. S. (2004) ‘Functional diversity of three different DsbA proteins from Neisseria meningitidis’, *Microbiology*. Microbiology Society, 150(9), pp. 2993–3000. doi: 10.1099/mic.0.27216-0.

Skrovanek, S., Diguilio, K., Bailey, R., Huntington, W., Urbas, R., Mayilvaganan, B., Mercogliano, G., Mullin, J. M., Skrovanek, S., Diguilio, K. and Mullin, J. M. (2014) ‘Zinc and

gastrointestinal disease', 5(4), pp. 496–513. doi: 10.4291/wjgp.v5.i4.496.

Slauch, J. M. and Silhavy, T. J. (1991) 'Genetic fusions as experimental tools.', *Methods in enzymology*, 204, pp. 213–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1658562 (Accessed: 30 April 2018).

Snyder, W. B., Davis, L. J., Danese, P. N., Cosma, C. L. and Silhavy, T. J. (1995) 'Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway.', *Journal of bacteriology*, 177(15), pp. 4216–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7635808 (Accessed: 27 March 2018).

van Stelten, J., Silva, F., Belin, D. and Silhavy, T. J. (2009) 'Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 325(5941), pp. 753–6. doi: 10.1126/science.1172221.

Takagi, H. (2010) 'The L -Cysteine / L -Cystine Shuttle System Provides Reducing Equivalents to the Periplasm in Escherichia coli * □', 285(23), pp. 17479–17487. doi: 10.1074/jbc.M109.081356.

Thede, G. L., Arthur, D. C., Edwards, R. A., Buelow, D. R., Wong, J. L., Raivio, T. L. and Glover, J. N. M. (2011) 'Structure of the periplasmic stress response protein CpxP.', *Journal of bacteriology*. American Society for Microbiology, 193(9), pp. 2149–57. doi: 10.1128/JB.01296-10.

Thomason, L. C., Costantino, N. and Court, D. L. (2007) 'E. coli Genome Manipulation by P1 Transduction', in *Current Protocols in Molecular Biology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., p. 1.17.1-1.17.8. doi: 10.1002/0471142727.mb0117s79.

Urdaneta, V. and Casadesús, J. (2017) 'Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts', *Frontiers in Medicine*. Frontiers Media SA, 4, p. 163. doi: 10.3389/FMED.2017.00163.

Vogt, S. L., Nevesinjac, A. Z., Humphries, R. M., Donnenberg, M. S., Armstrong, G. D. and Raivio, T. L. (2010) 'The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic Escherichia coli bundle-forming pilus', *Molecular Microbiology*, 76(5), pp. 1095–1110. doi: 10.1111/j.1365-2958.2010.07145.x.

Vogt, S. L. and Raivio, T. L. (2012) 'Just scratching the surface: An expanding view of the Cpx envelope stress response', *FEMS Microbiology Letters*, 326(1), pp. 2–11. doi: 10.1111/j.1574-6968.2011.02406.x.

Vollmer, W., Blanot, D. and De Pedro, M. A. (2008) 'Peptidoglycan structure and architecture', *FEMS Microbiology Reviews*, 32(2), pp. 149–167. doi: 10.1111/j.1574-6976.2007.00094.x.

Wang, D. and Fierke, C. A. (2013) 'The BaeSR regulon is involved in defense against zinc toxicity in E. coli', *Metallomics*. The Royal Society of Chemistry, 5(4), p. 372. doi: 10.1039/c3mt20217h.

Weatherspoon-Griffin, N., Yang, D., Kong, W., Hua, Z. and Shi, Y. (2014) 'The CpxR/CpxA two-component regulatory system up-regulates the multidrug resistance cascade to facilitate

Escherichia coli resistance to a model antimicrobial peptide.', *The Journal of biological chemistry*, 289(47), pp. 32571–82. doi: 10.1074/jbc.M114.565762.

Whitfield, C., Szymanski, C. M. and Aebi, M. (2015) *Eubacteria*, *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. doi: 10.1101/GLYCOBIOLOGY.3E.021.

Van Wielink, J. E. and Duine, J. A. (1990) 'How big is the periplasmic space?', *Trends in Biochemical Sciences*. Elsevier Current Trends, 15(4), pp. 136–137. doi: 10.1016/0968-0004(90)90208-S.

Wong, J. L. (2015) 'Cellular role of the inhibitor-chaperone CpxP in'.

Wong, J. L. (no date) 'unpublished observation'.

Wooley, R. E. and Blue, J. L. (1975) 'In-vitro effect of edta-tris-lysozyme solutions on selected pathogenic bacteria.', *Journal of medical microbiology*, 8(1), pp. 189–94. doi: 10.1099/00222615-8-1-189.

Yamamoto, K. and Ishihama, A. (2005) 'Transcriptional Response of Escherichia coli to External Zinc', 187(18), pp. 6333–6340. doi: 10.1128/JB.187.18.6333.

Yamamoto, K., Oshima, T., Nonaka, G., Ito, H. and Ishihama, A. (2011) 'Induction of the Escherichia coli cysK gene by genetic and environmental factors', (1987). doi: 10.1111/j.1574-6968.2011.02364.x.

Zhang, G., Meredith, T. C. and Kahne, D. (2013) 'On the essentiality of lipopolysaccharide to

Gram-negative bacteria', *Current Opinion in Microbiology*. Elsevier Current Trends, 16(6), pp. 779–785. doi: 10.1016/J.MIB.2013.09.007.

Zhao, B. and Houry, W. A. (2010) 'Acid stress response in enteropathogenic gammaproteobacteria: an aptitude for survival.', *Biochemistry and cell biology = Biochimie et biologie cellulaire*, 88(2), pp. 301–14. doi: 10.1139/o09-182.

Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., Nguyen, M. Q., Yeh, P. and Miller, J. H. (2015) 'Synergistic interactions of vancomycin with different antibiotics against Escherichia coli: trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type E. coli.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology, 59(1), pp. 276–81. doi: 10.1128/AAC.03502-14.

Ravio, T and Zhu, T. unpublished findings.