Cellular role of the inhibitor-chaperone CpxP in *Escherichia coli*

by

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Abstract

Bacteria must sense stress signals and adapt accordingly in order to survive. In Gram-negative bacteria, the envelope is the first to encounter adverse environmental conditions and contains signal transduction systems to relay information from the periplasm to the cytoplasm. The Cpx two-component system consists of the inner membrane sensor kinase CpxA and the cytoplasmic response regulator CpxR. In the presence of envelope stress, CpxA autophosphorylates and phosphorylates CpxR. Phosphorylated CpxR modulates the transcription of over 100 genes, including the chaperone CpxP. CpxP is a small periplasmic protein that inhibits the Cpx pathway when over-expressed and exhibits weak chaperone activity *in vitro.* In the absence of stress, CpxP is thought to interact directly with the sensing domain of CpxA and prevent activation of the Cpx pathway. In the presence of stress such as misfolded pilin proteins, CpxP is titrated away from CpxA and degraded, freeing CpxA to sense stress and activate the pathway. Paralogues of CpxP bind metal ions and modulate signal transduction from the periplasm to the cytoplasm in response to metal-binding. Excess zinc and copper induce the Cpx pathway in *E. coli* and *Salmonella enterica* but iron chelation induces the Cpx pathway in *Vibrio cholerae*. We sought to test whether the Cpx system in *E. coli* could also respond to metal limitation. We show that the expression of *cpxP* is elevated when cells are grown in metal-limited conditions and this increase is reversed by the addition of exogenous zinc. The sensor kinase CpxA and CpxP are dispensable for the activation of *cpxP* expression by chelation or its reversal by zinc. We assessed the impact of Cpx mutations on agar containing toxic levels of zinc and discovered that deletion of *cpxP* in the laboratory strain of *E. coli* results in zinc-resistance that is complemented by the over-expression of *cpxP in trans*. Over-expression of *cpxP* strongly decreased the expression of the zinc efflux protein *zntA*, which remained high at

toxic levels of zinc in the *cpxP* mutant. Deletion of either *cpxA* or *cpxR* did not phenocopy the zinc resistance of the *cpxP* mutant, though the expression of *cpxP* is low in these genetic backgrounds. Mutational analysis of CpxP demonstrated that residues that mediate the interaction between CpxP and CpxA are also important for the zinc-related function of CpxP. Unexpectedly, activation of the Cpx pathway by over-expressing NlpE in enteropathogenic *E. coli* conferred zinc resistance but insertional inactivation of *cpxP* did not. Therefore, the expression of *cpxP* appears to be toxic in the presence of high levels of zinc and the expression of Cpx-regulated factors other than *cpxP* may be required to mediate zinc resistance under these conditions. We hypothesized that CpxP binds zinc to alter intracellular zinc trafficking. To test our hypothesis, we performed inductively-coupled plasma mass spectrometry on purified CpxP protein and showed that CpxP binds zinc in non-stoichiometric amounts. This observation confirmed that CpxP, as the prototypical member of a family of extracytoplasmic signal transduction proteins, is a metal-binding protein like its paralogues. Finally, to gain a better understanding of how inhibition of the Cpx pathway is maintained, we performed a genetic screen for Cpx-regulated envelope-localized genes that inhibit the Cpx pathway upon over-expression. We identified *nuoF*, the soluble NADH-binding component of ComplexI and *efeB,* a heme peroxidase. NuoF and EfeB are novel *cpxA*-, *cpxR*-, and *cpxP*-dependent inhibitors of the Cpx pathway. NuoF did not require a functional Complex I and EfeB did not require the EfeU heme permease to inhibit the Cpx pathway. We speculate that the over-expression of NuoF and EfeB alter the accumulation of an unidentified cellular metabolite that induces the Cpx response. Our work shows that the non-essential auxiliary regulator CpxP integrates protein-folding, metal, and metabolic signals and relays them to the sensor kinase CpxA to modulate Cpx activity, promoting bacterial adaptation to multiple stresses.

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LIST OF SYMBOLS, NOMENCLATURE AND ABBREVIATIONS

Symbols

Δ Deletion at the specified genetic locus

: Interruption of the genetic locus by insertion

Abbreviations

Amp: ampicillin

ATP: adenosine triphosphate

CD: circular dichroism

CDF: cation diffusion facilitator family

Cam: chloramphenicol

CAMP: cationic antimicrobial peptides

DNA: deoxyribonucleic acid

GST: glutathione-*S*-transferase

IM: inner membrane

IPGT: isopropyl-β-D-thiogalactopyranoside

Kan: kanamycin

LPS: lipopolysaccharide

MBP: maltose-binding protein

MFS: major facilitator superfamily

NRAMP: natural resistance associated macrophage protein

OM: outer membrane

OMP: outer membrane protein

PDZ: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)

PE: phosphatidylethanolamine

PG: peptidoglycan

PMF: proton-motive force

PPIase: peptidyl-prolyl isomerase

RNA: ribonucleic acid

RNAP: RNA polymerase

RND: resistance, nodulation, and division

RR: response regulator

SK: sensor kianse

sRNA: small RNA

TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine

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

ZIP: ZRT (zinc-regulated transporter), IRT-like (iron-regulated transporter) proteins

Chapter 1: General Introduction

1.1. Introduction

Bacterial survival depends on adapting to constantly changing environmental conditions, including changes in temperature, pH, osmolarity, nutrient availability, light, cell density, microbial community structure, and the presence of host cells. In Gram-negative bacteria, the envelope is the first structure to encounter environmental changes and contains sensory proteins that initiate signal transduction events that lead to physiological changes and adaptation. Commensal *Escherichia coli* are Gram-negative bacteria found in the lower intestines of mammalian hosts as part of a community of microbial species known as normal microflora. The gut microflora function to protect the host from infection by pathogenic species of bacteria such as *Campylobacter jejuni* (85) and appear to also have roles in protecting human hosts from metabolic syndrome and gastrointestinal disease (178). Commensal *E. coli* isolated from cow feedlots exhibited the ability to elaborate a range of surface appendages, including various pili and flagella that aid *E. coli* in forming biofilms *in vitro* (300). There are many pathogenic species of *E. coli* that possess the ability to cause disease. One such species is enteropathogenic *Escherichia coli* (EPEC) that infects the small intestine of human hosts and causes diarrheal illness (142). EPEC is responsible for as much as 3-15% of infant deaths worldwide in children under the age of five (131) where access to sanitized water is limited and, therefore, the treatment of diarrheal illness is not as effective as in the developed world. The ability of EPEC to cause disease is partly mediated by the expression of genes found on a plasmid known as the EPEC adherence factor (EAF) plasmid (143) which contains the genes required for the elaboration of the bundle-forming pilus, an appendage that aids EPEC in the initial stages of attachment to host cells, and its regulators (52, 71, 172) . A chromosomal locus that contains the genes required for the expression of a type III secretion system and its effectors called the

EPEC attachment and effacement factor locus (*eae*) also contributes to EPEC pathogenesis by mediating close contact with host cells and effacement of the gut microvilli (59). Commensal *E. coli* lack the EAF plasmid and *eae* locus but share a common ancestor with EPEC and other pathogenic species of *E. coli* and, therefore, possess the potential to acquire multiple genetic elements that might endow this strain with the ability to cause disease through horizontal gene transfer (139).

1.2. The Gram-negative envelope

The envelope is comprised of the outer membrane, inner or cytoplasmic membrane, and the periplasmic space between (182) (reviewed in (40)) and is the site of protein secretion, nutrient import, waste and toxin export, respiration, and virulence factor assembly, amongst others. The periplasmic space is a macromolecule-dense site of protein-folding and degradation, energy generation and utilization, and protein and solute trafficking

1.2.1. The outer membrane

The outer membrane is an asymmetrical bilayer, consisting of an extracellular lipopolysaccharide leaflet and a periplasmic lipid leaflet. Lipopolysaccharide consists of a modified sugar covalently linked to lipid tails called lipid A, an inner core of sugar residues proximal to lipid A, an outer core of sugars distal to lipid A, and repeating units of sugars called the O-antigen. The lipopolysaccharide repels cationic antimicrobial peptides (CAMPs) (82, 91) and modifications to lipid A aid immune evasion in animal hosts (117). The outer membrane also houses the trimeric β-barrel outer membrane proteins or porins (OMPs) that allow the passive diffusion of solutes in and out of the cell, the export machinery required for the insertion and secretion of outer membrane and extracellular proteins, and a number of associated lipoproteins (reviewed by (232) and (308)). The outer membrane, as the first Gram-negative

barrier to the environment, ensures that the cell is both permeable to nutrients but protected against antimicrobial molecules.

1.2.2. The inner membrane

The inner membrane bounds the cytoplasm and maintains tight control on the molecules that enter or exit the cell. Components of the aerobic respiratory chain reside in the inner membrane, including the 14-gene NADH dehydrogenase I (Nuo) or Complex I. The identity and organization of the *nuo* locus in *E. coli* is conserved in the mitochondrial complex of the same name in eukaryotes (292), highlighting the importance of Nuo for energy generation across domains of life. Another NADH dehydrogenase, NADH dehydrogenase II, is encoded by the *ndh* gene and is expressed only under aerobic conditions (79). The *nuo* operon is expressed under both anaerobic and aerobic conditions and, unlike Ndh, the Nuo complex pumps protons from the cytoplasm to the periplasm to produce proton motive force (PMF) (163). The repression of *ndh* during anaerobic growth is mediated by the iron-containing transcriptional regulator Fnr which uses a bound ferrous iron to sense redox conditions within the cell and repress its target genes (79, 257). Electrons are passed from NADH dehydrogenases to the quinone pool and on to terminal oxidases, depending on the nutrients and oxygen available. The resulting proton gradient generated by the proton-pumping members of the respiratory chain drives ATP generation by the F_0-F_1 ATP synthase (reviewed in (220)). The proteins in the inner membrane reflect the conditions bacteria experience at a given time and the composition changes to ensure energy is available for the cell to function under all conditions.

Additionally, the inner membrane is the site of protein secretion and targeting but the need to secrete essential envelope components must be balanced with maintaining the selective permeability of the inner membrane. The SecYEG translocon and the YidC insertase

independently and cooperatively function to fold and insert proteins destined for the envelope or the extracellular space. SecYEG contains a 16 x 25 Å cavity through which nascent proteins are threaded from the cytoplasm in to the periplasm, the inner membrane, or to chaperones that will usher the proteins to their final destination. The protein translocation channel is closed to the periplasm in the crystal structure, perhaps requiring higher order oligomerization or substrate to open the membrane cavity (21). YidC cooperates with the Sec translocon to insert membrane proteins into the inner membrane; however, YidC can also insert proteins independently of Sec. The structure of one paralogue of YidC in *Bacillus subtilis* (YidC2) confirms that YidC functions as monomer to insert single-spanning membrane proteins such as Pf3 coat protein using a hydrophilic groove in the C-terminal cytoplasmic cavity (129). This cavity is estimated to hold 2000 $Å^3$ and molecular dynamics simulations suggest that a combination of hydrophobic residues and lipid tails from the membrane occlude the extracellular opening of YidC. The presence of substrate is thought to displace the hydrophobic "plug" from the YidC channel (130). Substrate release is facilitated by ATP hydrolysis via SecA for the Sec translocon and the combined forces of hydrophobic interactions between membrane proteins and the membrane and proton motive force (PMF) in YidC (12, 130). Thus, regulated translocation mediated by SecYEG and YidC ensure that envelope-destined proteins are secreted across the cytoplasmic membrane without compromising the barrier function of the cytoplasmic membrane.

1.2.3. The periplasm

The aqueous space between the inner and outer membranes is oxidizing compared to the cytoplasm and devoid of ATP. The periplasm contains the thin peptidoglycan (PG) layer that maintains cell shape and turgor pressure, as well as the biosynthetic and lytic enzymes required for PG re-modeling for the purposes of cell division or to resist changes in osmolarity. Solutes

and macromolecules imported into the cell also must cross the periplasm to reach the appropriate receptor for active import into the cytoplasm. The import of maltose, histidine, and oligopeptides, for instance, is mediated by a set of transporters in the inner membrane that hydrolyze ATP and use a conserved set of soluble periplasmic solute-binding proteins to maintain the specificity of the transporter for its respective solute (92). There are numerous inner membrane efflux proteins and tripartite pumps that span the envelope to mediate the active transport of antimicrobial compounds and endogenous metabolic wastes that would be toxic to the cell if accumulated (186, 212). Additionally, the chaperones and proteases required to elaborate porins, adhesins, virulence structures, and lipoproteins destined for the outer membrane function in the periplasm (9, 164). Thus, the periplasm is the site of cell shape maintenance and protein, solute, and waste trafficking between the two membranes of Gram-negative bacteria.

1.3. Envelope stress responses

The complexity of the envelope and the essentiality of the cellular functions found there necessitate regulatory systems that can sense disturbances to envelope processes and respond accordingly. The envelope stress responses monitor the envelope through inner membrane sensory proteins that transduce extracytoplasmic signals to the cytoplasm. Some sensor proteins are assisted by auxiliary regulators that sense a specific aspect of envelope homeostasis and transduce the signal to the sensor protein, thereby ensuring that signals in the outer membrane or periplasm can reach the appropriate signal transducer in the inner membrane (Figure 1-1).

1.3.1. Outer membrane vesiculation

Gram-negative bacteria release vesicles bounded by outer membrane components containing select periplasmic and outer membrane cargo, including virulence factors and signalling molecules (88, 113, 168, 171, 216). However, outer membrane vesiculation also

enables the cell to physically remove toxic substrates from the cell. Disruption of genes involved in periplasmic proteolysis, OMP expression, or LPS biosynthesis increases vesiculation in *E. coli* (121, 167) . Stresses that impact the Gram-negative envelope induce the production of OMVs, though the precise mechanisms bacteria employ to select OMV cargo remains unknown.

1.3.2. The σ^E stress response

The extracytoplasmic sigma factor σ^E is sequestered at the inner membrane by the integral membrane anti-sigma factor RseA (133, 176). RseA is cleaved at least three times before σ^E is released to the cytoplasm to transcribe its regulon. The first cleavage is performed in the periplasm by the integral membrane protease DegS. To initiate the proteolytic cascade, misfolded OMPs expose a conserved YxF motif that binds the post-synaptic density PSD95, *Drosophila* disc large tumor suppressor Dlg1, and zonula occludens 1 zo-1 (PDZ) domain of DegS, a substrate-binding and oligomerization domain commonly found in proteases, to allosterically activate DegS and promote the cleavage of RseA (31, 254, 301). However, full σ^E activation is only achieved when DegS cleaves RseA and when the inhibition by another periplasmic protein RseB is removed (31). In the presence of aberrant LPS subunits, RseB is titrated from the C-terminal domain of RseA, stimulating the periplasmic PDZ domain of the integral membrane protease RseP to cleave RseA in the membrane (146, 266). The activation of σ^E, therefore, occurs only when both DegS and RseB alter their interactions with RseA in the presence of a compromised outer membrane. Finally, the ClpXP protease complex captures the N-terminal RseA fragment released from the inner membrane by RseP cleavage and partially degrades RseA in the cytoplasm to release σ^E, though other cytoplasmic proteases can also participate in this process (58) (Figure 1-1).

Release of σ^E in the cytoplasm results in increased expression of chaperones, lipoproteins, and proteases required for OMP biogenesis, including *surA,* and *degP (36, 193, 236)*. Similarly, the expression of the LPS chaperones and assembly factors LptA and LptB are also controlled by σ^E, confirming the role of σ^E in mediating responses to aberrant LPS (255). Additionally, σ^E regulates a set of small RNAs (sRNAs) that post-transcriptionally decrease the expression of OMPs (53, 271) and the abundant lipoprotein Lpp (83). The expression of sRNAs during envelope stress enables the cell to rapidly reduce the amount of problematic protein components from the cell envelope (282). These studies demonstrate the specificity of σ^E and its regulators for monitoring and maintaining outer membrane homeostasis at both the transcriptional and post-transcriptional level.

The σ^E pathway is critical for the virulence of a number of pathogens. In *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), σ^E is not essential and loss of σ^E is associated with increased sensitivity to reactive oxygen species and antimicrobial peptides, and a decreased capacity to infect mice (98). The expression of the outer membrane lipoprotein *smpA* is activated by the σ^E response and contributes to the ability of *S. enterica* to infect mice, though the biochemical function of *smpA* is unknown (144). Similarly, the causative agent of tuberculosis, *Mycobacterium tuberculosis,* requires σ^E for survival in human and murine macrophages (157). Thus, the activation of the σ^E envelope stress response aids bacterial survival in animal hosts.

1.3.3. The phage shock protein (Psp) response

The Psp response mediates bacterial adaptation to a number of stresses generally thought to affect the inner membrane, including ethanol, high temperature, infection by filamentous bacteriophages (22), stationary phase survival (293), defective Sec- or YidC-mediated secretion

(108), and over-expression of secretins and mutations in ATP synthase (165). Unlike the σ^E pathway, the molecular details of Psp signalling are unclear. Extracytoplasmic signals are integrated at the inner membrane by the positive regulators of *psp* expression, PspB and PspC, though the induction of the Psp response by the over-expression of some secretins does not require either of these components (166). PspG and PspD are additional regulators of the *psp* response with unknown contributions to signalling that are located in and peripheral to the inner membrane, respectively (80, 149) . The current model for induction of *pspA* expression posits that soluble PspA binding to cytoplasmic PspF prevents the activation of *pspA* expression in the absence of stress. During inner membrane stress, PspA is recruited to the membrane regulators PspB and PspC (Figure 1-1), releasing PspF to activate the transcription of *pspA* via its interaction with the nitrogen-responsive sigma factor σ^{54} (16, 110) . The activation of *pspA* expression assists in the maintenance of cellular proton motive force (PMF), which is essential for the generation of energy and transport of molecules across the cytoplasmic membrane (16). Interestingly, the localization of PspA depends on the presence of negatively-charged phospholipids and the cell shape maintenance proteins MreB and RodZ, suggesting that PspA is recruited to the divisome to maintain inner membrane homeostasis during cell division (111). In agreement with the observations that Psp maintains membrane integrity in *E. coli*, *S.* Typhimurium *pspA* mutants are attenuated in natural resistance-associated macrophage protein 1 (NRAMP-1)-positive mice due to the ability of *pspA* to promote the PMF-dependent uptake of essential divalent cations in the metal-poor environment of the murine host (114). Mutations in *pspC* result in a loss of viability when the food-borne pathogen *Yersinia enterocolitica* expresses the Ysc type III secretion system that is not dependent on the expression of *pspA* (43), suggesting that Psp regulon members other than PspA contribute to cell viability during the

expression of virulence factors. Thus, Psp signalling depends on a series of complex protein-protein interactions between membrane and cytoplasmic proteins that impact virulence.

1.3.4. Two-component signal transduction

One of the most common ways bacteria sense signals and effect a response is through two-component signal transduction (TCS). The canonical two-component system consists of a sensor histidine kinase (SK) in the inner membrane and a cytoplasmic response regulator (RR). Upon signal-sensing, a SK will sense extracellular signals and autophosphorylate in the cytoplasm at a conserved histidine residue (96). The SK then phosphorylates its cognate RR in the cytoplasm at a conserved aspartate residue, stimulating a conformational change in the RR that results in differential transcription of target genes (151). SKs usually also act as phosphatases toward their respective RR in the absence of stress to ensure that signalling does not occur in the absence of a specific signal (107). TCS are found in all domains of life but are not present in animals, making them an attractive target for antimicrobial development (68, 127). The following envelope stress responses employ the ability of SKs to integrate extracellular signals at the inner membrane and generate a physiological response via phosphotransfer reactions to response regulators (Figure 1-1).

1.3.5. The regulator of capsular synthesis (Rcs) phosphorelay

The Rcs phosphorelay consists of a hybrid sensor histidine kinase RcsC and the response regulator RcsB (262) (Figure 1-1). RcsC senses changes in osmolarity (251), antibiotic-induced changes to peptidoglycan (134), and the over-expression of the outer membrane lipoprotein RcsF (66, 155). Upon sensing its cognate signals, RcsC phosphorylates the histidine phosphotransfer protein RcsD and RcsD transfers the phosphate to RcsB in the cytoplasm. Phosphorylated RcsB activates the expression of colanic acid production, required for

exopolysaccharide synthesis in *E. coli* (70), genes involved in flagellar biosynthesis (61), the lipoprotein sorting protein *lolA* (268), the sRNA *rprA* which increases the translation of the stationary phase sigma factor *rpoS*, and the *rcs* operon (55). Another response regulator, RcsA requires an interaction with RcsB for stability and assists in the transcription of the *rcs* regulon (69). Basal transcription of cell wall genes by RcsB is required for intrinsic resistance to the antibiotics cefsulodin and amdinocillin (134) and activation of Rcs both attenuates and enhances the virulence of multiple pathogens by changing bacterial responses to osmolarity (20, 57, 64, 289), suggesting that the Rcs pathway responds to changes at the cell surface that reinforce the barrier function of the cell.

1.3.6. The bacterial adaptive response (Bae) two-component system

The BaeSR two-component system (TCS) was identified as a regulator of the periplasmic chaperone *spy* in response to indole, spheroplasting, and the over-expression of the P-pilus pilin PapG (225), all signals that contribute to envelope stress. In addition to *spy*, Bae regulates the expression of resistance-nodulation-and-division (RND) and major facilitator superfamily (MFS) efflux pump proteins, including *mdtABCD (10)*, *acrD* and *tolC*, as well as genes involved in motility, solute transport, and other two-component regulatory systems (187). BaeR-mediated expression of *mdt, acrD,* and *tolC* results in resistance to multiple antibiotics, including a number of β-lactams, the aminocoumarin novobiocin, and the bile component deoxycholate (10). Deletion of *tolC* results in activation of *spy* expression that is coordinately mediated by the Bae and Cpx TCS (235). An attractive model for BaeSR activation posits that endogenous metabolites accumulate in the absence of efflux to activate the Bae response (Figure 1-1). Though exogenous indole induces BaeSR and indole accumulates in stationary phase in *E. coli,* the accumulation of intracellular indole does not induce *mdt* (135), suggesting that BaeS does

not activate the expression of efflux in response to accumulated intracellular indole. Similarly, the accumulation of intracellular acetate or ethanol did not induce *spy* expression (235). The BaeSR TCS, therefore, assists in the efflux of an as-of-yet unidentified cellular metabolite that causes envelope stress upon accumulation.

1.3.7. The conjugative pilus expression (Cpx) two-component system

Expression of *spy* is also controlled by the Cpx TCS (228). The Cpx TCS was first identified as a set of mutations that negatively affected the expression of the conjugative F pilus in *E. coli* (170). The SK CpxA senses envelope stress and transduces the signal to the CpxR RR by phosphorylation (39, 226). Phosphorylated CpxR modulates the transcription of numerous genes in the inner membrane and envelope, including chaperones and proteases, genes involved in cell wall biogenesis, solute transport, energy generation, and genes of unknown function (221, 229) . The periplasmic inhibitor of the Cpx response, *cpxP*, is strongly induced upon Cpx activation and is used as a reporter for Cpx activity (51). The structure and function of CpxP is discussed in more detail in Section 1.4.

1.3.7.1. Classical inducers of the Cpx pathway are involved in envelope protein folding

The signals that induce the Cpx system are diverse and the precise biochemical cue sensed by CpxA is unknown. The 'classical' Cpx inducing cues are thought to result in envelope protein misfolding and mislocalization (Figure 1-2). Over-expression of the hybrid periplasmic LamB-LacZ-PhoA protein in *E. coli* results in envelope stress via unknown mechanisms but likely aggregates in the absence of high levels of chaperones and proteases, such as DegP (39). DegP is a dual chaperone-protease that forms large oligomers that may span the entire envelope to degrade a wide variety of envelope substrates (106, 128, 256). Constitutive gain-of-function mutations in *cpxA*, the *cpxA** alleles, produced high enough levels of *degP* to suppress the

toxicity of LamB-LacZ-PhoA expression in *E. coli* *(39, 226)* . Subsequent studies showed that the heterologous expression of the uropathogenic *E. coli* (UPEC) P-pilin subunits PapE and PapG or the bundle-forming pilus subunit BfpA from enteropathogenic *E. coli* (EPEC) in the laboratory strain *E. coli* K-12 also activated the Cpx pathway (102, 184). Over-expression of pilins in the absence of their chaperones is thought to result in protein aggregation that induces the Cpx pathway. Similarly, growth in alkaline medium is hypothesized to cause protein-misfolding through an unknown mechanism and also induces the Cpx pathway in *Shigella sonnei* and *E. coli* (42, 181).

Over-expression of the outer membrane lipoprotein *nlpE* is another Cpx activating signal. When over-expressed, NlpE may misfold in a similar manner to that of pilin subunits. Alternatively, the outer membrane lipoprotein may mislocalize to the inner membrane when over-expressed to directly interact with CpxA and activate the pathway (93, 253) . The outer membrane-proximal N-terminal domain of NlpE contains a conserved CXXC motif and resembles bacterial lipocalins while the C-terminal domain contains an oligosaccharyl-binding fold (93). NlpE senses surface adhesion through an unknown mechanism and activates Cpx during adhesion to hydrophobic surfaces (194). The contribution of each of the domains of NlpE to its function is not known. However, the NlpE homologue in *V. cholerae* consists of only the N-terminal lipocalin domain and does not induce the Cpx pathway upon over-expression in that organism (250). As with other Cpx-inducing cues, over-expression of *nlpE* is thought to lead to misfolding of *nlpE* to activate the Cpx pathway.

Two signals without a direct link to protein-folding that induce the Cpx pathway are the accumulation of the PG precursor lipid II and changes to the phospholipid composition of the inner membrane. Lipid II is a precursor of PG and the surface-exposed enterobacterial common

antigen (ECA) and is flipped from the cytoplasm to the periplasm by the MurJ flippase (245). Accumulation of lipid II at the inner membrane activates the Cpx and σ^E pathways, confirming that this signal affects the outer membrane as well (42). Similarly, mutants unable to synthesize phosphatidylethanolamine (PE) express only phosphatidylglycerol and cardiolipin in the inner membrane (49). Inactivation of PE biosynthesis results in high-levels of *degP* transcription mediated by CpxR (175), suggesting that the Cpx system may sense changes in membrane composition and up-regulate protein-folding factors. Interestingly, cardiolipin and phosphatidylglycerol are essential for SecYEG function and aid in dimer formation during assembly of the translocon in the membrane (67). Thus activation of the Cpx system may aid in maintaining envelope protein homeostasis during secretion-related stress from an altered inner membrane.

The idea that the Cpx system senses and responds to envelope protein misfolding is supported by the activation of chaperones and proteases that contribute to survival during exposure to Cpx-related stresses. For example, mutation of the alkaline-inducible *cpxP* gene product results in alkaline sensitivity, suggesting that CpxR up-regulates *cpxP* expression to aid survival in alkaline conditions (42) (Figure 1-2). Certain signals that induce the Cpx pathway, however, do not induce the expression of genes that affect survival under those conditions and call into question the physiological relevance of the Cpx response to a particular process. For example, *E. coli* exposed to excess copper up-regulated a number of genes of unknown function that are regulated by CpxR (298). The authors proposed that copper causes protein misfolding, perhaps by interfering with redox-sensitive disulfide bond formation in the periplasm. However, single deletions of the uncharacterized *y*-genes identified by Yamamoto and colleagues (291) as CpxR-regulated copper homeostasis genes does not result in copper sensitivity (221) and

induction of the Cpx system by copper occurred at a concentration significantly higher than the concentration of copper required to activate copper efflux (120). Taken together, these observations suggest that the Cpx system may not respond to excess copper ions *per se* but to a secondary effect of excess copper on the envelope. Some inducing cues, therefore, may lead to a Cpx-activating signal but do not represent a direct input to the Cpx system.

1.3.7.2. CpxA-independent signals and new inducing cues

Though the classical view of two-component systems posits that signals enter via the sensor kinase, the Cpx system senses and responds to a signal that does not require CpxA. Entry into stationary phase induces Cpx in a *cpxA*-independent manner, implicating the CpxR response regulator in aiding cellular changes required for stationary phase growth (51, 296). Perhaps cellular metabolites that accumulate by the end of exponential phase growth require Cpx for turnover (295). How these metabolites are sensed and how CpxR can sense growth phase in the absence of CpxA is unknown.

A number of 'new' inducing signals have been characterized in a variety of organisms, expanding the possible cellular roles for Cpx in the cell. Notably, mutation of the efflux protein *tolC* induces the Cpx TCS in a *cpxA*-dependent manner (235), suggesting that an endogenous substrate for TolC-dependent pumps may accumulate in the absence of efficient efflux to induce the pathway. Similarly, mutation of the *vexAB* and *vexGH* RND efflux pumps in *Vibrio cholerae* results in activation of the Cpx pathway. The Cpx-mediated increase in Vex expression does not result in increased resistance to antimicrobial compounds, however, suggesting that Cpx-mediated RND efflux functions to rid the cell of an endogenous metabolic waste (269). Since Cpx was not required to colonize the infant mouse intestine (250), Cpx may be required to efflux a specific metabolic waste that accumulates in the late stages of infection (269).

Severe defects in lipid A modification also activate the Cpx response. Combined *waaC lpxM* or *waaC lpxL* mutations result in loss of lipid A glycosylation and activation of the Cpx and σ^E stress responses (125). These observations implicate the Cpx response in sensing severe LPS defects and the role of Cpx might be to assist σ^E in the maintenance of outer membrane protein homeostasis.

Intriguingly, a role for the Cpx system in responding to altered metal homeostasis is emerging. A number of Cpx-regulated chaperones, proteases, and *y*-genes were up-regulated in the presence of zinc in *E. coli* TG1 and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (72, 137, 217, 299). In agreement with these observations, mutants that lack the Cpx-regulated chaperone *spy* exhibited decreased doubling times upon exposure to excess zinc in minimal medium (287). In contrast to *E. coli* and *Salmonella,* the *V. cholerae* Cpx system is activated by iron chelation and up-regulates a number of iron-related genes upon *cpxR* over-expression, suggesting that the Cpx system assists in surviving low iron conditions. However, the *cpxR* mutant was only sensitive to low iron conditions when forced to respire, connecting the iron-responsive role of Cpx to bacterial respiration (3). In *E. coli*, however, a number of iron and heme transporters are strongly down-regulated upon Cpx activation (229), demonstrating that some functions of the Cpx system may be species-specific.

1.3.7.3. The Cpx system is connected to other signal transduction systems through connector proteins

Envelope stress responses do not function in a vacuum and the integration of multiple cellular signals is key to bacterial survival. The small inner membrane protein MzrA connects CpxAR to the EnvZ/OmpR TCS. Constitutively active *cpxA** alleles increased *mzrA* expression, resulting in decreased expression of the porins LamB and MalE. MzrA interacts directly with the

EnvZ sensor kinase, possibly to modulate activation of kinase activity and limit the ability of OmpR to modulate porin expression in response to osmolarity (65). The Cpx system has also been shown to repress the synthesis of the adhesive protein curlin in response to osmolarity independently of MzrA (112, 222). Therefore, the Cpx response does not respond to changes in osmolarity solely through MzrA, but the action of MzrA on the Cpx response might ensure that changes to OMP expression are specific to surviving Cpx-related stresses.

Another protein connects the stationary phase survival sigma factor and the Cpx system. In *S.* Typhimurium, the σ^S-regulated protein Cpx-activating connector-like factor CacA activates the Cpx pathway upon over-expression. The expression of CacA connects the Cpx two-component system to the regulator of stationary phase survival, σ^S, in an uncharacterized fashion. The use of connector proteins to regulate multiple TCS enables the cell to modulate the expression of multiple regulons simultaneously, ensuring a rapid response to changing environmental conditions.

1.4. CpxP is an auxiliary regulator of the Cpx system

In addition to connector proteins, many TCS possess auxiliary regulators that communicate specific signals to the sensor kinase to tailor pathway activity to certain conditions. For instance, the lipoprotein NlpE activates the Cpx TCS to communicate surface adhesion (194). The small periplasmic protein CpxP is also an auxiliary regulator of the Cpx TCS (Figure 1-2). Over-expression of *cpxP* results in a strong decrease in Cpx activity that is not replicated by other Cpx-regulated folding factors (24). CpxP inhibits the Cpx pathway through the sensor kinase CpxA, probably through a direct interaction between negative charges in the periplasmic sensing domain of CpxA and positive charges on the concave surface of CpxP (228, 277, 305). Though CpxP inhibits Cpx activity, deletion of *cpxP* results in only a slight increase in Cpx

activity (227), suggesting that other inhibitors may limit Cpx activation in the absence of *cpxP*. Furthermore, *cpxP* is not required for activation of the Cpx system (51), supporting the auxiliary role of CpxP in Cpx pathway activation. Expression of CpxP increases dramatically upon Cpx activation but high-levels of CpxP protein do not immediately dampen Cpx activation, suggesting that CpxP performs some other stress-responsive function in the periplasm during Cpx-related stress. According to this model, the inhibitor activity of CpxP is inactivated by stress, releasing CpxP from CpxA to respond to stress in another fashion (51). Thus, CpxP may exist in two distinct states: inhibitor and periplasmic stress responder.

The stress-responsive activity is proposed to be its ATP-independent chaperone activity, though CpxP possesses relatively weak chaperone activity *in vitro* as compared to the paralogous chaperone Spy. While Spy prevents the thermal aggregation of the model substrate malate dehydrogenase (MDH) at sub-stoichiometric concentrations, CpxP exerts a similar effect on MDH only when present in double the molar concentration of MDH (224). The *in vivo* substrates of CpxP are not known but CpxP appears to have a unique role in pilus biogenesis. Expression of *cpxP* and *degP* was required to prevent the toxic accumulation of the fibrillar tip subunit PapE and the adhesin PapG from the UPEC P-pilus when expressed heterologously in *E. coli* K-12 (99). In a revised model for CpxP-mediated activation of the Cpx pathway, CpxP is titrated from the sensing domain of CpxA by misfolded proteins via its chaperone activity and ushers the proteins to DegP. There, CpxP is degraded by DegP, either separately or concurrently with misfolded proteins (24, 102). In agreement with this hypothesis, *cpxP* is also required for the expression of the bundle-forming pilus (BFP) in enteropathogenic *E. coli* (EPEC) in which DegP also degrades the major pilin BfpA (99, 283). However, the binding of misfolded pilin substrates by CpxP has not been demonstrated and the catalytic activity of CpxP remains

uncharacterized. The dual role of CpxP as a signaling molecule and chaperone for adhesive pili may explain the important role of *cpxP* in certain strains of UPEC for urogenital tract colonization (47).

Homologues of CpxP are identified by the presence of two conserved LTXXQ motifs (Pfam 07813) and these include Spy in *E. coli*, ZraP in *S.* Typhimurium, and CnrX in the metal-resistant bacterium *Cupriavidus metallidurans*. Of the homologues, only CpxP and Spy share significant sequence identity (29%) but ZraP and CnrX share structural homology with CpxP (7, 275) (Figure 1-4). CpxP is a mainly α-helical dimer, where each protomer forms bent hooked hairpins. The resulting concave face of the dimer is remarkably positively-charged while the convex surface is mostly negatively-charged with hydrophobic patches (270, 305). The conserved LTXXQ motifs are located at the poles of each monomer and hydrogen bonds between the glutamine residues of each monomer stabilize the dimer. The contribution of the conserved LTXXQ motifs to CpxP function is unknown but mutation of Q_{55} or Q_{128} abolishes expression of *cpxP* in the presence of *degP* (24), suggesting that the glutamine residues contribute to protein stability.

Spy shares a remarkably similar structure with CpxP but has no known signal transduction role in the cell. Patches of hydrophobic residues on the concave surface of Spy are proposed to participate directly in the chaperone activity of the protein while multiple residues that line the concave surface of Spy, including an alanine within the N-terminal LTXXQ motif, may also participate in substrate-binding (223, 224). Though *spy* expression increases in the presence of sodium tungstate (135), zinc (287, 299) , and copper (120, 298), no metal ions of physiological significance were detected in the Spy structure.

In contrast, ZraP expression is induced by excess zinc and over-expression of ZraP inhibits the zinc-responsive ZraSR TCS (140, 189). Upon zinc-binding, ZraP is proposed to oligomerize into decamers (7), though a zinc-binding site is yet to be identified and the contribution of zinc-binding to function remains uncharacterized. Nevertheless, ZraP is also an ATP-independent chaperone that exhibits activity against thermally denatured MDH with similar efficacy as Spy and contributes to polymixin B resistance in *S.* Typhimurium. The third α-helix that contains the absolutely conserved LTPEQ motif in CpxP and Spy was disordered in the ZraP structure and is thought to interfere with the ability of CpxP and Spy to form higher order oligomers than dimers (7).

Though CnrX and CpxP share only 6% sequence identity, the proteins share similar protein architecture. CnrX (cobalt and nickel resistance) binds nickel and cobalt in the periplasm of *C. metallidurans* and binds an anti-sigma factor CnrY to facilitate the release of a sigma factor CnrH. Once free in the cytoplasm, CnrH associates with RNA polymerase (RNAP) to activate the expression of an RND efflux pump, CnrCBA, that captures and pumps cobalt and nickel into the extracellular space (75, 275). Nickel- and cobalt-sensing in the periplasm ensure that cells are primed to export potentially toxic heavy metal ions before they accumulate in the cytoplasm and damage cellular constituents. The genome sequence of *C. metallidurans* reveals multiple mechanisms for heavy metal resistance, including RND, cation diffusion facilitator (CDF), and P-type ATPase efflux pumps that extrude multiple heavy metals from the cell, as well as multiple sigma factors and putative TCS that are both known and predicted to regulate heavy metal efflux from this organism (105). Thus, CpxP is structurally homologous to the metal-binding and signal transduction proteins ZraP and CnrX, suggesting that CpxP could also bind a metal to affect its known activities (Figure 1-4).

1.5. Bacterial metal homeostasis

Metals serve important and often essential roles in protein structure and activity. While the metals used by a particular bacterium will differ according to niche, enteric pathogens require a subset of metals for conserved processes such as central metabolism, respiration, and transcription. It has become increasingly clear that mammalian hosts chelate metals as an antimicrobial response to infection and that bacteria have evolved sophisticated mechanisms to maintain metal homeostasis at the host-pathogen interface and in the range of environments in which they live.

1.5.1. Iron

Iron is the most abundant transition metal in living cells. However, iron is subject to oxidation by molecular oxygen and can participate in the Fenton or Haber-Weiss reactions to produce dangerous reactive oxygen species that can damage lipids, proteins, and DNA (86). The advent of a mainly oxygenic atmosphere resulted in the limited availability of bioactive iron and the susceptibility of iron-containing enzymes to oxidation (reviewed by (101)). Additionally, the oxidized ferric ion exhibits low solubility at neutral pH, limiting usable iron in the environment. Bacteria have developed strategies to acquire iron in metal-poor environments, convert iron into the metabolically-active form, and to repair oxidatively-damaged iron-containing proteins.

1.5.1.1. Cellular iron is incorporated into proteins in many forms

Bacteria exploit the redox potential of iron by incorporating iron into cofactors and proteins that preclude or regulate the interaction of iron with oxygen. The ubiquitous iron sulfur (Fe-S) clusters are one of the most abundant cofactors in enzymes and likely the most abundant form of iron in cells. Fe-S clusters contribute to a variety of bacterial processes, including respiration, photosynthesis, and nitrogen fixation. The *isc* genes are conserved in eukaryotic

mitochondria where they are used to assemble the Fe-S clusters required for electron transport and ATP-generation (180). The Isc proteins catalyze the formation of Fe-S clusters on a scaffold protein and then insert the cluster into a client protein (192, 264, 273) and are considered the "housekeeping" proteins for Fe-S cluster biogenesis. In contrast, the expression of the *suf* genes is induced by oxidative stress or iron starvation (198) and can replace the function of *isc* (265), suggesting that Suf proteins can synthesize and assemble Fe-S clusters when the cell is stressed. OxyR, an oxidative stress-responsive transcription factor and integration host factor IHF can bind to the promoter of *suf* and activate expression of the operon in the presence of specific stressors (136, 304). The regulation of *suf* expression, however, is tightly linked with that of *isc. isc* expression also increases during oxidative stress and the induction of *iscR*, a transcriptional regulator, activates the expression of *sufA*, the scaffold protein for *suf*-mediated Fe-S cluster biogenesis (303). Through the coordinated expression and induction of the different Fe-S cluster biogenesis operons, the cell can ensure that essential processes requiring Fe-S clusters can be made and function under all conditions.

Iron is also incorporated into respiratory enzymes as heme, including cytochromes in *B. subtilis* (62), and *c*-type cytochromes in *Rhodobacter capsulatus* (17) and *Bradyrhizobium japonicum* (272). Heme is comprised of a protoporphyrin ring bound to ferrous iron coordinated by six nitrogen atoms. The iron atom readily undergoes oxidation (138) which is especially important in cytochromes during the transfer of electrons between protein complexes. Bacterial hemoglobins perform detoxification functions in pathogenic bacteria. In *Mycobacterium bovis*, a truncated hemoglobin encoded by *glbN* aids cells in surviving NO-induced stress, a key antimicrobial defense in human phagocytes, by converting NO to nitrate and protecting the aerobic respiratory complexes from poisoning (195). The single-domain Cgb

globin of *Campylobacter jejuni* performs a similar function, though the regulator responsible for *cgb* transcription is not able to sense NO directly, suggesting that another signal is required or a secondary signal resulting from nitrosative damage must be sensed before the globin is expressed (252). Genes for hemoglobins are reportedly found in 65% of all bacterial genomes, 76% of gammaproteobacterial genomes, and up to 92% of all betaproteobacterial genomes (281). In the psycrophilic gammaproteobacterium *Pseudoalteromonas haloplanktis*, a hemoglobin mediates resistance to reactive nitrogen species (RNS) (37), suggesting the need for hemoglobins to detoxify RNS is evolutionarily conserved and not restricted to pathogenic species of bacteria.

1.5.1.2 Iron acquisition is regulated by Fur

Because iron is required for diverse processes, bacteria have evolved transport systems to acquire iron under metal-limiting conditions. Siderophores are high-affinity iron capture proteins that are synthesized and secreted by bacterial cells during iron starvation to scavenge iron. The model for siderophore-mediated iron uptake involves the interaction of an outer membrane receptor specific for an iron-bound siderophore with a long envelope-spanning protein called TonB that energizes siderophore import (286) (Figure 1-3). TonB is also required for the transport of vitamin B12 and responsible for susceptibility to multiple *E. coli*-derived antibacterial compounds called colicins (11, 46). TonB interacts with two inner membrane proteins, ExbB and ExbD, which help stabilize TonB and assemble the inner membrane TonB-ExbBD complex (4, 115, 249). Energy derived from PMF is stored by the TonB-ExbBD complex and transduced to outer membrane receptors to drive the import of siderophores into the periplasm (132). Once in the periplasm, periplasmic-binding proteins can usher siderophore-iron complexes to inner membrane transporters that actively transport the complexes into the cytoplasm. Dedicated enzymes hydrolyze iron from the siderophores, freeing iron for the cell's

metabolic needs (147, 213, 260). Iron uptake, therefore, is the product of coordinated protein-protein interactions that cross the envelope to energize the transport of essential iron compounds into bacteria for metabolic use.

The expression of the numerous proteins required to maintain iron homeostasis is regulated by the ferric uptake regulator Fur (87) (Figure 1-3). Fur exists as a homodimer with an N-terminal winged-helix DNA-binding domain (DBD) and a C-terminal dimerization domain. Fur binds a zinc ion in the dimerization domain required for the structural integrity and oligomerization of the protein (215). In *Pseudomonas aeruginosa*, the residues that coordinate the structural zinc ion are two histidines and two glutamates whereas two cysteines coordinate the zinc ion in *E. coli* (206). In *E. coli*, metal-binding in the regulatory site in the C-terminal domain of Fur results in conformational changes throughout the protein and folding of an N-terminal α-helix proximal to the DBD. Upon formation of the α-helix, the DBD is reoriented in a manner that is competent to recognize and repress target promoters and to communicate with the C-terminal dimerization domain (206). Fur, therefore, requires two different metal species to perform its regulatory function in the cell and the coordination chemistry of the two different metal ions diverges between bacterial species. Though the significance of the difference in residues is not known, the use of cysteine residues in *E. coli* could signify a role for Fur in sensing redox conditions in the cell as well as iron levels, whereas the histidines used in *Pseudomonas* could sense pH conditions (158, 206). Further comparative studies are required to elucidate the significance of the different coordination chemistries employed by Fur in different bacteria.

Fur regulates the expression of siderophore biosynthesis, secretion, and import genes, the *tonB exbBD* genes, metabolic genes that aid the cell in surviving iron starvation, and genes that

regulate motility (244) (Figure 1-3). While iron-bound Fur (Fur-Fe) was originally thought to be the only conformation competent to bind DNA, examples of apo-Fur regulation in *Campylobacter jejuni* and *Helicobacter pylori* present a new paradigm for Fur-mediated virulence gene regulation. In *C. jejuni*, Fur-Fe represses the expression of iron acquisition and oxidative stress genes while apo-Fur represses the expression of *dsbB*, a disulfide bond exchange protein, and *rrc*, a rubredoxin-containing protein induced by oxidative stress (25). In *H. pylori*, apo-Fur directly represses the expression of a *c*-type cytochrome that contains a heme group and a Ni/Fe hydrogenase required to metabolize molecular hydrogen (29). Apo-Fur represses the expression of specific iron-containing proteins, perhaps because the expression of those proteins in the absence of sufficient iron to produce cofactors is detrimental to the cell during iron starvation. Conversely, apo-Fur activates the expression of a single transcriptional unit in *E. coli*: the *ymgA-ariR* operon which encodes for genes involved in regulating biofilm formation and acid-stress (244). Possibly, pathogenic bacteria have acquired the ability to regulate specific virulence genes with apo-Fur (244). Extraintestinal *E. coli* (ExPEC) requires Fur to sense and respond to human serum (97) while *V. cholerae* does not appear to use apo-Fur regulation (174), though methods to study this new mode of regulation are just now emerging.

Fur can also exert post-transcriptional effects on gene expression through the repression of the sRNA *ryhB* (160). *rhyB* decreases the expression of multiple targets by binding target mRNAs along with the RNA chaperone Hfq, facilitating the degradation of transcripts that assist in iron utilization (50, 161, 162). In general, *rhyB* expression results in a decrease in iron-containing proteins and an increase in intracellular iron pools in a response collectively known as iron-sparing (104, 161) (Figure 1-3). The coordinated action of Fur and *rhyB* ensures the efficient and balanced uptake and usage of iron according to the extracellular conditions.

1.5.2. Zinc

Zinc is the second most abundant metal in living organisms after iron. Due to a completely filled *d* valence shell, zinc cannot participate in redox reactions like iron or copper. In eukaryotes, zinc is necessary for the stability and proper folding of the ubiquitous and abundant zinc-finger transcription factors and other zinc-binding proteins required for the tight and sophisticated control of gene expression. In bacteria and archaea, however, zinc is mostly associated with peptidases and proteases, likely selected as both a catalytic and structural cofactor that promotes the folding of a wide variety of substrates. Bacteria possess a number of conserved and unique transporters to regulate intracellular zinc flux and maintain zinc homeostasis.

1.5.2.1. Zinc is essential and zinc uptake is mediated by Zur

Zinc is imported for use in a variety of processes, including the biogenesis of PG (141), proteolysis (248), and ribosomal biogenesis (200). A number of L,D-transpeptidases, D,D-carboxypeptidases, and muramidases contain zinc ions that are essential for both folding and function (26, 95, 129, 207, 240, 246, 307). In *Neisseria gonorrheae*, a zinc-binding metallopeptidase that degrades PG is required for expression of the type IV pilus, presumably to ensure the pilus can cross the envelope (261). Of increasing clinical importance are the zinc-dependent metallo-β-lactamases which require two zinc ions for activity and cause widespread β-lactamase resistance via plasmid transmission (reviewed in (116)). Similarly, numerous metalloproteases depend on zinc for activity and folding. NleC is a type III secretion system effector in EPEC and EHEC that cleaves p65 in the human nuclear NF-κB complex to subvert host immune responses during infection but cannot cleave p65 *in vitro* in the absence of zinc (148). Similarly, proteins that catalyze bacterial transcription and translation initiation and

elongation require zinc as a structural cofactor (27, 81, 89). Thus, zinc is required for conserved processes across all domains of life, such as transcription, but also for unique bacterial processes, such as PG re-modelling.

Zinc uptake is mediated by a number of transporters with differing affinities for zinc in the periplasm. ZnuABC is a high-affinity ABC-transporter that couples the transport of zinc into the cytoplasm with the hydrolysis of ATP (202). ZnuA is a periplasmic zinc-binding protein that binds zinc with a K_D of less than 20nM and shuttles zinc to the inner membrane transport channel protein ZnuB where conformational changes in both proteins facilitate the release of zinc to the transporter (32, 302). ZnuA can take zinc from a lower-affinity zinc transporter, ZinT, during zinc starvation in *Salmonella enterica* to increase the efficiency of zinc uptake (100, 211). There are two zinc-binding sites in ZnuA: a high affinity site comprised of a conserved histidine and aspartate residue (302) and a second site that may have lower affinity for zinc (32, 100, 302). High affinity zinc transport mediated by ZnuABC is especially important in the host where zinc is chelated by metallothioneins and Ca^{2+}-binding proteins such as calmodulin (15). *S.* Typhimurium lacking *znuABC* is attenuated in mice (28) and is being considered for the production of live cell vaccines (201, 209). Similarly, *H. ducreyi* and *Brucella abortus* lacking *znuA* were significantly less virulent in animal models of infection (123, 145). ZnuABC, therefore, ensures that bacteria can obtain zinc in the metal-poor host environment during infection.

The lower-affinity zinc importer ZinT binds zinc, cadmium, and nickel, though a *zinT* mutant requires zinc for growth in defined medium (119). ZinT consists of a lipocalin-like domain and a helical domain with a cadmium ion bound by three histidine residues that connect the two domains of ZinT (44). The physiological role of *zinT* remains unclear because ZinT

appears to be important only under conditions of severe zinc depletion in lab strains of *E. coli* that also lack *znuABC* (73, 211) and may have a general stress-related role (44). However, *zinT* is required for adhesion in enterohaemorraghic *E. coli* (EHEC) (63) and *zinT* mutants display decreased *csgA* expression, suggesting *zinT* might play a specific role in zinc acquisition to facilitate adherence.

ZupT is a ZIP-family (zinc-regulated transporters (ZRT), iron-regulated transporter (IRT)-like protein) transporter that increases cellular sensitivity to zinc upon over-expression (77). Unlike ZnuABC and ZinT, ZupT can bind multiple metals (78), suggesting that ZupT can function in the uptake of multiple metals under metal ion depletion. In support of its minor role in zinc acquisition, *zupT* mutants of *S.* Typhimurium are not attenuated like *znuA* mutants but exhibit a fitness defect in competition experiments with wild-type *S. enterica* in mice (30). In contrast, *C. metallidurans* ZupT is likely the main zinc importer during zinc starvation and no ZnuABC homologue is encoded (124). Thus, organisms possessing a system for high-affinity zinc import do not require ZupT-mediated transport for survival in moderate zinc depletion. The expression of zinc uptake is regulated by the cytoplasmic regulator Zur (zinc uptake regulator) (202) which binds zinc with femtomolar sensitivity and the metal-bound protein binds the promoters of *znuABC* and *zinT* to repress the expression of zinc uptake transporters (197, 203) (Figure 1-3). Pools of intracellular zinc are estimated to be in the micromolar range but the vast majority of intracellular zinc is bound by proteins, thereby limiting the pools of 'free' zinc through the activity of Zur (197). Zur is part of the same family of transcriptional regulators as Fur and possesses the same conserved structure: an N-terminal winged helix DBD, a C-terminal dimerization domain, and a hinge region between the N- and C-terminal domains (150). The mechanism of protein activation upon metal-binding, however, is distinct. Mutational analysis of

zur in *Streptomyces coelicolor*, which contains three zinc-binding sites, suggests that differential zinc-binding to two of the three sites can modulate promoter-binding activity. Binding of zinc to the inter-domain loop repositions the DBD for promoter binding but zinc-binding to the dimerization domain seems to be required to fully repress sensitive targets like *znuA*, suggesting that cooperation between the zinc-binding sites is required for the robust repression of targets in zinc-replete conditions (247). In *Mycobacterium tuberculosis* Zur (FurB), there are three zinc-binding sites, including a regulatory site in the hinge region hypothesized to help position the domains relative to one another upon zinc-binding. One zinc ion binds two cysteines from the DBD (C126 and C129) and is essential for protein structure. Another zinc binds at two cysteines in the dimerization domain (C86 and C89) where each cysteine is contributed by independent CXXC motifs. The third site consists of three histidine residues and one glutamate but the physiological relevance of zinc-binding at this site is unknown (150). In contrast, *E. coli* Zur contains only two zinc-binding sites: a tightly-bound structural zinc coordinated by four cysteines and a second regulatory site (197). Zur, therefore, is subject to fine regulation at each of its zinc-binding sites, allowing for highly sensitive regulation of its promoters in response to intracellular zinc concentration.

The Zur regulon is not confined to the aforementioned zinc transporters but also includes stress-responsive ribosomal proteins. A common signature for ribosomal proteins is the zinc ribbon motif (278) and zinc depletion induces Zur-mediated de-repression of ribosomal proteins that do not contain zinc ribbon motifs. Zinc starvation, therefore, de-represses the expression of ribosomal proteins that replace the action of zinc-requiring ribosomes during zinc starvation (154, 200). Similarly, *Neisseria meningitidis* Zur regulates proteins predicted to modify tRNAs that could improve the efficiency of translation during zinc starvation (205). Transcriptomic and

computational analyses of *zur*-mutant actinobacteria suggest that zinc-dependent alcohol dehydrogenases and a putative oxidreductase may be under the control of Zur (241). Zur mediates the repression of oxidative stress response genes in *Anabaena spp.*, connecting the activity of Zur with multiple stress-responsive transcription factors (243). Zur, therefore, primarily represses zinc transport but also represses the expression of genes required for translation, metabolism, and stress responses that could be beneficial during zinc limitation. For pathogens, the acquisition of zinc is critical and, accordingly, *zur* mutants are impaired for virulence in the plant pathogen *Xanthomonas campestris* (267) and in the intracellular pathogen *Listeria monocytogenes* during mouse infection (38, 54) . In both cases, a primary means of attenuation is the accumulation of intracellular zinc in the absence of *zur.* The maintenance of zinc homeostasis through the action of Zur, therefore, is critical for pathogens infecting hosts in both plants and animals.

1.5.2.2. Excess zinc is toxic and zinc efflux is mediated by ZntR

As is the case with iron, zinc is both essential but toxic at high levels. A precise mechanism for zinc toxicity in bacteria is not known but a number of mechanisms in specific species have been proposed. In *E. coli,* zinc likely interferes with thiol-binding to Fe-S cluster cofactors in metabolic enzymes, leading to metabolic collapse and cell death (297). Intriguingly, Xu and colleagues did not observe an effect on the expression of the multiple Fe-S cluster-containing NADH dehydrogenase complex I (Nuo) *in vivo*, though zinc can prevent activity of the purified complex *in vitro* by blocking proton-pumping and indirectly stopping electron transfer (242). Similarly, zinc and other divalent cations can bind to cytochrome *bd* and arrest bacterial aerobic respiration (13), which has the potential to cause toxicity in situations where *E. coli* would be forced to respire. In *Streptococcus pneumoniae*, zinc binds to the secreted

manganese acquisition protein PsaA, preventing the acquisition of manganese and sensitizing the bacterium to oxidative stress in murine macrophages (169). Unlike redox-active metals like iron and copper, zinc does not participate directly in the production of reactive oxygen species (ROS) but competes with other metals and cofactors for protein-binding, thereby preventing protein function and resulting in toxicity.

Though the mammalian host carefully prevents bacterial metal acquisition, there is evidence that the immune system may compartmentalize excess zinc as an antimicrobial response. Upon phagocytosis of *M. tuberculosis*, murine macrophages produce a bactericidal "zinc burst" in phagosomes, likely released from cytosolic metallothioneins through some unknown mechanism. The intracellular accumulation of zinc in *M. tuberculosis* induces the expression of P-type ATPase transporters to efflux zinc from cells (19). Indeed, *M. tuberculosis*-infected murine macrophages accumulate up to 456.8μM Zn^{2+} 24 hours post-infection (284), suggesting that phagosomal zinc accumulation may be an important immune response during infection. In agreement with these observations, zinc deficiency may be detrimental to immune function and treatment of a number of diarrheal pathogens and viruses with zinc may decrease the severity of illness (219, 258, 291). Intestinal bacteria may encounter high levels of zinc during host zinc supplementation to treat disease or as a preventative measure to stimulate immune function.

In *E. coli*, the major zinc efflux protein ZntA mediates high affinity capture of zinc and transport out of the cytoplasm and inactivation of *zntA* results in sensitivity to zinc, cadmium, cobalt, and nickel (14, 231). The structure of ZntA from *Shigella sonnei* was recently solved, revealing a unique mechanism for zinc capture and transport to the periplasm as compared to its structural homologue CopA. A funnel lined with negative charges ushers a captured zinc ion in

the cytoplasm toward the conserved methionine residue where, once zinc is bound, a phenylalanine blocks re-entry of zinc into the cytosol. Conformational changes associated with ATP-binding and hydrolysis moves zinc to a high-affinity cysteine at a CPC motif and the high affinity site is actively exposed to the periplasm to extrude zinc from the membrane-embedded cysteine (290). The path of zinc in the periplasm to the outside of the cell remains unclear, but could perhaps be mediated by porins or uncharacterized outer membrane proteins and metallochaperones. P-type ATPases such as ZntA are attractive drug targets because they are widely conserved in bacteria and plants but are not present in animals (290).

The zinc efflux protein YiiP/FieF was first characterized as a putative zinc transporter of the cation diffusion facilitator (CDF) family with pH-dependent binding properties resulting from the protonation or deprotonation of a conserved histidine required to bind zinc and cadmium (34). One group proposed that excess iron could induce expression of *yiiP* in *E. coli* and that YiiP could measurably efflux iron from the cytoplasm during iron stress, however these authors also observed active efflux of zinc in reconstituted proteoliposomes and that *yiiP* is not under the control of the iron-responsive transcriptional regulator Fur (78). Subsequent structural studies showed that YiiP is a membrane-embedded homodimer that binds to zinc on the cytoplasmic side of a membrane-embedded six-helix bundle and that the zinc is likely captured from the cytoplasm by a C-terminal metallochaperone-like domain (152). The site of active zinc transport is in the membrane but zinc is captured in the cytoplasm at the C-terminus of the protein. A flexible loop that links the transmembrane domains to the C-terminal domains of the dimers maintains dimerization and responds to zinc-binding at a regulatory site in the cytoplasm by acting as a hinge that swings open or closed depending on whether zinc is bound to the protein (153). Thus, zinc-binding to the metallochaperone domain in the cytoplasm allosterically

activates YiiP to transport zinc out of the cytoplasm and this mechanism is likely conserved amongst members of the CDF transporter family. The site of zinc transport in the human YiiP homologue is almost identical to the bacterial homologue, save for the substitution of an aspartate with a histidine (94). Therefore, the amino acids that bind zinc in YiiP have been selected for with high specificity. Unlike ZntA, the activity of YiiP is driven by a proton gradient established by a histidine residue that binds both zinc and, depending on the side of the membrane to which it is exposed, a proton (84). These structural studies, therefore, strongly argue that YiiP is a zinc and not an iron transporter. However, relatively little is known about the regulation of *yiiP* expression and a better understanding of the conditions in which *yiiP* is expressed will supplement the biochemical studies.

ZitB is also a CDF-family zinc-transporter that can mediate the efflux of zinc from *zntA* mutant cells (74). Similar to YiiP, proton antiport also drives zinc efflux in ZitB (34). However, whereas the expression of *yiiP* is activated by unknown means in the presence of excess zinc, *zitB* expression appears to be constitutive and ZitB may be the 'housekeeping' zinc efflux pump that can mediate resistance to transient zinc shock in *E. coli* (288).

The MerR-like transcriptional regulator ZntR mediates the expression of zinc efflux proteins. ZntR activates the expression of itself and *zntA* in the presence of a threshold amount of zinc proposed to be 100μM in *E. coli* (23), though ZntR binds zinc at femtomolar amounts (197) (Figure 1-3). Apo-ZntR is competent to bind DNA and, like MerR, unwinds and widens the minor groove of DNA at the *zntA* promoter. Upon zinc-binding, ZntR relaxes the DNA winding, placing the -35 and -10 promoter elements in a geometry that is suitable for RNA polymerase binding, thereby activating expression of *zntA* (196). In the absence of *zntR*, the

minimal inhibitory concentration (MIC) for zinc in *E. coli* TG1 is 750 μM whereas the MIC for wild-type *E. coli* is 2.2mM zinc, confirming that ZntR mediates zinc tolerance in *E. coli* (23). The variety of zinc efflux proteins and the transcriptional regulator ZntR are critical for bacterial survival in high levels of zinc and the structural and mechanistic details behind zinc efflux have emerged only recently. Metal excess is generally rare but the conservation of zinc efflux systems in pathogenic species of bacteria suggests that zinc efflux serves an important function in pathogens.

1.5.3. Copper

Copper, like zinc, is thought to be entirely bound by proteins in the cytoplasm of cells (280). Copper is an important cofactor for bacterial respiratory enzymes (45, 177) and is therefore important for bacterial growth but bacteria have also developed systems to detoxify the cell of excess copper.

1.5.3.1. Copper aids electron transport

Copper is incorporated into the *E. coli* ubiquinol oxidase (1). Ubiquinol oxidases are part of the heme-copper oxidase family that includes homologues of bacterial and mitochondrial cytochrome c. Ubiquinol oxidase takes two electrons from ubiquinol and reduces molecular oxygen in cooperation with cytochrome bo_3 while also pumping protons into the periplasm to contribute to PMF (6). Treatment of ubiquinol oxidase with cyanide results in reduction of the copper center and release of the copper-cyanide complex, resulting in a loss of ubiquionol oxidase activity (279). These observations demonstrate the importance of copper in the oxidation of ubiquinone in *E. coli*.

1.5.3.2. Copper is toxic and the export of copper is regulated by CueR and CusR

The copper requirements of other species of bacteria are less clear. *E. coli*, for instance, encodes no known copper import system but possesses multiple copper export systems. The P-type ATPase CopA binds cuprous copper at an N-terminal CXXC motif and is essential for copper resistance (56, 190, 230). However, mutation of the copper-binding cysteines does not prevent ATP hydrolysis by CopA nor affect copper sensitivity in *E. coli.* Substitutions in the N-terminal CXXC motif decreased the threshold amount of copper required to activate the enzyme, suggesting a potential regulatory function for this motif *in vivo* or improper conditions to see an effect of cysteine replacement (56). In contrast, replacement of the conserved intramembrane CPC motif of CopA results in copper sensitivity, implicating these residues in Cu^{+} binding (56), which is also consistent with binding of zinc in ZntA at the cognate residues of the CPC motif (290). In some bacterial genera and in *Saccharomyces cerevisiae*, CopA cooperates with copper metallochaperones to capture copper in the cytoplasm, including *Synechosystis sp.*, *Neisseria meningitidis,* and *Enterococcus hirae* while some bacterial species use only the ATPase, as in *E. coli* and *M. tuberculosis*. Intriguingly, obligate parasites such as *Borrelia burgdorferi, Mycoplasma spp.*, and *Chlamydia spp.* do not encode either copper chaperones or transporting ATPases (109). In contrast, the photosynthetic bacterium *Synechosystis* PCC 6803 requires copper for the function of plastocyanin in low-iron conditions, which transfers electrons from cytochrome b_6f to photosystem I inside thylakoids and the import of copper into thylakoids is mediated by the CPx-type ATPase PacS and the metallochaperone Atx1 (274). The complexity of copper partitioning into multiple cellular compartments in cyanobacteria perhaps necessitates the use of a copper metallochaperone to aid copper transport. Thus, the use of metallochaperones to facilitate copper trafficking is genus-specific and may reflect a difference in copper trafficking in certain organisms.

CopA expression is repressed by the MerR-family transcriptional regulator CueR and copper prevents CueR binding to the *copA* promoter (263) (Figure 1-3). CueR may also be denoted as CopR (210), though CueR seems to be more widely used. Similar to the structure of ZntR, CueR binds copper at the dimer interface and coordinates copper with two cysteine residues. A conserved serine bridges the dimerization interface and contacts the metal-binding loop, providing a microenvironment that can only accommodate Cu^{+} and shielding the ion from solvent to prevent oxidation (33). The expression of CopA, therefore, is sensitively regulated by the highly cuprous ion-specific transcriptional regulator CueR.

Another copper-transporting efflux pump in *E. coli* is encoded by Cu-sensing CusCFBA resistance-nodulation-and-division (RND) efflux pump, which drives copper out of the cytoplasm. CusA is the inner membrane protein that captures copper and transports it out of the cytoplasm, CusC connects CusA to CusB, the membrane fusion protein, and CusF binds periplasmic copper, presumably to usher periplasmic copper to the CusCBA efflux pump for export (60) (Figure 1-3). CusF was recently shown to take Cu^{+} from the heavy metal-binding domain of CopA to usher the ion to CusCBA for efflux (199), providing the first mechanism for the specific export of toxic metal ions from the cytoplasm to the extracellular space (Figure 1-3). CusF binds Cu^{+} at the periplasmic interface of CopA to ensure that cuprous ion is sequestered away from potential targets of toxicity at the membrane. The expression of the *cus* locus is controlled by a copper-sensing two-component system CusSR (179) which also activates the expression of the "copper sponge" PcoE that can further sequester toxic copper from cellular macromolecules (306).

Despite the potential for toxicity of Cu^{+} for producing reactive oxygen species, cuprous ion is only stable under reducing anaerobic conditions and copper is more toxic to *E. coli* in the

absence of oxygen (197). Under aerobic conditions, *E. coli* requires CopA to efflux copper from the cytoplasm and the multicopper oxidase CueO to oxidize Cu^{+} in the periplasm (74, 197) (Figure 1-3). CueO consists of three connected azurin-like domains and a unique methionine-rich helix which lies proximal to a cluster of copper-binding sites and is thought to modulate metal-specificity (233). CueO has been shown to directly oxidize Cu^{+} *in vitro* but can also oxidize catecholate siderophores to prevent the reduction of Cu^{2+} to Cu^{+} by apo-siderophores (76, 122), demonstrating that CueO can act both directly and indirectly to prevent Cu^{+} formation in the periplasm. Under anaerobic conditions, deletion of *cusR* led to increased copper accumulation, suggesting that Cus-mediated efflux is important in the absence of oxygen. Despite clear evidence for oxygen-based regulation of the *cop* and *cus* genes, the oxygen-sensing regulators ArcB and Fnr were not required for the differential regulation of the copper resistance genes (197).

Copper is employed as an antimicrobial in phagosomes against *M. tuberculosis* infection (237) and CueO is required for copper tolerance in *M. tuberculosis* (238), providing evidence for the necessity of copper efflux during pathogenicity. Similarly, *S.* Typhimurium lacking *cueO* is unable to colonize the liver and spleen of mice (2), demonstrating the importance of periplasmic copper detoxification during mouse colonization in this organism.

1.5.4. Other metals

A number of metals play key roles in bacterial metabolism and respiration but are significantly less abundant than iron, zinc, and copper. Manganese is especially important for surviving oxidative stress and is incorporated into *E. coli* superoxide dismutase (118), accumulates in *Deinococcus radiodurans* to promote resistance to high-levels of radiation (41), and is crucial for the resistance of *S. pneumoniae* to oxidative and nitrosative killing (191). In

order to obtain manganese, *E. coli* uses the inner membrane NRAMP transporter MntH (156). During oxidative stress, the transcriptional regulator OxyR increases *mntH* expression to increase manganese uptake and manganese metallates normally iron-containing enzymes to protect the proteins from oxidative damage (5). Instead of degrading or inhibiting the expression of iron-containing proteins, manganese replacement renders these proteins resistant to oxidative damage and ensures that the cell simply has to re-metallate proteins with iron rather than synthesizing them *de novo*. Because manganese is a relatively non-competitive metal compared to iron (285), manganese protection of cellular enzymes during oxidative stress facilitates an efficient return to homeostatic conditions. *mntH* expression is also under the control of Fur (204), reinforcing the connection between manganese, iron, and oxidative homeostasis in cells. Intriguingly, lactic acid bacteria and *B. burgdorferi* do not require iron but use manganese in potentially iron-requiring enzymes instead (8, 218, 276), further exemplifying the adaptive role manganese plays in bacterial metabolism.

Nickel is incorporated into hydrogenases, ureases, and other metabolic enzymes in multiple bacteria (reviewed in (18)) and the uptake of nickel is mediated by an ABC transporter encoded by the *nikABCDE* genes (183, 194). The *nik* operon is regulated by the transcriptional regulator NikR (214). The periplasmic nickel-binding protein NikA binds nickel at an unusual hydrophobic pocket with five amino acid ligands, including two tryptophan residues, with low affinity (K_D in the micromolar range). Under anaerobic conditions when *nikA* gene expression is induced, the up to 23 000 copies of NikA present per cell may overcome the low binding affinity for nickel (90). Nickel enhances virulence gene expression in enteric pathogens. *Helicobacter pylori* requires nickel for the maturation of urease, a virulence determinant required for full gastric invasion (188). The addition of nickel to planktonically-grown *E. coli* promotes biofilm

formation mediated by an increase in curli biosynthesis (208), though precisely how nickel affects curli biosynthesis remains unknown. The peptidyl-prolyl isomerase (PPIase) SlyD binds nickel ions at the catalytic PPIase site, which ablates chaperone function (159). SlyD can bind the HycE subunit of the Ni/Fe hydrogenase in *E. coli* and facilitate both the membrane insertion and folding of HycE (35), thereby promoting expression of the Ni/Fe hydrogenase.

As with other metals, nickel excess may be toxic and cells efflux nickel through the resistance to cobalt and nickel RcnA nickel/cobalt efflux pump (234). The expression of RcnA is repressed by the nickel-sensing transcriptional regulator RcnR (103). The cadmium, zinc, and nickel CznCBA RND efflux pump in *H. pylori* regulates the activation of urease in the cytoplasm by controlling which metal is present to charge the protein. In the presence of nickel, urease is activated but efflux of nickel will prevent urease activation. Urease charged with cadmium and zinc is inactive and efflux of these metals will prevent the competitive inhibition of urease (259). Thus, Czn-mediated metal efflux represents a post-translational mechanism of virulence regulation by modulating the activity of urease.

Cobalt is a constituent of vitamin B12 and is a structural requirement for nitrogen fixation and nitrate reduction in diazotrophic bacteria (185). Vitamin B12 is required to initiate a complicated post-transcriptional signaling cascade for utilization of phosphatidylethanolamine in the mammalian intestine by *Enteroccous faecalis* and *Listeria monocytogenes (48, 173)*. The *nhlF* gene of *Rhodococcus rhodocrous* encodes a nitrile hydratase that can confer cobalt uptake upon *E. coli* (126). Cobalt efflux may be mediated by zinc or copper transporters in the inner membrane, including ZntA and CopA (14, 230). Cobalt is effluxed with some specificity by the *Cupriavidus metallidurans* cadmium, zinc, and cobalt efflux pump CzcP (239), and *E. coli*

YiiP/FieF (294). The mechanisms of cobalt toxicity are not known but bacterial zinc transporters appear to be able to efflux both ions without compromising bacterial survival.

1.6. Research Objectives

The transcriptomic studies that suggest excess metals induce the Cpx pathway in *E. coli* did not explore the role of the Cpx pathway in responding to increases in intracellular copper or zinc. The auxiliary regulator CpxP shares homology with proteins that bind metals and integrates periplasmic signals and is therefore an excellent candidate for a signalling protein that could sense metals and communicate those signals to CpxA (Figure 1-4). Additionally, though the structure of CpxP is known, a full understanding of how CpxP interacts with CpxA to inhibit the Cpx pathway is still missing. This work sought to understand how CpxP interacts with metals or with other proteins to mediate signal transduction to the Cpx pathway through three main objectives:

1. To determine the effect of metal limitation on Cpx pathway activity in *E. coli* under standard laboratory conditions and to examine whether the Cpx pathway senses a specific metal ion species. Specifically, we wished to examine the role of the inhibitor CpxP on Cpx metal-sensing, if any.
2. To determine whether full-length CpxP purified under native conditions binds a metal ion and to determine the effects of metal-binding on the inhibitor and chaperone activities of CpxP *in vitro* and *in vivo.*
3. Finally, we sought to characterize novel regulators of the Cpx pathway that functioned analogously to CpxP in an attempt to further characterize the negative regulation of Cpx activity by CpxP.

1.7. Figures

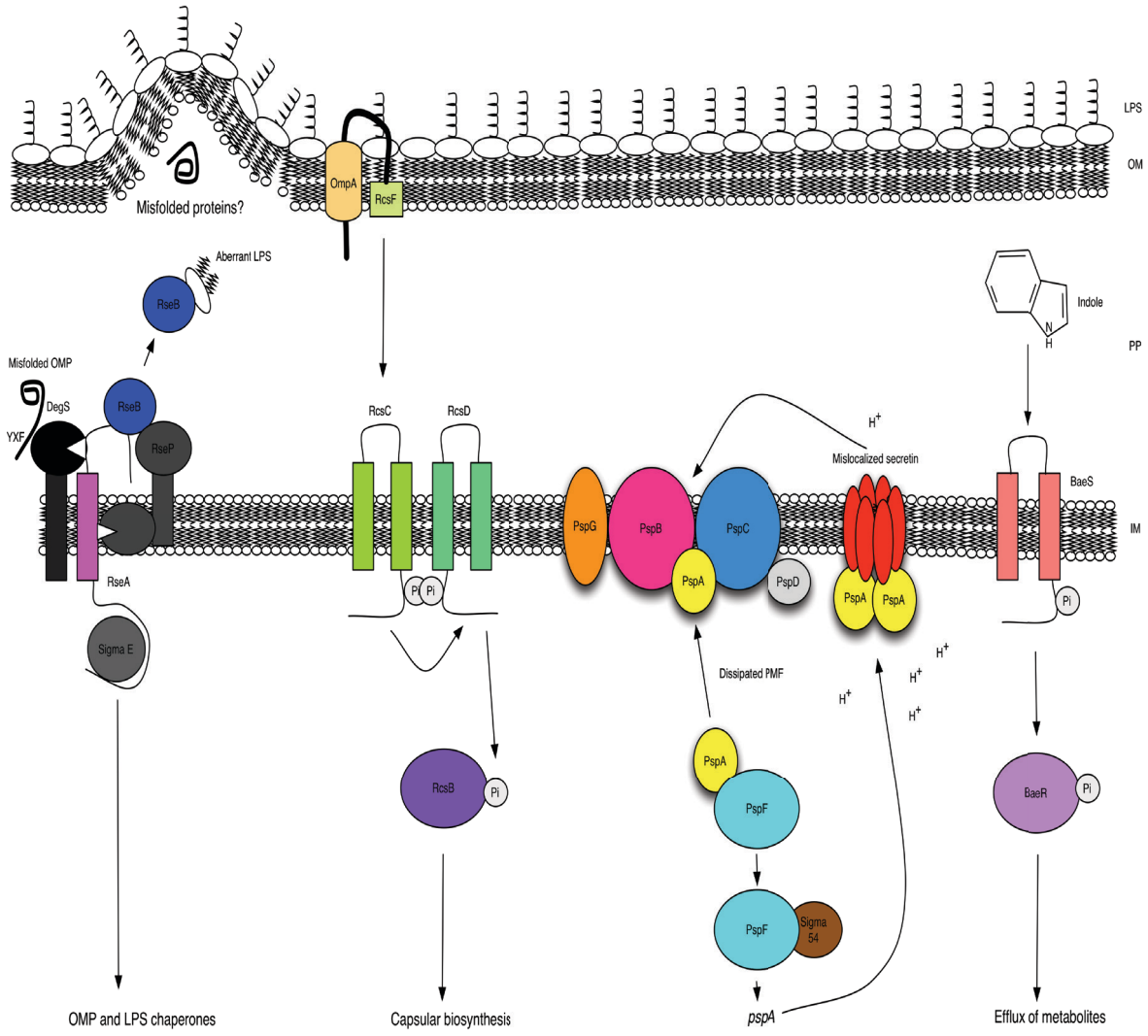


Figure 1-1: Envelope stress responses sense stress and modulate gene expression to facilitate adaptation.

Outer membrane vesicles form through an unknown mechanism and select specific cargo to remove from the envelope during stress. The σ^E response senses both misfolded OMPs and off-pathway LPS subunits to activate the expression of chaperones that assist in achieving outer

membrane homeostasis. The Rcs phopshorelay senses peptidoglycan and osmotic stress and up-regulates the synthesis of colanic acid to produce capsule in *E. coli.* The Psp response senses inner membrane stresses and activates the expression *pspA,* a protein thought to help reinforce the PMF. The Bae TCS senses noxious chemicals and metabolites and increases cellular efflux to ensure the envelope is not damaged. The Cpx TCS is also an envelope stress response and is diagrammed in Figure 1-2. LPS denotes lipopolysaccharide, OM denotes outer membrane, PP denotes periplasm, and IM denotes inner membrane.

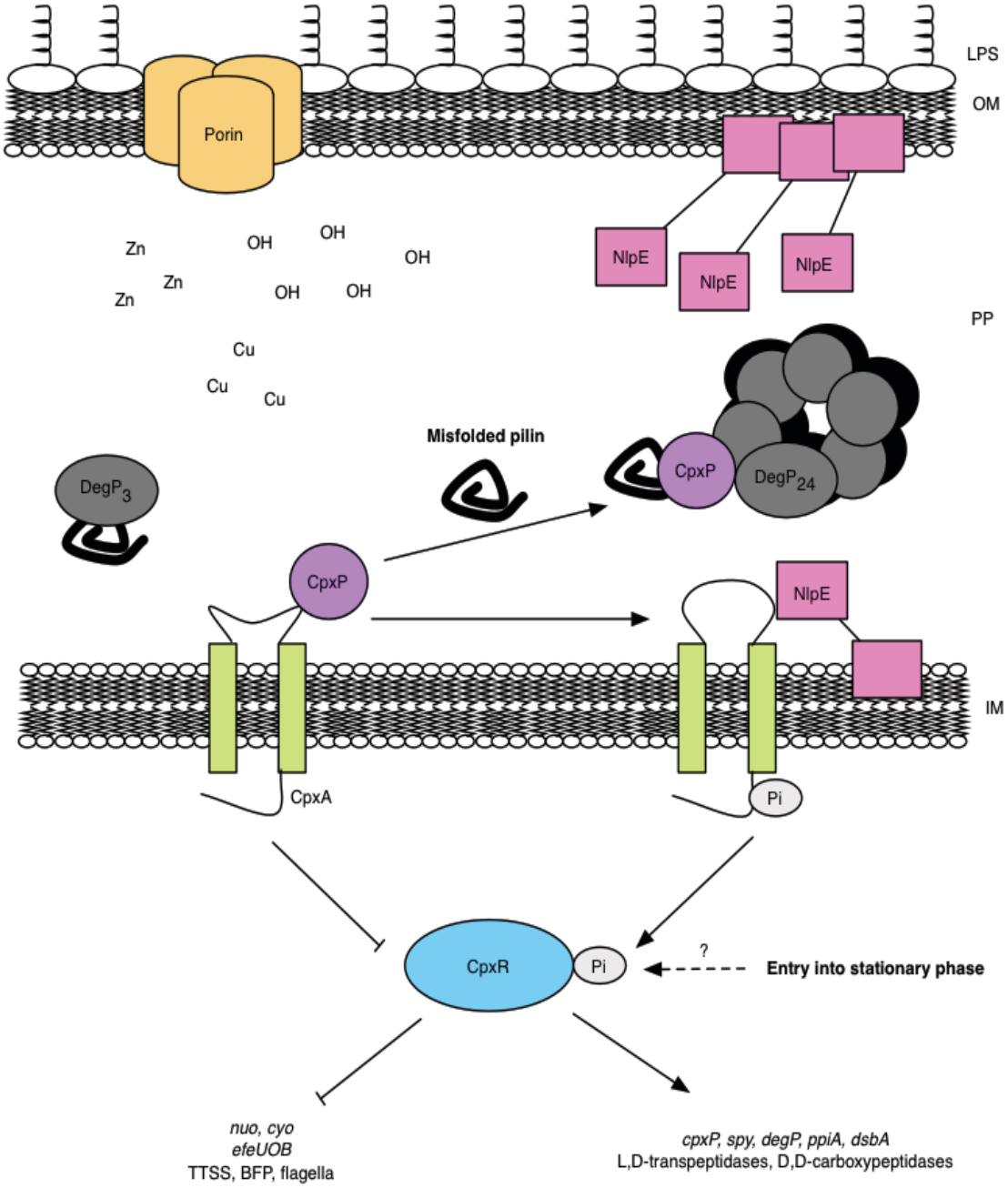

LPS
OM
Porin
Zn
Zn
Zn
OH
OH
OH
OH
OH
NlpE
NlpE
NlpE
PP
Cu
Cu
Cu
Misfolded pilin
DegP3
CpxP
DegP24
CpxP
NlpE
IM
CpxA
Pi
CpxR
Pi
?
Entry into stationary phase
nuo, cyo
efeUOB
TTSS, BFP, flagella
cpxP, spy, degP, ppiA, dsbA
L,D-transpeptidases, D,D-carboxypeptidases

Figure 1-2: The Cpx envelope stress response senses diverse environmental signals and modulates the expression of envelope-localized gene products.

The sensor kinase CpxA senses alkaline pH, over-expression of the lipoprotein nlpE, misfolded pilin subunits, and excess copper and zinc ions. CpxA likely interacts directly with the periplasmic inhibitor CpxP in the absence of stress. When an inducing signal is present, CpxP is titrated from CpxA, perhaps by its chaperone activity, and is degraded by the oligomerized protease-chaperone DegP. In the absence of CpxP, CpxA senses stress, autophosphorylates, and phosphorylates CpxR. Phosphorylated CpxR up-regulates the expression of chaperones, proteases, and cell wall remodelling enzymes while down-regulating the expression of components of the aerobic respiratory chain, iron acquisition systems, and virulence structures. CpxR can also change gene expression in response to growth independently of CpxA through an unknown mechanism. LPS denotes lipopolysaccharide, OM denotes outer membrane, PP denotes periplasm, IM denotes inner membrane.

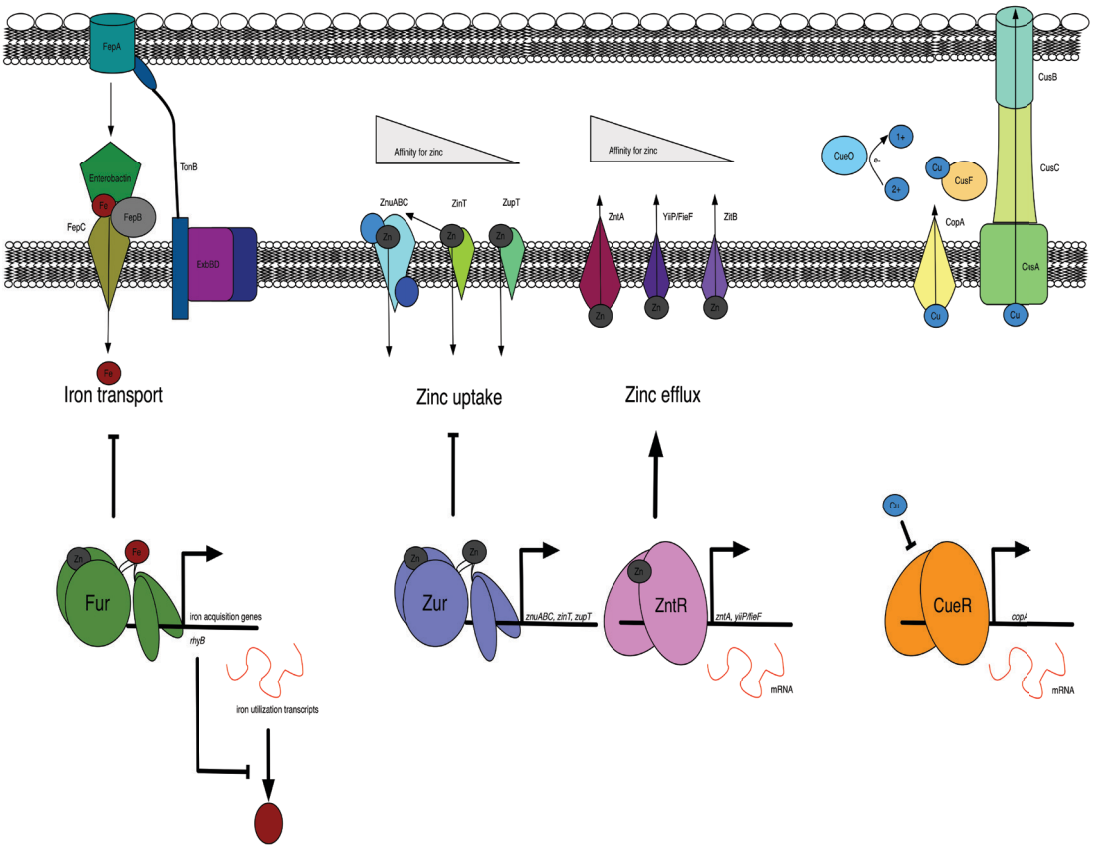


Figure 1-3: The regulators of iron, zinc, and copper homeostasis ensure that essential metals are obtained when needed and toxic excess is removed from the cell.

Fur represses the expression of iron acquisition genes, including the genes required for the synthesis and transport of the siderophore enterobactin. Fur also represses the sRNA rhyB responsible for modulating the stability of transcripts required for iron utilization. Zur and ZntR modulate zinc uptake and efflux, respectively. No outer membrane proteins for zinc export are known in Enterobacteriaceae. CueR regulates the expression of the copper efflux protein CopA while CusR (not shown) regulates the expression of the RND efflux pump CusCBA that also effluxes copper. CueR also controls the expression of the multi-copper oxidase CueO. The periplasmic solute-binding protein CusF takes copper from CopA and shuttles it to CusCBA for export.

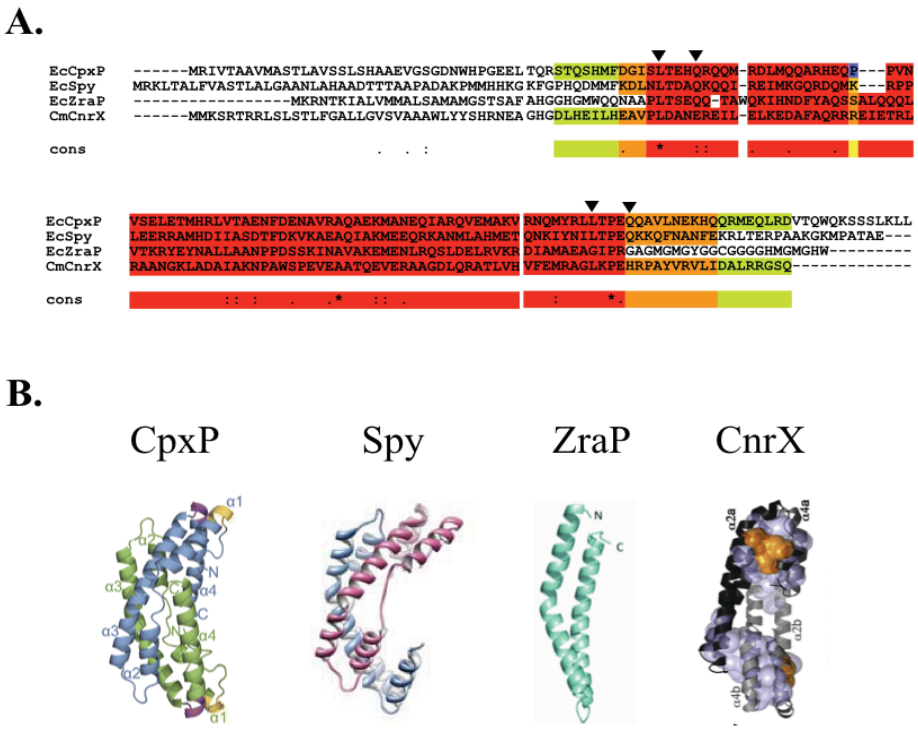


Figure 1-4: CpxP homologues are identified by the conserved LTXXQ motifs and similar α-helical structure.

A. CpxP shares significant sequence similarity only with Spy. Alignment of E. coli (Ec) CpxP, Spy, and ZraP and C. metallidurans (Cm) CnrX, generated using T-coffee. A high degree of sequence similarity is indicated in red and orange while poor conservation is indicated by blue and yellow shading. The L and Q residues of the conserved LTXXQ motifs are marked with black arrowheads. 'cons' denotes degree of conservations, '*' denotes absolutely conserved residues, '.' denotes similar residues, ':' denotes very similary residues. **B**. Cartoon representations of the crystal structures from *E. coli* CpxP (270) and Spy (224), *S. Typhimurium ZraP (7)*, and *C. metallidurans* CnrX (275).

1.8. References

1. **Abramson J., S. Riistama, G. Larsson, A. Jasaitis, M. Svensson-Ek, L. Laakkonen, A. Puustinen, S. Iwata, and M. Wikström.** 2000. The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site. Nature Structural Molecular Biology **7**:910.

2. **Achard M. E. S., J. J. Tree, J. A. Holden, K. R. Simpfendorfer, O. L. C. Wijburg, R. A. Strugnell, M. A. Schembri, M. J. Sweet, M. P. Jennings, and A. G. McEwan.** 2010. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. Infect Immun **78**:2312-2319.

3. **Acosta N., S. Pukatzki, and T. L. Raivio.** 2014. The *Vibrio cholerae* Cpx envelope stress response senses and mediates adaptation to low iron. J Bacteriol . **197(2)**: 226-76.

4. **Ahmer B. M., M. G. Thomas, R. A. Larsen, and K. Postle.** 1995. Characterization of the *exbBD* operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and stability. J Bacteriol **177**:4742-4747.

5. **Anjem A., S. Varghese, and J. A. Imlay.** 2009. Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. Mol Microbiol **72**:844-858.

6. **Anraku Y., and R. B. Gennis.** 1987. The aerobic respiratory chain of *Escherichia coli*. Trends in Biochemical Sciences **12**:262-266.

7. **Appia-Ayme C., A. Hall, E. Patrick, S. Rajadurai, T. A. Clarke, and G. Rowley.** 2012. ZraP is a periplasmic molecular chaperone and a repressor of the zinc-responsive two-component regulator ZraSR. Biochem J **442**:85-93.

8. **Archibald F.** 1986. Manganese: its acquisition by and function in the lactic acid bacteria. Crit Rev Microbiol **13**:63-109.

9. **Bakker D., C. E. Vader, B. Roosendaal, F. R. Mooi, B. Oudega, and F. K. de Graaf.** 1991. Structure and function of periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. Mol Microbiol **5**:875-886.

10. **Baranova N., and H. Nikaido.** 2002. The *baeSR* two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. J Bacteriol **184**:4168-4176.

11. **Bassford P. J., C. Bradbeer, R. J. Kadner, and C. A. Schnaitman.** 1976. Transport of vitamin B12 in *tonB* mutants of *Escherichia coli*. J Bacteriol **128**:242-247.

12. **Bauer B. W., T. Shemesh, Y. Chen, and T. A. Rapoport.** 2014. A "push and slide" mechanism allows sequence-insensitive translocation of secretory proteins by the SecA ATPase. Cell **157**:1416-1429.

13. **Beard S. J., M. N. Hughes, and R. K. Poole.** 1995. Inhibition of the cytochrome bd-terminated NADH oxidase system in *Escherichia coli* K-12 by divalent metal cations. FEMS Microbiol Lett **131**:205-210.

14. **Beard S. J., R. Hashim, J. Membrillo-Hernández, M. N. Hughes, and R. K. Poole.** 1997. Zinc(II) tolerance in *Escherichia coli* K-12: evidence that the *zntA* gene (o732) encodes a cation transport ATPase. Mol Microbiol **25**:883-891.

15. **Becker K. W., and E. P. Skaar.** 2014. Metal limitation and toxicity at the interface between host and pathogen. FEMS Microbiol Rev .

16. **Becker L. A., I. -S. Bang, M. -L. Crouch, and F. C. Fang.** 2005. Compensatory role of PspA, a member of the phage shock protein operon, in *rpoE* mutant Salmonella enterica serovar Typhimurium. Mol Microbiol **56**:1004-1016.

17. **Beckman D. L., D. R. Trawick, and R. G. Kranz.** 1992. Bacterial cytochromes c biogenesis. Genes Dev **6**:268-283.

18. **Boer J. L., S. B. Mulrooney, and R. P. Hausinger.** 2014. Nickel-dependent metalloenzymes. Arch Biochem Biophys **544**:142-152.

19. **Botella H., P. Peyron, F. Levillain, R. Poincloux, Y. Poquet, I. Brandli, C. Wang, L. Tailleux, S. Tilleul, G. M. Charrière, S. J. Waddell, M. Foti, G. Lugo-Villarino, Q. Gao, I. Maridonneau-Parini, P. D. Butcher, P. R. Castagnoli, B. Gicquel, C. de Chastellier, and O. Neyrolles.** 2011. Mycobacterial P(1)-type ATPases mediate resistance to zinc poisoning in human macrophages. Cell Host Microbe **10**:248-259.

20. **Bouchart F., G. Boussemart, A. -F. Prouvost, V. Cogez, E. Madec, O. Vidal, B. Delrue, J. -P. Bohin, and J. -M. Lacroix.** 2010. The virulence of a *Dickeya dadantii* 3937 mutant devoid of osmoregulated periplasmic glucans is restored by inactivation of the RcsCD-RcsB phosphorelay. J Bacteriol **192**:3484-3490.

21. **Breyton C., W. Haase, T. A. Rapoport, W. Kühlbrandt, and I. Collinson.** 2002. Three-dimensional structure of the bacterial protein-translocation complex SecYEG. Nature **418**:662-665.

22. **Brissette J. L., L. Weiner, T. L. Ripmaster, and P. Model.** 1991. Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. J Mol Biol **220**:35-48.

23. **Brocklehurst K. R., J. L. Hobman, B. Lawley, L. Blank, S. J. Marshall, N. L. Brown, and A. P. Morby.** 1999. ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. Mol Microbiol **31**:893-902.

24. **Buelow D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J Bacteriol **187**:6622-6630.

25. **Butcher J., S. Sarvan, J. S. Brunzelle, J. -F. Couture, and A. Stintzi.** 2012. Structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation. Proc Natl Acad Sci U S A **109**:10047-10052.

26. **Büttner F. M., S. Zoll, M. Nega, F. Götz, and T. Stehle.** 2014. Structure-Function Analysis of Staphylococcus aureus Amidase Reveals the Determinants of Peptidoglycan Recognition and Cleavage. J Biol Chem **289**:11083-11094.

27. **Caly D. L., P. W. O'Toole, and S. A. Moore.** 2010. The 2.2-Å structure of the HP0958 protein from *Helicobacter pylori* reveals a kinked anti-parallel coiled-coil hairpin domain and a highly conserved ZN-ribbon domain. J Mol Biol **403**:405-419.

28. **Campoy S., M. Jara, N. Busquets, A. M. Pérez De Rozas, I. Badiola, and J. Barbé.** 2002. Role of the high-affinity zinc uptake *znuABC* system in *Salmonella enterica* serovar Typhimurium virulence. Infect Immun **70**:4721-4725.

29. **Carpenter B. M., J. J. Gilbreath, O. Q. Pich, A. M. McKelvey, E. L. Maynard, Z. -Z. Li, and D. S. Merrell.** 2013. Identification and characterization of novel *Helicobacter pylori* apo-Fur-regulated target genes. J Bacteriol **195**:5526-5539.

30. **Cerasi M., J. Z. Liu, S. Ammendola, A. J. Poe, P. Petrarca, M. Pesciaroli, P. Pasquali, M. Raffatellu, and A. Battistoni.** 2014. The ZupT transporter plays an important role in zinc homeostasis and contributes to *Salmonella enterica* virulence. Metallomics **6**:845-853.

31. **Chaba R., B. M. Alba, M. S. Guo, J. Sohn, N. Ahuja, R. T. Sauer, and C. A. Gross.** 2011. Signal integration by DegS and RseB governs the σ^E-mediated envelope stress response in *Escherichia coli*. Proc Natl Acad Sci U S A **108**:2106-2111.

32. **Chandra B. R., M. Yogavel, and A. Sharma.** 2007. Structural analysis of ABC-family periplasmic zinc binding protein provides new insights into mechanism of ligand uptake and release. J Mol Biol **367**:970-982.

33. **Changela A., K. Chen, Y. Xue, J. Holschen, C. E. Outten, T. V. O'Halloran, and A. Mondragón.** 2003. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science **301**:1383-1387.

34. **Chao Y., and D. Fu.** 2004. Thermodynamic studies of the mechanism of metal binding to the *Escherichia coli* zinc transporter YiiP. J Biol Chem **279**:17173-17180.

35. **Chung K. C. C., and D. B. Zamble.** 2011. The *Escherichia coli* metal-binding chaperone SlyD interacts with the large subunit of [NiFe]-hydrogenase 3. FEBS Lett **585**:291-294.

36. **Connolly L., A. De Las Penas, B. M. Alba, and C. A. Gross.** 1997. The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. Genes Dev **11**:2012-2021.

37. **Coppola D., D. Giordano, M. Tinajero-Trejo, G. di Prisco, P. Ascenzi, R. K. Poole, and C. Verde.** 2013. Antarctic bacterial haemoglobin and its role in the protection against nitrogen reactive species. Biochim Biophys Acta **1834**:1923-1931.

38. **Corbett D., J. Wang, S. Schuler, G. Lopez-Castejon, S. Glenn, D. Brough, P. W. Andrew, J. S. Cavet, and I. S. Roberts.** 2012. Two zinc uptake systems contribute to the full virulence of *Listeria monocytogenes* during growth in vitro and in vivo. Infect Immun **80**:14-21.

39. **Cosma C. L., P. N. Danese, J. H. Carlson, T. J. Silhavy, and W. B. Snyder.** 1995. Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope-associated stresses. Mol Microbiol **18**:491-505.

40. **Costerton J. W., J. M. Ingram, and K. J. Cheng.** 1974. Structure and function of the cell envelope of Gram-negative bacteria. Bacteriol Rev **38**:87-110.

41. **Daly M. J., E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai, A. Venkateswaran, M. Hess, M. V. Omelchenko, H. M. Kostandarithes, K. S. Makarova, L. P. Wackett, J. K. Fredrickson, and D. Ghosal.** 2004. Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science **306**:1025-1028.

42. **Danese P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*. J Bacteriol **180**:5875-5884.

43. **Darwin A. J., and V. L. Miller.** 2001. The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth in vitro when the Ysc type III secretion system is produced. Mol Microbiol **39**:429-444.

44. **David G., K. Blondeau, M. Schiltz, S. Penel, and A. Lewit-Bentley.** 2003. YodA from *Escherichia coli* is a metal-binding, lipocalin-like protein. J Biol Chem **278**:43728-43735.

45. **Davidson V. L., and L. H. Jones.** 1996. Electron transfer from copper to heme within the methylamine dehydrogenase--amicyanin--cytochrome c-551i complex. Biochemistry **35**:8120-8125.

46. **Davies J. K., and P. Reeves.** 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group B. J Bacteriol **123**:96-101.

47. **Debnath I., J. P. Norton, A. E. Barber, E. M. Ott, B. K. Dhakal, R. R. Kulesus, and M. A. Mulvey.** 2013. The Cpx stress response system potentiates the fitness and virulence of uropathogenic *Escherichia coli*. Infect Immun **81**:1450-1459.

48. **DebRoy S., M. Gebbie, A. Ramesh, J. R. Goodson, M. R. Cruz, A. van Hoof, W. C. Winkler, and D. A. Garsin.** 2014. Riboswitches. A riboswitch-containing sRNA controls gene expression by sequestration of a response regulator. Science **345**:937-940.

49. **DeChavigny A., P. N. Heacock, and W. Dowhan.** 1991. Sequence and inactivation of the *pss* gene of *Escherichia coli*. Phosphatidylethanolamine may not be essential for cell viability. J Biol Chem **266**:5323-5332.

50. **Desnoyers G., A. Morissette, K. Prévost, and E. Massé.** 2009. Small RNA-induced differential degradation of the polycistronic mRNA iscRSUA. EMBO J **28**:1551-1561.

51. **DiGiuseppe P. A., and T. J. Silhavy.** 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol **185**:2432-2440.

52. **Donnenberg M. S., J. A. Girón, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol Microbiol **6**:3427-3437.

53. **Douchin V., C. Bohn, and P. Bouloc.** 2006. Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*. J Biol Chem **281**:12253-12259.

54. **Dowd G. C., P. G. Casey, M. Begley, C. Hill, and C. G. M. Gahan.** 2012. Investigation of the role of ZurR in the physiology and pathogenesis of *Listeria monocytogenes*. FEMS Microbiol Lett **327**:118-125.

55. **Ebel W., and J. E. Trempy.** 1999. *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions To activate its own expression. J Bacteriol **181**:577-584.

56. **Fan B., and B. P. Rosen.** 2002. Biochemical characterization of CopA, the *Escherichia coli* Cu(I)-translocating P-type ATPase. J Biol Chem **277**:46987-46992.

57. **Ferrières L., A. Thompson, and D. J. Clarke.** 2009. Elevated levels of sigma S inhibit biofilm formation in *Escherichia coli*: a role for the Rcs phosphorelay. Microbiology **155**:3544-3553.

58. **Flynn J. M., I. Levchenko, R. T. Sauer, and T. A. Baker.** 2004. Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. Genes Dev **18**:2292-2301.

59. **Francis C. L., A. E. Jerse, J. B. Kaper, and S. Falkow.** 1991. Characterization of interactions of enteropathogenic *Escherichia coli* O127:H6 with mammalian cells in vitro. J Infect Dis **164**:693-703.

60. **Franke S., G. Grass, C. Rensing, and D. H. Nies.** 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol **185**:3804-3812.

61. **Fredericks C. E., S. Shibata, S. -I. Aizawa, S. A. Reimann, and A. J. Wolfe.** 2006. Acetyl phosphate-sensitive regulation of flagellar biogenesis and capsular biosynthesis depends on the Rcs phosphorelay. Mol Microbiol **61**:734-747.

62. **Fridén H., and L. Hederstedt.** 1990. Role of His residues in *Bacillus subtilis* cytochrome b558 for haem binding and assembly of succinate: quinone oxidoreductase (complex II). Mol Microbiol **4**:1045-1056.

63. **Gabbianelli R., R. Scotti, S. Ammendola, P. Petrarca, L. Nicolini, and A. Battistoni.** 2011. Role of ZnuABC and ZinT in *Escherichia coli* O157:H7 zinc acquisition and interaction with epithelial cells. BMC Microbiol **11**:36.

64. **García-Calderón C. B., M. García-Quintanilla, J. Casadesús, and F. Ramos-Morales.** 2005. Virulence attenuation in *Salmonella enterica rcsC* mutants with constitutive activation of the Rcs system. Microbiology **151**:579-588.

65. **Gerken H., E. S. Charlson, E. M. Cicirelli, L. J. Kenney, and R. Misra.** 2009. MzrA: a novel modulator of the EnvZ/OmpR two-component regulon. Mol Microbiol **72**:1408-1422.

66. **Gervais F. G., and G. R. Drapeau.** 1992. Identification, cloning, and characterization of *rcsF*, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation *ftsZ84* in *Escherichia coli* K-12. J Bacteriol **174**:8016-8022.

67. **Gold V. A. M., A. Robson, H. Bao, T. Romantsov, F. Duong, and I. Collinson.** 2010. The action of cardiolipin on the bacterial translocon. Proc Natl Acad Sci U S A **107**:10044-10049.

68. **Gotoh Y., Y. Eguchi, T. Watanabe, S. Okamoto, A. Doi, and R. Utsumi.** 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. Curr Opin Microbiol **13**:232-239.

69. **Gottesman S., and V. Stout.** 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. Mol Microbiol **5**:1599-1606.

70. **Gottesman S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J Bacteriol **162**:1111-1119.

71. **Gómez-Duarte O. G., and J. B. Kaper.** 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. Infect Immun **63**:1767-1776.

72. **Graham A. I., G. Sanguinetti, N. Bramall, C. W. McLeod, and R. K. Poole.** 2012. Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of the bacterial response to zinc reveals transient behaviour of the Fur and SoxS regulators. Microbiology **158**:284-292.

73. **Graham A. I., S. Hunt, S. L. Stokes, N. Bramall, J. Bunch, A. G. Cox, C. W. McLeod, and R. K. Poole.** 2009. Severe zinc depletion of *Escherichia coli*: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins. J Biol Chem **284**:18377-18389.

74. **Grass G., B. Fan, B. P. Rosen, S. Franke, D. H. Nies, and C. Rensing.** 2001. ZitB (YbgR), a member of the cation diffusion facilitator family, is an additional zinc transporter in *Escherichia coli*. J Bacteriol **183**:4664-4667.

75. **Grass G., C. Grosse, and D. H. Nies.** 2000. Regulation of the *cnr* cobalt and nickel resistance determinant from *Ralstonia* sp. strain CH34. J Bacteriol **182**:1390-1398.

76. **Grass G., K. Thakali, P. E. Klebba, D. Thieme, A. Müller, G. F. Wildner, and C. Rensing.** 2004. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. J Bacteriol **186**:5826-5833.

77. **Grass G., M. D. Wong, B. P. Rosen, R. L. Smith, and C. Rensing.** 2002. ZupT is a Zn(II) uptake system in *Escherichia coli*. J Bacteriol **184**:864-866.

78. **Grass G., S. Franke, N. Taudte, D. H. Nies, L. M. Kucharski, M. E. Maguire, and C. Rensing.** 2005. The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. J Bacteriol **187**:1604-1611.

79. **Green J., and J. R. Guest.** 1994. Regulation of transcription at the *ndh* promoter of *Escherichia coli* by FNR and novel factors. Mol Microbiol **12**:433-444.

80. **Green R. C., and A. J. Darwin.** 2004. PspG, a new member of the *Yersinia enterocolitica* phage shock protein regulon. J Bacteriol **186**:4910-4920.

81. **Grishin N. V.** 2000. C-terminal domains of *Escherichia coli* topoisomerase I belong to the zinc-ribbon superfamily. J Mol Biol **299**:1165-1177.

82. **Groisman E. A., C. Parra-Lopez, M. Salcedo, C. J. Lipps, and F. Heffron.** 1992. Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. Proc Natl Acad Sci U S A **89**:11939-11943.

83. **Guo M. S., T. B. Updegrove, E. B. Gogol, S. A. Shabalina, C. A. Gross, and G. Storz.** 2014. MicL, a new σE-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein. Genes Dev **28**:1620-1634.

84. **Gupta S., J. Chai, J. Cheng, R. D'Mello, M. R. Chance, and D. Fu.** 2014. Visualizing the kinetic power stroke that drives proton-coupled zinc(II) transport. Nature.

85. **Haag L. M., A. Fischer, B. Otto, R. Plickert, A. A. Kühl, U. B. Göbel, S. Bereswill, and M. M. Heimesaat.** 2012. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. PLoS One **7**:e35988.

86. **Halliwell B., and J. M. Gutteridge.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J **219**:1-14.

87. **Hantke K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol Gen Genet **182**:288-292.

88. **Haurat M. F., J. Aduse-Opoku, M. Rangarajan, L. Dorobantu, M. R. Gray, M. A. Curtis, and M. F. Feldman.** 2011. Selective sorting of cargo proteins into bacterial membrane vesicles. J Biol Chem **286**:1269-1276.

89. **Härd T., A. Rak, P. Allard, L. Kloo, and M. Garber.** 2000. The solution structure of ribosomal protein L36 from *Thermus thermophilus* reveals a zinc-ribbon-like fold. J Mol Biol **296**:169-180.

90. **Heddle J., D. J. Scott, S. Unzai, S. -Y. Park, and J. R. H. Tame.** 2003. Crystal structures of the liganded and unliganded nickel-binding protein NikA from *Escherichia coli*. J Biol Chem **278**:50322-50329.

91. **Helander I. M., Y. Kato, I. Kilpeläinen, R. Kostiainen, B. Lindner, K. Nummila, T. Sugiyama, and T. Yokochi.** 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. Eur J Biochem **237**:272-278.

92. **Higgins C. F., I. D. Hiles, K. Whalley, and D. J. Jamieson.** 1985. Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent transport systems. EMBO J **4**:1033-1039.

93. **Hirano Y., M. M. Hossain, K. Takeda, H. Tokuda, and K. Miki.** 2007. Structural studies of the Cpx pathway activator NlpE on the outer membrane of *Escherichia coli*. Structure **15**:963-976.

94. **Hoch E., W. Lin, J. Chai, M. Hershfinkel, D. Fu, and I. Sekler.** 2012. Histidine pairing at the metal transport site of mammalian ZnT transporters controls Zn^{2+} over Cd^{2+} selectivity. Proc Natl Acad Sci U S A **109**:7202-7207.

95. **Hoyland C. N., C. Aldridge, R. M. Cleverley, M. -C. Duchêne, G. Minasov, O. Onopriyenko, K. Sidiq, P. J. Stogios, W. F. Anderson, R. A. Daniel, A. Savchenko, W.**

Vollmer, and R. J. Lewis. 2014. Structure of the LdcB LD-carboxypeptidase reveals the molecular basis of peptidoglycan recognition. Structure **22**:949-960.

96. **Huang J. M., Y. F. Wei, Y. H. Kim, L. Osterberg, and H. R. Matthews.** 1991. Purification of a protein histidine kinase from the yeast *Saccharomyces cerevisiae*. The first member of this class of protein kinases. J Biol Chem **266**:9023-9031.

97. **Huja S., Y. Oren, D. Biran, S. Meyer, U. Dobrindt, J. Bernhard, D. Becher, M. Hecker, R. Sorek, and E. Z. Ron.** 2014. Fur is the master regulator of the extraintestinal pathogenic *Escherichia coli* response to serum. MBio **5**.

98. **Humphreys S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts.** 1999. The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella typhimurium*. Infect Immun **67**:1560-1568.

99. **Humphries R. M., T. P. Griener, S. L. Vogt, G. L. Mulvey, T. Raivio, M. S. Donnenberg, P. I. Kitov, M. Surette, and G. D. Armstrong.** 2010. N-acetyllactosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic *Escherichia coli*. Mol Microbiol **76**:1111-1126.

100. **Ilari A., F. Alaleona, G. Tria, P. Petrarca, A. Battistoni, C. Zamparelli, D. Verzili, M. Falconi, and E. Chiancone.** 2014. The Salmonella enterica ZinT structure, zinc affinity and interaction with the high-affinity uptake protein ZnuA provide insight into the management of periplasmic zinc. Biochim Biophys Acta **1840**:535-544.

101. **Imlay J. A.** 2006. Iron-sulphur clusters and the problem with oxygen. Mol Microbiol **59**:1073-1082.

102. **Isaac D. D., J. S. Pinkner, S. J. Hultgren, and T. J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc Natl Acad Sci U S A **102**:17775-17779.

103. **Iwig J. S., J. L. Rowe, and P. T. Chivers.** 2006. Nickel homeostasis in *Escherichia coli* - the *rcnR-rcnA* efflux pathway and its linkage to NikR function. Mol Microbiol **62**:252-262.

104. **Jacques J. -F., S. Jang, K. Prévost, G. Desnoyers, M. Desmarais, J. Imlay, and E. Massé.** 2006. RyhB small RNA modulates the free intracellular iron pool and is essential for normal growth during iron limitation in *Escherichia coli*. Mol Microbiol **62**:1181-1190.

105. **Janssen P. J., R. Van Houdt, H. Moors, P. Monsieurs, N. Morin, A. Michaux, M. A. Benotmane, N. Leys, T. Vallaeys, A. Lapidus, S. Monchy, C. Médigue, S. Taghavi, S. McCorkle, J. Dunn, D. van der Lelie, and M. Mergeay.** 2010. The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. PLoS One **5**:e10433.

106. **Jiang J., X. Zhang, Y. Chen, Y. Wu, Z. H. Zhou, Z. Chang, and S. -F. Sui.** 2008. Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. Proc Natl Acad Sci U S A **105**:11939-11944.

107. **Jin T., and M. Inouye.** 1993. Ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the signaling domain of the hybrid *Escherichia coli* transmembrane receptor, Taz1. J Mol Biol **232**:484-492.

108. **Jones S. E., L. J. Lloyd, K. K. Tan, and M. Buck.** 2003. Secretion defects that activate the phage shock response of *Escherichia coli*. J Bacteriol **185**:6707-6711.

109. **Jordan I., D. A. Natale, and M. Y. Galperin.** 2000. Copper chaperones in bacteria: association with copper-transporting ATPases. Trends in Biochemical Sciences **25**:480-481.

110. **Jovanovic G., L. Weiner, and P. Model.** 1996. Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress-induced psp operon. J Bacteriol **178**:1936-1945.

111. **Jovanovic G., P. Mehta, L. Ying, and M. Buck.** 2014. Anionic lipids and the cytoskeletal proteins MreB and RodZ define the spatio-temporal distribution and function of membrane stress controller PspA in *Escherichia coli*. Microbiology **160**:2374-2386.

112. **Jubelin G., A. Vianney, C. Beloin, J. -M. Ghigo, J. -C. Lazzaroni, P. Lejeune, and C. Dorel.** 2005. CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. J Bacteriol **187**:2038-2049.

113. **Kadurugamuwa J. L., and T. J. Beveridge.** 1996. Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. J Bacteriol **178**:2767-2774.

114. **Karlinsey J. E., M. E. Maguire, L. A. Becker, M. -L. V. Crouch, and F. C. Fang.** 2010. The phage shock protein PspA facilitates divalent metal transport and is required for virulence of *Salmonella enterica* sv. Typhimurium. Mol Microbiol **78**:669-685.

115. **Karlsson M., K. Hannavy, and C. F. Higgins.** 1993. ExbB acts as a chaperone-like protein to stabilize TonB in the cytoplasm. Mol Microbiol **8**:389-396.

116. **Karsisiotis A. I., C. F. Damblon, and G. C. K. Roberts.** 2014. A variety of roles for versatile zinc in metallo-β-lactamases. Metallomics **6**:1181-1197.

117. **Kawahara K., H. Tsukano, H. Watanabe, B. Lindner, and M. Matsuura.** 2002. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. Infect Immun **70**:4092-4098.

118. **Keele B. B., J. M. McCord, and I. Fridovich.** 1970. Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. J Biol Chem **245**:6176-6181.

119. **Kershaw C. J., N. L. Brown, and J. L. Hobman.** 2007. Zinc dependence of zinT (*yodA*) mutants and binding of zinc, cadmium and mercury by ZinT. Biochem Biophys Res Commun **364**:66-71.

120. **Kershaw C. J., N. L. Brown, C. Constantinidou, M. D. Patel, and J. L. Hobman.** 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. Microbiology **151**:1187-1198.

121. **Kesty N. C., and M. J. Kuehn.** 2004. Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles. J Biol Chem **279**:2069-2076.

122. **Kim C., W. W. Lorenz, J. T. Hoopes, and J. F. Dean.** 2001. Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J Bacteriol **183**:4866-4875.

123. **Kim S., K. Watanabe, T. Shirahata, and M. Watarai.** 2004. Zinc uptake system (*znuA* locus) of *Brucella abortus* is essential for intracellular survival and virulence in mice. J Vet Med Sci **66**:1059-1063.

124. **Kirsten A., M. Herzberg, A. Voigt, J. Seravalli, G. Grass, J. Scherer, and D. H. Nies.** 2011. Contributions of five secondary metal uptake systems to metal homeostasis of *Cupriavidus metallidurans* CH34. J Bacteriol **193**:4652-4663.

125. **Klein G., B. Lindner, W. Brabetz, H. Brade, and S. Raina.** 2009. *Escherichia coli* K-12 Suppressor-free Mutants Lacking Early Glycosyltransferases and Late Acyltransferases: minimal

lipopolysaccharide structure and induction of envelope stress response. J Biol Chem **284**:15369-15389.

126. **Komeda H., M. Kobayashi, and S. Shimizu.** 1997. A novel transporter involved in cobalt uptake. Proc Natl Acad Sci U S A **94**:36-41.

127. **Koretke K. K., A. N. Lupas, P. V. Warren, M. Rosenberg, and J. R. Brown.** 2000. Evolution of two-component signal transduction. Mol Biol Evol **17**:1956-1970.

128. **Krojer T., J. Sawa, E. Schäfer, H. R. Saibil, M. Ehrmann, and T. Clausen.** 2008. Structural basis for the regulated protease and chaperone function of DegP. Nature **453**:885-890.

129. **Kumar A., S. Kumar, D. Kumar, A. Mishra, R. P. Dewangan, P. Shrivastava, S. Ramachandran, and B. Taneja.** 2013. The structure of Rv3717 reveals a novel amidase from *Mycobacterium tuberculosis*. Acta Crystallogr D Biol Crystallogr **69**:2543-2554.

130. **Kumazaki K., S. Chiba, M. Takemoto, A. Furukawa, K. -I. Nishiyama, Y. Sugano, T. Mori, N. Dohmae, K. Hirata, Y. Nakada-Nakura, A. D. Maturana, Y. Tanaka, H. Mori, Y. Sugita, F. Arisaka, K. Ito, R. Ishitani, T. Tsukazaki, and O. Nureki.** 2014. Structural basis of Sec-independent membrane protein insertion by YidC. Nature **509**:516-520.

131. **Lanata C. F., C. L. Fischer-Walker, A. C. Olascoaga, C. X. Torres, M. J. Aryee, R. E. Black, and Child Health Epidemiology Reference Group of the World Health Organization and UNICEF.** 2013. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. PLoS One **8**:e72788.

132. **Larsen R. A., M. G. Thomas, and K. Postle.** 1999. Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol Microbiol **31**:1809-1824.

133. **De Las Peñas A., L. Connolly, and C. A. Gross.** 1997. The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. Mol Microbiol **24**:373-385.

134. **Laubacher M. E., and S. E. Ades.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J Bacteriol **190**:2065-2074.

135. **Leblanc S. K. D., C. W. Oates, and T. L. Raivio.** 2011. Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli.* J Bacteriol **193**:3367-3375.

136. **Lee J. -H., W. -S. Yeo, and J. -H. Roe.** 2004. Induction of the *sufA* operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: involvement of OxyR, IHF and an unidentified oxidant-responsive factor. Mol Microbiol **51**:1745-1755.

137. **Lee L. J., J. A. Barrett, and R. K. Poole.** 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J Bacteriol **187**:1124-1134.

138. **Lehninger A., Nelson, D. L., and M. M. Cox.** 2008. Lehninger Principles Of Biochemistry. W. H. Freeman and Sons. New York, New York.

139. **Leimbach A., J. Hacker, and U. Dobrindt.** 2013. *E. coli* as an all-rounder: the thin line between commensalism and pathogenicity. Curr Top Microbiol Immunol **358**:3-32.

140. **Leonhartsberger S., A. Huber, F. Lottspeich, and A. Böck.** 2001. The *hydH/G* Genes from *Escherichia coli* code for a zinc and lead responsive two-component regulatory system. J Mol Biol **307**:93-105.

141. **Lessard I. A., and C. T. Walsh.** 1999. Mutational analysis of active-site residues of the enterococcal D-ala-D-Ala dipeptidase VanX and comparison with *Escherichia coli* D-ala-D-Ala ligase and D-ala-D-Ala carboxypeptidase VanY. Chem Biol **6**:177-187.

142. **Levine M., D. Nalin, R. Hornick, E. Bergquist, D. Waterman, C. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. The Lancet **311**:1119-1122.

143. **Levine M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J Infect Dis **152**:550-559.

144. **Lewis C., H. Skovierova, G. Rowley, B. Rezuchova, D. Homerova, A. Stevenson, A. Sherry, J. Kormanec, and M. Roberts.** 2008. Small outer-membrane lipoprotein, SmpA, is regulated by sigmaE and has a role in cell envelope integrity and virulence of *Salmonella enterica* serovar Typhimurium. Microbiology **154**:979-988.

145. **Lewis D. A., J. Klesney-Tait, S. R. Lumbley, C. K. Ward, J. L. Latimer, C. A. Ison, and E. J. Hansen.** 1999. Identification of the *znuA*-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. Infect Immun **67**:5060-5068.

146. **Lima S., M. S. Guo, R. Chaba, C. A. Gross, and R. T. Sauer.** 2013. Dual molecular signals mediate the bacterial response to outer-membrane stress. Science **340**:837-841.

147. **Lin H., M. A. Fischbach, D. R. Liu, and C. T. Walsh.** 2005. In vitro characterization of salmochelin and enterobactin trilactone hydrolases IroD, IroE, and Fes. J Am Chem Soc **127**:11075-11084.

148. **Li W., Y. Liu, X. Sheng, P. Yin, F. Hu, Y. Liu, C. Chen, Q. Li, C. Yan, and J. Wang.** 2014. Structure and mechanism of a type III secretion protease, NleC. Acta Crystallogr D Biol Crystallogr **70**:40-47.

149. **Lloyd L. J., S. E. Jones, G. Jovanovic, P. Gyaneshwar, M. D. Rolfe, A. Thompson, J. C. Hinton, and M. Buck.** 2004. Identification of a new member of the phage shock protein response in *Escherichia coli*, the phage shock protein G (PspG). J Biol Chem **279**:55707-55714.

150. **Lucarelli D., S. Russo, E. Garman, A. Milano, W. Meyer-Klaucke, and E. Pohl.** 2007. Crystal structure and function of the zinc uptake regulator FurB from *Mycobacterium tuberculosis*. J Biol Chem **282**:9914-9922.

151. **Lukat G. S., B. H. Lee, J. M. Mottonen, A. M. Stock, and J. B. Stock.** 1991. Roles of the highly conserved aspartate and lysine residues in the response regulator of bacterial chemotaxis. J Biol Chem **266**:8348-8354.

152. **Lu M., and D. Fu.** 2007. Structure of the zinc transporter YiiP. Science **317**:1746-1748.

153. **Lu M., J. Chai, and D. Fu.** 2009. Structural basis for autoregulation of the zinc transporter YiiP. Nat Struct Mol Biol **16**:1063-1067.

154. **Maciąg A., E. Dainese, G. M. Rodriguez, A. Milano, R. Provvedi, M. R. Pasca, I. Smith, G. Palù, G. Riccardi, and R. Manganelli.** 2007. Global Analysis of the Mycobacterium tuberculosis Zur (FurB) Regulon. J Bacteriol **189**:730-740.

155. **Majdalani N., and S. Gottesman.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu Rev Microbiol **59**:379-405.

156. **Makui H., E. Roig, S. T. Cole, J. D. Helmann, P. Gros, and M. F. Cellier.** 2000. Identification of the *Escherichia coli* K-12 Nramp orthologue (MntH) as a selective divalent metal ion transporter. Mol Microbiol **35**:1065-1078.

157. **Manganelli R., M. I. Voskuil, G. K. Schoolnik, and I. Smith.** 2001. The *Mycobacterium tuberculosis* ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol Microbiol **41**:423-437.

158. **Maret W.** 2004. Zinc and sulfur: a critical biological partnership. Biochemistry **43**:3301-3309.

159. **Martino L., Y. He, K. L. D. Hands-Taylor, E. R. Valentine, G. Kelly, C. Giancola, and M. R. Conte.** 2009. The interaction of the *Escherichia coli* protein SlyD with nickel ions illuminates the mechanism of regulation of its peptidyl-prolyl isomerase activity. FEBS J **276**:4529-4544.

160. **Massé E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A **99**:4620-4625.

161. **Massé E., C. K. Vanderpool, and S. Gottesman.** 2005. Effect of RyhB Small RNA on Global Iron Use in *Escherichia coli*. J Bacteriol **187**:6962-6971.

162. **Massé E., F. E. Escorcia, and S. Gottesman.** 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes Dev **17**:2374-2383.

163. **Matsushita K., T. Ohnishi, and H. R. Kaback.** 1987. NADH-ubiquinone oxidoreductases of the *Escherichia coli* aerobic respiratory chain. Biochemistry **26**:7732-7737.

164. **Matsuyama S., T. Tajima, and H. Tokuda.** 1995. A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. EMBO J **14**:3365-3372.

165. **Maxson M. E., and A. J. Darwin.** 2004. Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the RpoE and Cpx extracytoplasmic stress responses. J Bacteriol **186**:4199-4208.

166. **Maxson M. E., and A. J. Darwin.** 2006. PspB and PspC of *Yersinia enterocolitica* are dual function proteins: regulators and effectors of the phage-shock-protein response. Mol Microbiol **59**:1610-1623.

167. **McBroom A. J., A. P. Johnson, S. Vemulapalli, and M. J. Kuehn.** 2006. Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. J Bacteriol **188**:5385-5392.

168. **McCaig W. D., A. Koller, and D. G. Thanassi.** 2013. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. J Bacteriol **195**:1120-1132.

169. **McDevitt C. A., A. D. Ogunniyi, E. Valkov, M. C. Lawrence, B. Kobe, A. G. McEwan, and J. C. Paton.** 2011. A molecular mechanism for bacterial susceptibility to zinc. PLoS Pathog **7**:e1002357.

170. **McEwen J., and P. Silverman.** 1980. Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions. Proc Natl Acad Sci U S A **77**:513-517.

171. **McMahon K. J., M. E. Castelli, E. García Vescovi, and M. F. Feldman.** 2012. Biogenesis of outer membrane vesicles in *Serratia marcescens* is thermoregulated and can be induced by activation of the Rcs phosphorelay system. J Bacteriol **194**:3241-3249.

172. **Mellies J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper.** 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Mol Microbiol **33**:296-306.

173. **Mellin J. R., M. Koutero, D. Dar, M. -A. Nahori, R. Sorek, and P. Cossart.** 2014. Riboswitches. Sequestration of a two-component response regulator by a riboswitch-regulated noncoding RNA. Science **345**:940-943.

174. **Miles S., B. M. Carpenter, H. Gancz, and D. S. Merrell.** 2010. *Helicobacter pylori* apo-Fur regulation appears unconserved across species. J Microbiol **48**:378-386.

175. **Mileykovskaya E., and W. Dowhan.** 1997. The Cpx two-component signal transduction pathway is activated in *Escherichia coli* mutant strains lacking phosphatidylethanolamine. J Bacteriol **179**:1029-1034.

176. **Missiakas D., M. P. Mayer, M. Lemaire, C. Georgopoulos, and S. Raina.** 1997. Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. Mol Microbiol **24**:355-371.

177. **Mogi T., H. Nakamura, and Y. Anraku.** 1994. Molecular structure of a heme-copper redox center of the *Escherichia coli* ubiquinol oxidase: evidence and model. J Biochem **116**:471-477.

178. **Moschen A. R., V. Wieser, and H. Tilg.** 2012. Dietary Factors: Major Regulators of the Gut's Microbiota. Gut Liver **6**:411-416.

179. **Munson G. P., D. L. Lam, F. W. Outten, and T. V. O'Halloran.** 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J Bacteriol **182**:5864-5871.

180. **Mühlenhoff U., and R. Lill.** 2000. Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. Biochim Biophys Acta **1459**:370-382.

181. **Nakayama S., and H. Watanabe.** 1995. Involvement of *cpxA*, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of *Shigella sonnei virF* gene. J Bacteriol **177**:5062-5069.

182. **Nanninga N.** 1970. Ultrastructure of the cell envelope of *Escherichia coli* B after freeze-etching. J Bacteriol **101**:297-303.

183. **Navarro C., L. F. Wu, and M. A. Mandrand-Berthelot.** 1993. The *nik* operon of *Escherichia coli* encodes a periplasmic binding-protein-dependent transport system for nickel. Mol Microbiol **9**:1181-1191.

184. **Nevesinjac A. Z., and T. L. Raivio.** 2005. The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic *Escherichia coli*. J Bacteriol **187**:672-686.

185. **Nicholas D. J., Kobayashi, M., and P. W. Wilson.** 1962. Cobalt requirement for inorganic nitrogen metabolism in microorganisms. Proc Natl Acad Sci U S A **48**:1537-1542.

186. **Nies D. H.** 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev **27**:313-339.

187. **Nishino K., T. Honda, and A. Yamaguchi.** 2005. Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system. J Bacteriol **187**:1763-1772.

188. **Nolan K. J., D. J. McGee, H. M. Mitchell, T. Kolesnikow, J. M. Harro, J. O'Rourke, J. E. Wilson, S. J. Danon, N. D. Moss, H. L. T. Mobley, and A. Lee.** 2002. In vivo behavior of a *Helicobacter pylori* SS1 nixA mutant with reduced urease activity. Infect Immun **70**:685-691.

189. **Noll M., K. Petrukhin, and S. Lutsenko.** 1998. Identification of a novel transcription regulator from Proteus mirabilis, PMTR, revealed a possible role of YJAI protein in balancing zinc in *Escherichia coli*. J Biol Chem **273**:21393-21401.

190. **Odermatt A., H. Suter, R. Krapf, and M. Solioz.** 1993. Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. J Biol Chem **268**:12775-12779.

191. **Ogunniyi A. D., L. K. Mahdi, M. P. Jennings, A. G. McEwan, C. A. McDevitt, M. B. Van der Hoek, C. J. Bagley, P. Hoffmann, K. A. Gould, and J. C. Paton.** 2010. Central role

of manganese in regulation of stress responses, physiology, and metabolism in *Streptococcus pneumoniae*. J Bacteriol **192**:4489-4497.

192. **Ollagnier-de-Choudens S., T. Mattioli, Y. Takahashi, and M. Fontecave.** 2001. Iron-Sulfur Cluster Assembly. J Biol Chem **276**:22604-22607.

193. **Onufryk C., M. -L. Crouch, F. C. Fang, and C. A. Gross.** 2005. Characterization of six lipoproteins in the sigmaE regulon. J Bacteriol **187**:4552-4561.

194. **Otto K., and T. J. Silhavy.** 2002. Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. Proc Natl Acad Sci U S A **99**:2287-2292.

195. **Ouellet H., Y. Ouellet, C. Richard, M. Labarre, B. Wittenberg, J. Wittenberg, and M. Guertin.** 2002. Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. Proc Natl Acad Sci U S A **99**:5902-5907.

196. **Outten C. E., F. W. Outten, and T. V. O'Halloran.** 1999. DNA Distortion Mechanism for Transcriptional Activation by ZntR, a Zn(II)-responsive MerR Homologue in *Escherichia coli*. J Biol Chem **274**:37517-37524.

197. **Outten F. W., D. L. Huffman, J. A. Hale, and T. V. O'Halloran.** 2001. The Independent *cue* and *cus* Systems Confer Copper Tolerance during Aerobic and Anaerobic Growth in *Escherichia coli*. J Biol Chem **276**:30670-30677.

198. **Outten F. W., O. Djaman, and G. Storz.** 2004. A *suf* operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. Mol Microbiol **52**:861-872.

199. **Padilla-Benavides T., A. M. George Thompson, M. M. McEvoy, and J. M. Argüello.** 2014. Mechanism of ATPase-mediated Cu+ Export and Delivery to Periplasmic Chaperones: The interaction of *Escherichia coli* CopA AND CusF. J Biol Chem **289**:20492-20501.

200. **Panina E. M., A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc Natl Acad Sci U S A **100**:9912-9917.

201. **Pasquali P., S. Ammendola, C. Pistoia, P. Petrucci, M. Tarantino, C. Valente, M. L. Marenzoni, G. Rotilio, and A. Battistoni.** 2008. Attenuated *Salmonella enterica* serovar Typhimurium lacking the ZnuABC transporter confers immune-based protection against challenge infections in mice. Vaccine **26**:3421-3426.

202. **Patzer S. I., and K. Hantke.** 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol Microbiol **28**:1199-1210.

203. **Patzer S. I., and K. Hantke.** 2000. The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. J Biol Chem **275**:24321-24332.

204. **Patzer S. I., and K. Hantke.** 2001. Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the *mntH* gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. J Bacteriol **183**:4806-4813.

205. **Pawlik M. -C., K. Hubert, B. Joseph, H. Claus, C. Schoen, and U. Vogel.** 2012. The zinc-responsive regulon of *Neisseria meningitidis* comprises 17 genes under control of a Zur element. J Bacteriol **194**:6594-6603.

206. **Pecqueur L., B. D'Autréaux, J. Dupuy, Y. Nicolet, L. Jacquamet, B. Brutscher, I. Michaud-Soret, and B. Bersch.** 2006. Structural changes of *Escherichia coli* ferric uptake regulator during metal-dependent dimerization and activation explored by NMR and X-ray crystallography. J Biol Chem **281**:21286-21295.

207. **Pennartz A., C. Généreux, C. Parquet, D. Mengin-Lecreulx, and B. Joris.** 2009. Substrate-induced inactivation of the *Escherichia coli* AmiD N-acetylmuramoyl-L-alanine amidase highlights a new strategy to inhibit this class of enzyme. Antimicrob Agents Chemother **53**:2991-2997.

208. **Perrin C., R. Briandet, G. Jubelin, P. Lejeune, M. -A. Mandrand-Berthelot, A. Rodrigue, and C. Dorel.** 2009. Nickel promotes biofilm formation by *Escherichia coli* K-12 strains that produce curli. Appl Environ Microbiol **75**:1723-1733.

209. **Pesciaroli M., F. Aloisio, S. Ammendola, C. Pistoia, P. Petrucci, M. Tarantino, M. Francia, A. Battistoni, and P. Pasquali.** 2011. An attenuated *Salmonella enterica* serovar Typhimurium strain lacking the ZnuABC transporter induces protection in a mouse intestinal model of *Salmonella* infection. Vaccine **29**:1783-1790.

210. **Petersen C., and L. B. Møller.** 2000. Control of copper homeostasis in *Escherichia coli* by a P-type ATPase, CopA, and a MerR-like transcriptional activator, CopR. Gene **261**:289-298.

211. **Petrarca P., S. Ammendola, P. Pasquali, and A. Battistoni.** 2010. The Zur-regulated ZinT protein is an auxiliary component of the high-affinity ZnuABC zinc transporter that facilitates metal recruitment during severe zinc shortage. J Bacteriol **192**:1553-1564.

212. **Piddock L. J. V.** 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol **4**:629-636.

213. **Pierce J. R., and C. F. Earhart.** 1986. *Escherichia coli* K-12 envelope proteins specifically required for ferrienterobactin uptake. J Bacteriol **166**:930-936.

214. **De Pina K., V. Desjardin, M. A. Mandrand-Berthelot, G. Giordano, and L. F. Wu.** 1999. Isolation and characterization of the *nikR* gene encoding a nickel-responsive regulator in *Escherichia coli*. J Bacteriol **181**:670-674.

215. **Pohl E., J. C. Haller, A. Mijovilovich, W. Meyer-Klaucke, E. Garman, and M. L. Vasil.** 2003. Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol Microbiol **47**:903-915.

216. **Pollack J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem Biophys Res Commun **38**:989-992.

217. **Pontel L. B., N. L. Scampoli, S. Porwollik, S. K. Checa, M. McClelland, and F. C. Soncini.** 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**:1659-1669.

218. **Posey J. E., and F. C. Gherardini.** 2000. Lack of a role for iron in the Lyme disease pathogen. Science **288**:1651-1653.

219. **Prasad A. S.** 2007. Zinc: mechanisms of host defense. J Nutr **137**:1345-1349.

220. **Price C. E., and A. J. Driessen.** 2010. Biogenesis of membrane bound respiratory complexes in *Escherichia coli*. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1803**:748-766.

221. **Price N. L., and T. L. Raivio.** 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol **191**:1798-1815.

222. **Prigent-Combaret C., E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, and C. Dorel.** 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. J Bacteriol **183**:7213-7223.

223. **Quan S., L. Wang, E. V. Petrotchenko, K. A. Makepeace, S. Horowitz, J. Yang, Y. Zhang, C. H. Borchers, and J. C. Bardwell.** 2014. Super Spy variants implicate flexibility in chaperone action. Elife **3**:e01584.

224. **Quan S., P. Koldewey, T. Tapley, N. Kirsch, K. M. Ruane, J. Pfizenmaier, R. Shi, S. Hofmann, L. Foit, G. Ren, U. Jakob, Z. Xu, M. Cygler, and J. C. A. Bardwell.** 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat Struct Mol Biol **18**:262-269.

225. **Raffa R. G., and T. L. Raivio.** 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol Microbiol **45**:1599-1611.

226. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

227. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

228. **Raivio T. L., M. W. Laird, J. C. Joly, and T. J. Silhavy.** 2000. Tethering of CpxP to the inner membrane prevents spheroplast induction of the Cpx envelope stress response. Mol Microbiol **37**:1186-1197.

229. **Raivio T. L., S. K. D. Leblanc, and N. L. Price.** 2013. The Escherichia coli Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. J Bacteriol **195**:2755-2767.

230. **Rensing C., B. Fan, R. Sharma, B. Mitra, and B. P. Rosen.** 2000. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci U S A **97**:652-656.

231. **Rensing C., B. Mitra, and B. P. Rosen.** 1997. The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. Proc Natl Acad Sci U S A **94**:14326-14331.

232. **Rigel N. W., and T. J. Silhavy.** 2012. Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria. Curr Opin Microbiol **15**:189-193.

233. **Roberts S. A., A. Weichsel, G. Grass, K. Thakali, J. T. Hazzard, G. Tollin, C. Rensing, and W. R. Montfort.** 2002. Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. Proc Natl Acad Sci U S A **99**:2766-2771.

234. **Rodrigue A., G. Effantin, and M. -A. Mandrand-Berthelot.** 2005. Identification of *rcnA* (*yohM*), a nickel and cobalt resistance gene in *Escherichia coli*. J Bacteriol **187**:2912-2916.

235. **Rosner J. L., and R. G. Martin.** 2013. Reduction of cellular stress by TolC-dependent efflux pumps in *Escherichia coli* indicated by BaeSR and CpxARP activation of *spy* in efflux mutants. J Bacteriol **195**:1042-1050.

236. **Rouvière P. E., and C. A. Gross.** 1996. SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. Genes Dev **10**:3170-3182.

237. **Rowland J. L., and M. Niederweis.** 2012. Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload. Tuberculosis (Edinburgh) **92**:202-210.

238. **Rowland J. L., and M. Niederweis.** 2013. A multicopper oxidase is required for copper resistance in *Mycobacterium tuberculosis*. J Bacteriol **195**:3724-3733.

239. **Scherer J., and D. H. Nies.** 2009. CzcP is a novel efflux system contributing to transition metal resistance in *Cupriavidus metallidurans* CH34. Mol Microbiol **73**:601-621.

240. **Schmelcher M., F. Waldherr, and M. J. Loessner.** 2012. *Listeria* bacteriophage peptidoglycan hydrolases feature high thermoresistance and reveal increased activity after divalent metal cation substitution. Appl Microbiol Biotechnol **93**:633-643.

241. **Schröder J., N. Jochmann, D. A. Rodionov, and A. Tauch.** 2010. The Zur regulon of *Corynebacterium glutamicum* ATCC 13032. BMC Genomics **11**:12.

242. **Schulte M., D. Mattay, S. Kriegel, P. Hellwig, and T. Friedrich.** 2014. Inhibition of the *Escherichia coli* respiratory Complex I by Zn^{2+}. Biochemistry .

243. **Sein-Echaluce V. C., A. González, M. Napolitano, I. Luque, F. Barja, M. L. Peleato, and M. F. Fillat.** 2014. Zur (FurB) is a key factor in the control of the oxidative stress response in *Anabaena* sp. PCC 7120. Environ Microbiol .

244. **Seo S. W., D. Kim, H. Latif, E. J. O'Brien, R. Szubin, and B. O. Palsson.** 2014. Deciphering Fur transcriptional regulatory network highlights its complex role beyond iron metabolism in *Escherichia coli*. Nat Commun **5**:4910.

245. **Sham L. -T., E. K. Butler, M. D. Lebar, D. Kahne, T. G. Bernhardt, and N. Ruiz.** 2014. Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. Science **345**:220-222.

246. **Shida T., H. Hattori, F. Ise, and J. Sekiguchi.** 2001. Mutational analysis of catalytic sites of the cell wall lytic N-acetylmuramoyl-L-alanine amidases CwlC and CwlV. J Biol Chem **276**:28140-28146.

247. **Shin J. -H., H. J. Jung, Y. J. An, Y. -B. Cho, S. -S. Cha, and J. -H. Roe.** 2011. Graded expression of zinc-responsive genes through two regulatory zinc-binding sites in Zur. Proc Natl Acad Sci U S A **108**:5045-5050.

248. **Simmonds S., and N. O. Toye.** 1967. The role of metal ions in the peptidase activity of *Escherichia coli* K-12. J Biol Chem **242**:2086-2093.

249. **Skare J. T., and K. Postle.** 1991. Evidence for a TonB-dependent energy transduction complex in *Escherichia coli*. Mol Microbiol **5**:2883-2890.

250. **Slamti L., and M. K. Waldor.** 2009. Genetic analysis of activation of the *Vibrio cholerae* Cpx pathway. J Bacteriol **191**:5044-5056.

251. **Sledjeski D. D., and S. Gottesman.** 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J Bacteriol **178**:1204-1206.

252. **Smith H. K., M. Shepherd, C. Monk, J. Green, and R. K. Poole.** 2011. The NO-responsive hemoglobins of *Campylobacter jejuni*: Concerted responses of two globins to NO and evidence in vitro for globin regulation by the transcription factor NssR. Nitric Oxide **25**:234-241.

253. **Snyder W. B., L. J. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy.** 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J Bacteriol **177**:4216-4223.

254. **Sohn J., R. A. Grant, and R. T. Sauer.** 2007. Allosteric activation of DegS, a stress sensor PDZ protease. Cell **131**:572-583.

255. **Sperandeo P., R. Cescutti, R. Villa, C. Di Benedetto, D. Candia, G. Dehò, and A. Polissi.** 2007. Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. J Bacteriol **189**:244-253.

256. **Spiess C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97**:339-347.

257. **Spiro S., R. E. Roberts, and J. R. Guest.** 1989. FNR-dependent repression of the ndh gene of *Escherichia coli* and metal ion requirement for FNR-regulated gene expression. Mol Microbiol **3**:601-608.

258. **Stafford S. L., N. J. Bokil, M. E. S. Achard, R. Kapetanovic, M. A. Schembri, A. G. McEwan, and M. J. Sweet.** 2013. Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper. Biosci Rep **33**.

259. **Stähler F. N., S. Odenbreit, R. Haas, J. Wilrich, A. H. M. Van Vliet, J. G. Kusters, M. Kist, and S. Bereswill.** 2006. The novel *Helicobacter pylori* CznABC metal efflux pump is required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. Infect Immun **74**:3845-3852.

260. **Stephens D. L., M. D. Choe, and C. F. Earhart.** 1995. *Escherichia coli* periplasmic protein FepB binds ferrienterobactin. Microbiology **141 (Pt 7)**:1647-1654.

261. **Stohl E. A., E. M. Dale, A. K. Criss, and H. S. Seifert.** 2013. *Neisseria gonorrhoeae* metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H_2O_2- and neutrophil-mediated killing. MBio **4**.

262. **Stout V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J Bacteriol **172**:659-669.

263. **Stoyanov J. V., J. L. Hobman, and N. L. Brown.** 2001. CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. Mol Microbiol **39**:502-511.

264. **Takahashi Y., and M. Nakamura.** 1999. Functional assignment of the ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster involved in the assembly of Fe-S clusters in *Escherichia coli*. J Biochem **126**:917-926.

265. **Takahashi Y., and U. Tokumoto.** 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem **277**:28380-28383.

266. **Tam C., and D. Missiakas.** 2005. Changes in lipopolysaccharide structure induce the sigma(E)-dependent response of *Escherichia coli*. Mol Microbiol **55**:1403-1412.

267. **Tang D. -J., X. -J. Li, Y. -Q. He, J. -X. Feng, B. Chen, and J. -L. Tang.** 2005. The zinc uptake regulator Zur is essential for the full virulence of *Xanthomonas campestris* pv. Campestris. Mol Plant Microbe Interact **18**:652-658.

268. **Tao K., S. -I. Narita, and H. Tokuda.** 2012. Defective lipoprotein sorting induces *lolA* expression through the Rcs stress response phosphorelay system. J Bacteriol **194**:3643-3650.

269. **Taylor D. L., X. R. Bina, L. Slamti, M. K. Waldor, and J. E. Bina.** 2014. Reciprocal regulation of resistance-nodulation-division efflux systems and the Cpx two-component system in *Vibrio cholerae*. Infect Immun **82**:2980-2991.

270. **Thede G. L., D. C. Arthur, R. A. Edwards, D. R. Buelow, J. L. Wong, T. L. Raivio, and J. N. M. Glover.** 2011. Structure of the periplasmic stress response protein CpxP. J Bacteriol **193**:2149-2157.

271. **Thompson K. M., V. A. Rhodius, and S. Gottesman.** 2007. SigmaE regulates and is regulated by a small RNA in *Escherichia coli*. J Bacteriol **189**:4243-4256.

272. **Thöny-Meyer L., P. James, and H. Hennecke.** 1991. From one gene to two proteins: the biogenesis of cytochromes b and c1 in *Bradyrhizobium japonicum*. Proc Natl Acad Sci U S A **88**:5001-5005.

273. **Tokumoto U., and Y. Takahashi.** 2001. Genetic analysis of the *isc* operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. J Biochem **130**:63-71.

274. **Tottey S., S. A. M. Rondet, G. P. M. Borrelly, P. J. Robinson, P. R. Rich, and N. J. Robinson.** 2002. A Copper Metallochaperone for Photosynthesis and Respiration Reveals Metal-specific Targets, Interaction with an Importer, and Alternative Sites for Copper Acquisition. J Biol Chem **277**:5490-5497.

275. **Trepreau J., E. Girard, A. P. Maillard, E. de Rosny, I. Petit-Haertlein, R. Kahn, and J. Covès.** 2011. Structural basis for metal sensing by CnrX. J Mol Biol **408**:766-779.

276. **Troxell B., and X. F. Yang.** 2013. Metal-dependent gene regulation in the causative agent of Lyme disease. Front Cell Infect Microbiol **3**:79.

277. **Tschauner K., P. Hörnschemeyer, V. S. Müller, and S. Hunke.** 2014. Dynamic Interaction between the CpxA Sensor Kinase and the Periplasmic Accessory Protein CpxP Mediates Signal Recognition in *E. coli.* PLoS One **9**:e107383.

278. **Tsiboli P., D. Triantafillidou, F. Franceschi, and T. Choli-Papadopoulou.** 1998. Studies on the Zn-containing S14 ribosomal protein from *Thermus thermophilus*. Eur J Biochem **256**:136-141.

279. **Tsubaki M., T. Mogi, H. Hori, M. Sato-Watanabe, and Y. Anraku.** 1996. Infrared and EPR studies on cyanide binding to the heme-copper binuclear center of cytochrome bo-type ubiquinol oxidase from *Escherichia coli*. Release of a CuB-cyano complex in the partially reduced state. J Biol Chem **271**:4017-4022.

280. **Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C., and T. V. O'Halloran.** 1999. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science. **284:** 805-808.

281. **Vinogradov S. N., D. Hoogewijs, X. Bailly, R. Arredondo-Peter, J. Gough, S. Dewilde, L. Moens, and J. R. Vanfleteren.** 2006. A phylogenomic profile of globins. BMC Evolutionary Biology **6**:31.

282. **Vogel J., and K. Papenfort.** 2006. Small non-coding RNAs and the bacterial outer membrane. Curr Opin Microbiol **9**:605-611.

283. **Vogt S. L., A. Z. Nevesinjac, R. M. Humphries, M. S. Donnenberg, G. D. Armstrong, and T. L. Raivio.** 2010. The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. Mol Microbiol **76**:1095-1110.

284. **Wagner D., J. Maser, I. Moric, N. Boechat, S. Vogt, B. Gicquel, B. Lai, J. -M. Reyrat, and L. Bermudez.** 2005. Changes of the phagosomal elemental concentrations by *Mycobacterium tuberculosis* Mramp. Microbiology **151**:323-332.

285. **Waldron K. J., and N. J. Robinson.** 2009. How do bacterial cells ensure that metalloproteins get the correct metal? Nat Rev Microbiol **7**:25-35.

286. **Wang C. C., and A. Newton.** 1971. An additional step in the transport of iron defined by the *tonB* locus of *Escherichia coli*. J Biol Chem **246**:2147-2151.

287. **Wang D., and C. A. Fierke.** 2013. The BaeSR regulon is involved in defense against zinc toxicity in *E. coli*. Metallomics **5**:372-383.

288. **Wang D., O. Hosteen, and C. A. Fierke.** 2012. ZntR-mediated transcription of zntA responds to nanomolar intracellular free zinc. J Inorg Biochem **111**:173-181.

289. **Wang D., S. S. Korban, and Y. Zhao.** 2009. The Rcs phosphorelay system is essential for pathogenicity in *Erwinia amylovora*. Mol Plant Pathol **10**:277-290.

290. **Wang K., O. Sitsel, G. Meloni, H. E. Autzen, M. Andersson, T. Klymchuk, A. M. Nielsen, D. C. Rees, P. Nissen, and P. Gourdon.** 2014. Structure and mechanism of Zn^{2+}-transporting P-type ATPases. Nature . **514**: 518-22.

291. **Wapnir R. A.** 2000. Zinc deficiency, malnutrition and the gastrointestinal tract. J Nutr **130**:1388S-1392S.

292. **Weidner U., S. Geier, A. Ptock, T. Friedrich, H. Leif, and H. Weiss.** 1993. The gene locus of the proton-translocating NADH: ubiquinone oxidoreductase in *Escherichia coli*. Organization of the 14 genes and relationship between the derived proteins and subunits of mitochondrial complex I. J Mol Biol **233**:109-122.

293. **Weiner L., and P. Model.** 1994. Role of an *Escherichia coli* stress-response operon in stationary-phase survival. Proc Natl Acad Sci U S A **91**:2191-2195.

294. **Wei Y., and D. Fu.** 2005. Selective metal binding to a membrane-embedded aspartate in the *Escherichia coli* metal transporter YiiP (FieF). J Biol Chem **280**:33716-33724.

295. **Wolfe A. J., N. Parikh, B. P. Lima, and B. Zemaitaitis.** 2008. Signal integration by the two-component signal transduction response regulator CpxR. J Bacteriol **190**:2314-2322.

296. **De Wulf P., O. Kwon, and E. C. Lin.** 1999. The CpxRA signal transduction system of *Escherichia coli*: growth-related autoactivation and control of unanticipated target operons. J Bacteriol **181**:6772-6778.

297. **Xu F. F., and J. A. Imlay.** 2012. Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify *Escherichia coli*. Appl Environ Microbiol **78**:3614-3621.

298. **Yamamoto K., and A. Ishihama.** 2005. Transcriptional response of *Escherichia coli* to external copper. Mol Microbiol **56**:215-227.

299. **Yamamoto K., H. Ogasawara, and A. Ishihama.** 2008. Involvement of multiple transcription factors for metal-induced *spy* gene expression in *Escherichia coli*. J Biotechnol **133**:196-200.

300. **Yang H. H., J. B. Morrow, D. Grasso, R. T. Vinopal, and B. F. Smets.** 2006. Intestinal versus external growth conditions change the surficial properties in a collection of environmental *Escherichia coli* isolates. Environ Sci Technol **40**:6976-6982.

301. **Yan N., and Y. Shi.** 2007. Allosteric activation of a bacterial stress sensor. Cell **131**:441-443.

302. **Yatsunyk L. A., J. A. Easton, L. R. Kim, S. A. Sugarbaker, B. Bennett, R. M. Breece, I. I. Vorontsov, D. L. Tierney, M. W. Crowder, and A. C. Rosenzweig.** 2008. Structure and metal binding properties of ZnuA, a periplasmic zinc transporter from *Escherichia coli*. J Biol Inorg Chem **13**:271-288.

303. **Yeo W. -S., J. -H. Lee, K. -C. Lee, and J. -H. Roe.** 2006. IscR acts as an activator in response to oxidative stress for the *suf* operon encoding Fe-S assembly proteins. Mol Microbiol **61**:206-218.

304. **Zheng M., X. Wang, L. J. Templeton, D. R. Smulski, R. A. LaRossa, and G. Storz.** 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J Bacteriol **183**:4562-4570.

305. **Zhou X., R. Keller, R. Volkmer, N. Krauss, P. Scheerer, and S. Hunke.** 2011. Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. J Biol Chem **286**:9805-9814.

306. **Zimmermann M., S. R. Udagedara, C. M. Sze, T. M. Ryan, G. J. Howlett, Z. Xiao, and A. G. Wedd.** 2012. PcoE--a metal sponge expressed to the periplasm of copper resistance *Escherichia coli*. Implication of its function role in copper resistance. J Inorg Biochem **115**:186-197.

307. **Zoll S., B. Pätzold, M. Schlag, F. Götz, H. Kalbacher, and T. Stehle.** 2010. Structural basis of cell wall cleavage by a staphylococcal autolysin. PLoS Pathog **6**:e1000807.

308. **Zückert W. R.** 2014. Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. Biochim Biophys Acta **1843**:1509-1516.

Chapter 2: Metal-limitation induces the Cpx two-component system in *Escherichia coli*

2.1. Introduction

Pathogenic bacteria must survive the metal-poor environment of both the human host and the environment during transmission. There are multiple mechanisms by which the human host chelates essential metals away from bacteria. Transferrin and lactoferrin sequester plasma iron, hemopexin scavenges free heme, while metallothioneins and the calcium-binding protein calprotectin sequester zinc from bacteria and parasites (14). Iron limitation affects biogenesis of iron-sulfur (Fe-S) clusters incorporated into respiratory and metabolic enzymes while zinc limitation affects cell wall biogenesis, proteolysis, and transcription (2, 14) . Bacteria must sense metal chelation in order to adapt accordingly.

Two-component signal transduction systems (TCS) sense environmental changes through an inner membrane sensor kinase and facilitate adaptation by changing gene expression through a cytoplasmic response regulator. The *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) PhoQ sensor kinase senses magnesium chelation during infection by binding magnesium ions directly. The bound magnesium ion mediates an interaction between a negatively-charged cluster of amino acids in PhoQ and the phospholipid head groups of the cytoplasmic membrane. In the absence of magnesium, the sensing domain of PhoQ is no longer held at the membrane and the conformational change is transduced to the cytoplasm through a conserved phosphotransfer to the response regulator PhoP, resulting in the expression of virulence genes (8).

The Cpx two-component system in *Escherichia coli* senses problems in envelope protein-folding. Over-expression of pilin subunits in the absence of their cognate chaperones results in Cpx activation (15, 27) , as well as the over-expression of the lipoprotein NlpE and growth in alkaline pH (9, 40) . The sensor kinase CpxA autophosphorylates upon sensing stress and

phosphorylates the response regulator CpxR (34). Activation of the Cpx TCS results in increased expression of chaperones, proteases, cell wall enzymes, and other genes associated with inner membrane function (36). Cpx activation also represses a number of virulence structures in enteropathogenic *E. coli* (EPEC), such as the type III secretion system (22, 23) and the bundle-forming pilus (44), which may be beneficial by diminishing the number of large proteinaceous envelope structures during stress. However, Cpx-mediated stress adaptation is important *in vivo* because *cpxR* was one of three response regulators required for colonization of the mouse intestine by commensal *E. coli* strain MP1 (18). CpxP is a third component of the Cpx signaling machinery, a periplasmic protein that inhibits Cpx activity in the absence of stress (35). CpxP is required for the colonization of the urogenital tract by some strains of uropathogenic *E. coli* (UPEC) (11). In *Vibrio cholerae*, the Cpx pathway senses distinct cues from that of *E. coli*, including increased salinity and perturbations to disulfide bonding. However, disruption of the outer membrane efflux component *tolC* activates the Cpx pathway in both organisms (37, 38). Recently, our lab demonstrated that the chelation of iron activated the *V. cholerae* Cpx pathway in a *cpxA*-dependent manner and that the addition of exogenous iron suppressed activation of the Cpx pathway by multiple chemical inducing cues (1).

Furthermore, numerous studies examining the effect of excess metal ions have suggested that the Cpx pathway is activated in the presence of excess metals. In *E. coli,* excess copper increases the expression of *cpxR* and a number of Cpx-regulated chaperones and proteases, suggesting that copper might induce protein mis-folding and induce the Cpx pathway to ameliorate the stress (16, 47). Excess zinc induces the expression of the periplasmic protein-folding factors *cpxP, spy*, and *ppiA* in *E. coli* and *Salmonella* (20, 31, 45, 48). CpxP and Spy share homology with the metal-binding signal transduction proteins ZraP from *S.* Typhimurium

and CnrX from *Cupriavidus metallidurans*, suggesting that Cpx-regulated chaperones may link the Cpx pathway to metal homeostasis.

Despite mounting transcriptomic evidence for a role of Cpx in sensing cellular metal status, the physiological significance of Cpx-mediated metal-sensing remains unknown. Therefore, we investigated whether metal chelation affects Cpx activity in *E. coli* and if metal-sensing depends on the auxiliary regulator CpxP. We demonstrate that growth in metal-limited medium activates *cpxP* expression and that the activation is reversed by the addition of exogenous zinc. Surprisingly, neither activation by chelation nor suppression by zinc required the sensor kinase CpxA or the inhibitor CpxP. These results directly contrast findings in *V. cholerae* where the CpxA sensor kinase is required for sensing iron chelation (1). However, activation by chelation depends on the master regulator of zinc homeostasis, Zur. When cellular zinc homeostasis is perturbed by growth in excess zinc, deletion of *cpxP* rendered cells zinc resistant by increasing the expression of the zinc efflux protein *zntA* whereas deletion of *cpxA* and *cpxR* had no effect on survival but strongly diminished *zntA* expression, even at toxic levels of zinc. Our findings suggest that metal-chelation induces *cpxP* expression in *E. coli* and that Cpx-mediated regulation of *cpxP* expression is important for bacterial adaptation to metal-limitation.

2.2. Materials and Methods

Bacterial strains and plasmids used in this study. All of the mutant strains and plasmids used in this study were constructed by standard genetic techniques. The strains and plasmids are listed in Table 2-1.

Acid washing of glassware. All glassware used to prepare, aliquot, and store metal-limited medium was acid-washed as follows: flasks and screw-cap bottles were filled to

approximately 10% volume with 1 M HNO_3 (Sigma) and swirled vigorously in a fumehood for one minute. The same volume of 1 M HNO_3 was rinsed over the sides and outside of the flask to scrub residual metal trace from all possible surfaces. Tubes were filled to approximately 10% volume with 1 M HNO_3 and vortexed in a fumehood for 30 seconds three times. Glass pipettes were used to pipette 1 M HNO_3 up and down vigorously for 30 seconds. All glassware was then rinsed with MilliQ five to six times or until a litmus strip placed on the inside surface of the glassware read between pH 5 and 6. Flasks were covered in foil, bottles and tubes were capped, and pipettes were placed in a canister then dried overnight at 80°C. Glassware was cooled to room temperature before use.

Media and growth conditions. All 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 were added in the following concentrations: 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, 50 μg/mL kanamycin, 3 μg/mL amikacin (all Sigma). 0.5 M stock solutions of metal salts were prepared fresh before each assay and used within 1 day of preparation (all Sigma, except for zinc sulfate – Baker & Adamson).

Metal-limited medium was prepared as follows: a 10-times concentrated stock of LB broth was prepared in an acid-washed flask and 5 g Chelex-100 resin (Bio-Rad) was added per 100 mL of medium prepared. The Chelex-LB mixture was left to stir at room temperature for 5-6 h then dialyzed against 10-times the volume of MilliQ water in an acid-washed screw-cap bottle overnight, using a 3000-5000 MW dialysis tube (Fisher). The next day, the medium was sterilized at 121°C for 30 minutes then stored at room temperature. All glassware used to store and aliquot metal-limited medium was acid-washed as described above to remove trace metal contamination.

Sodium phosphate-buffered LB was prepared by adding 10 mL 1 M dibasic sodium phosphate to 100 mL sterile LB broth (pH 8.0) or 791 µL 1 M dibasic sodium phosphate and 9.21 mL 1 M monobasic sodium phosphate to 100 mL sterile LB broth (pH 5.8).

β-galactosidase assays. Single isolated colonies of each strain were inoculated into 2 mL of LB broth with the appropriate antibiotics in quintuplicate and grown overnight, shaking at 225 rpm in a test tube rack at 37°C. The next morning, cultures were diluted 1:50 into fresh LB broth plus antibiotics and grown for 3.5 h in the same fashion. The indicated amount of metal salts or the same volume of MilliQ water was added and cultures were returned to the incubator for an additional hour. β-galactosidase activity was measured in a microtitre plate as described previously (39).

Gradient plate assays. Gradient plates were prepared using agar containing 10 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 20 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (both Sigma) and 1.5 mM zinc sulfate, as described previously (3). Briefly, petri dishes propped on a 8 mm prop and 20 mL of LB agar containing only IPTG and X-gal was poured to cover the bottom of the plate. The agar was left to dry at room temperature. Once dry, the prop was removed and 20 mL of agar containing IPTG, X-gal, and 1.5 mM zinc sulfate was poured into the same plate. Plates were dried at least 20 h at room temperature and used within 3 days of preparation. Using a disposable sterile inoculating loop, a single isolated colony was streaked in a straight line across the entire plate, along the gradient of zinc and incubated overnight at 37°C. Plates were imaged using the Chemi-Doc Imager (Bio-Rad).

Zinc plate assays. 1 mM zinc sulfate (final concentration) was added from a 1 M stock solution prepared fresh that day to LB agar. 10 µM IPTG and 20 µg/mL X-gal was also added before plates were poured. Plates were dried at room temperature for at least 20 h, stored at 4°C, and

used within one week of preparation. Single isolated colonies were inoculated into 2 mL LB broth plus antibiotics in duplicate and grown in a test tube rack overnight at 37°C shaking at 225 rpm. The next morning, 5 µL aliquots standardized to the lowest OD_{600} were spotted onto dried 1 mM zinc sulfate-containing plates and dried upright at room temperature for approximately 1 h. Plates were incubated upside down at 37°C. The next day, plates were imaged on a Chemi-Doc Imager (Bio-Rad) to assess luminescence or the Start-It Colony Counter (Fisher) to assess growth.

2.3. Results

2.3.1. Metal chelation induces *cpxP* transcription

To test whether metal limitation activates the Cpx pathway in *E. coli*, we prepared metal-limited LB medium using the non-specific divalent cation chelator Chelex-100 and examined the activity of a *cpxP'-'lacZ* transcriptional reporter after growth in untreated or chelated LB. The expression of *cpxP* in *E. coli* is strictly dependent on *cpxR* (9) and is not known to be regulated by any other transcription factors. Accordingly, *cpxP* expression is often used as a measure of Cpx signaling. In metal-limited LB, Cpx activity was elevated 3.7-fold as compared to activity in untreated LB (Figure 2-1). Therefore, metal-limitation activates the expression of the periplasmic inhibitor-chaperone *cpxP*, similar to the activation of a *cpxP-lux* reporter in *V. cholerae* (1).

2.3.2. Zinc reverses the metal chelation-induced expression of *cpxP*

We wondered whether the absence of a specific metal ion was being sensed by the Cpx pathway. Iron chelation induces the Cpx system in *V. cholerae* (1) while excess zinc and copper induced the Cpx pathway in *E. coli* and *Salmonella* (20, 31, 48). Manganese is not expected to affect Cpx activity but can partially substitute for iron or copper in transcriptional regulators,

metabolic enzymes, and superoxide dismutases (24, 41, 43). Therefore, we added different metal salts to bacteria grown in metal-limited LB and measured *cpxP'-'lacZ* transcription. The addition of Cu^{2+} had no effect on the ability of metal chelation to increase *cpxP* transcription, while the addition of Fe^{2+} or Mn^{2+} resulted in a modest 1.5-fold decrease as compared to the MilliQ water control (Figure 2-1). Addition of Zn^{2+}, however, resulted in a dramatic 23-fold decrease in *cpxP* transcription (Figure 2-1), suggesting that zinc can reverse the elevated levels of *cpxP* transcription induced by metal-limitation. No metals affected *cpxP'-'lacZ* expression when added to bacteria grown in metal-replete LB medium (Figure 2-1), suggesting that basal levels of *cpxP* expression are not responsive to excess metals at the concentration tested.

2.3.3. Zinc reverses the induction of *cpxP* expression by other Cpx activating signals

The *E. coli* Cpx system is thought to sense protein-misfolding in the Gram-negative envelope, however the precise biochemical cue sensed by the CpxA sensor kinase is unknown. Over-expression of *nlpE* or *pap* subunits and growth in alkaline pH requires the CpxA sensor kinase to activate the Cpx pathway while entry into stationary phase requires only CpxR (12). We wondered whether the activation of *cpxP* transcription by other, known Cpx inducing signals might also be reversed by zinc. The addition of exogenous zinc decreased *cpxP'-'lacZ* activity by over-expression of either *nlpE* or *bfpA* but did not suppress activation by alkaline pH nor entry into stationary phase (Figure 2-2A). The decrease in *cpxP'-'lacZ* transcription is only observed after one hour of treatment at high levels of zinc (between 300 and 400μM zinc sulfate) (Figure 2-2B), though we did not observe large decreases in growth in the zinc-treated samples as compared to the MilliQ controls in our β-galactosidase assays (data not shown). Therefore, the addition of exogenous zinc reverses the increase in *cpxP* expression induced by metal limitation.

2.3.4. The metal status of the cell is sensed independently of the CpxA sensor kinase and the auxiliary regulator CpxP

Based on the ability of zinc to suppress activation of *cpxP'-'lacZ* expression by metal chelation and other known Cpx inducing signals, we wondered if known Cpx inducing cues activate the response by chelating zinc. If Cpx inducing cues do indeed chelate zinc, then we would expect metal chelation and zinc to be sensed by the sensor kinase, in a *cpxA*-dependent manner. Therefore, we examined the *cpxP'-'lacZ* activity of wild-type, *cpxA, cpxP*, and *cpxR* mutant strains in metal-limited medium in the presence or absence of zinc. As shown previously, growth in metal-limited LB resulted in 3.5-fold activation of *cpxP'-'lacZ* expression, as compared to growth in untreated LB (Figure 2-3). Further, addition of zinc decreased this activation 33.6-fold (Figure 2-3). In a *cpxA* deletion strain, metal chelation resulted in a 4.3-fold increase in *cpxP* transcription as compared to the same mutant grown in untreated LB (Figure 2-3). As observed in the wild-type strain, addition of zinc decreased *cpxP'-'lacZ* activity 33.5-fold in the *cpxA* mutant. Therefore, the CpxA sensor kinase is not required for activation of *cpxP* transcription by metal limitation nor its reversal by zinc. In a *cpxP* mutant, metal chelation activated *cpxP'-'lacZ* expression 5.0-fold in metal-limited LB as compared to growth in untreated LB and addition of zinc decreased expression 283-fold. This observation demonstrates CpxP is not required for the changes in *cpxP* transcription observed upon metal chelation or in response to zinc. Only a 2.6-fold increase in *cpxP* expression was observed in metal-limited medium in the absence of *cpxR* and the addition of zinc did not significantly change reporter activity in this background (Student's t-test, p-value = 0.5922). As mentioned previously, the expression of *cpxP* is dependent on *cpxR* (9) and metal limitation did not exert a significant effect in the absence of the only known transcriptional regulator of *cpxP* expression.

2.3.5. Regulation of *cpxP* expression by zinc requires the Zur transcriptional regulator

That the Cpx system does not sense chelation through the sensor kinase is surprising. We hypothesized that another zinc-responsive regulator may activate *cpxP* expression in response to chelation and zinc. The BaeSR two-component system senses noxious metabolites in the envelope and up-regulates the expression of efflux pumps to remove toxic compounds from the cell (37). The BaeS sensor kinase senses excess zinc and sodium tungstate (19, 48) . Additionally, Cpx and Bae systems coordinately regulate the expression of the periplasmic chaperone *spy* (33), demonstrating that the BaeR and CpxR regulons partially overlap. Similarly, the σ^E envelope stress response activates the expression of *degP* in the presence of excess zinc in EPEC (26) and the Cpx response also activates the expression of *degP* (10). To test whether other metal-responsive envelope stress signaling pathways regulate *cpxP* expression in response to metal status, we examined *cpxP'-'lacZ* activity in wild-type and *baeR* mutants and found no difference in Cpx activation in response to metal limitation or zinc (Figure 2-4A, left panel). Similarly, we compared *cpxP'-'lacZ* activity to the σ^{-E}-dependent reporter *rpoHp3-lacZ* in the presence of zinc and observed activation of only *cpxP'-'lacZ* expression (Figure 2-4A, right panel), suggesting that zinc does not activate the σ^{-E} pathway at the concentrations required to activate the Cpx pathway in *E. coli* K-12. Notably, we added 300μM zinc sulfate (final concentration) to cultures in these assays to circumvent any anomalous physiological effects exerted by excess zinc, as we have previously shown that only high levels of zinc can reverse the activation of *cpxP'-'lacZ* expression (Figure 2-3B) and other labs have observed an increase in Cpx-regulated transcript abundance in the presence of as little as 200μM zinc (20, 26). Thus, it is unlikely that other metal-sensing envelope stress responses are involved in the observed changes

in *cpxP'-'lacZ* expression observed during growth in metal-limited medium or in the presence of zinc.

The most sensitive zinc-responsive transcription factor in the cell is the zinc-uptake regulator Zur (29). Zur represses the expression of zinc-uptake transporters, including the high-affinity ZnuABC importer that is required for virulence of enteric pathogens *in vivo* (7, 30) . To investigate the role of Zur in regulation of *cpxP'-'lacZ* expression in response to metals, we deleted *zur* and examined β-galactosidase levels under various conditions (Figure 2-4B). In untreated LB, the deletion of *zur* had no effect on *cpxP'-'lacZ* expression in the absence or presence of exogenously added zinc sulfate (Figure 2-4B). Similarly, when the Cpx response was induced by NlpE over-expression, the presence of an intact *zur* allele had no impact on *cpxP* expression (Figure 2-4B). In contrast, when zinc sulfate was added to strains that were over-expressing NlpE, a reduction in *cpxP'-'lacZ* expression was observed in wild-type but not *zur* mutant cells (Figure 2-4B). Therefore, deletion of the master regulator of zinc homeostasis affects the ability of zinc to reverse the increase in *cpxP* expression mediated by activation of the Cpx response.

2.3.6. Deletion or over-expression of *cpxP* alters zinc resistance and zinc trafficking

The observation that *cpxP* expression is affected by zinc suggests that the function of *cpxP* is connected to cellular levels of zinc. To determine whether the function of *cpxP* is connected to metals, we examined the ability of *cpxP* mutants to grow in the presence of zinc. Consistent with our observation that *cpxP* expression is inhibited by zinc, we found that a *cpxP* deletion mutant grew much better than the isogenic wild-type strain in the presence of high levels zinc on LB agar containing a gradient of zinc (Figure 2-5). The wild-type strain grew approximately 50mm across the diameter of the plate while the *cpxP* mutant strain grew nearly

the entire 100mm diameter of the plate (Figure 2-5). In contrast, complementation of the *cpxP* mutation by over-expression of the high-copy pCpxP plasmid *in trans* restored zinc sensitivity to the *cpxP* mutant (Figure 2-5). These observations indicate that the *cpxP* mutant is zinc-resistant and may alter intracellular zinc levels when exposed to high levels of zinc to avoid toxicity.

To begin to understand the zinc-resistance phenotype of the *cpxP* mutant, we examined the effect of *cpxP* deletion and over-expression on the expression of a gene under the control of the zinc-responsive transcriptional activator ZntR. ZntR activates the expression of the P_I-type ATPase *zntA* which catalyzes the high-affinity active transport of zinc out of the cytoplasm (4). We constructed a luminescent *zntA* reporter (*zntA-lux*) by fusing the promoter of *zntA* to the *luxCDABE* genes that mediate light production, as described previously (32). We transformed wild-type and *cpxP* mutant cells with the *zntA-lux* reporter and examined luminescence when cells were grown on LB agar containing a gradient of zinc. In the wild-type strain, *zntA-lux* expression increased with increasing amounts of zinc sulfate and saturated at the point of cell death (Figure 2-5). Similarly, luminescence increased in the *cpxP* mutant with increasing amounts of zinc sulfate but remained high over the entire diameter of the plate (Figure 2-5). In contrast, *zntA-lux* expression in the complemented mutant resulted in a strong decrease in *zntA-lux* expression, even at toxic levels of zinc (Figure 2-5). These observations suggest that over-expression of *cpxP* decreases zinc efflux mediated by ZntA, even at toxic levels of zinc.

2.3.7. The Cpx system affects zinc trafficking in *E. coli* K-12

Our data suggest that *cpxP* expression and function are linked to cellular zinc status (Figure 2-5). We wondered whether this function could be ascribed solely to CpxP or if these results might reflect a broader role for the Cpx envelope stress response. Accordingly, we transformed wild-type, *cpxA*, *cpxP*, and *cpxR* mutants with the *zntA-lux* reporter and examined

the growth and luminescence of transformants on LB agar containing a gradient of zinc. As observed previously, the wild-type strain grew approximately half the diameter of the plate while the *cpxP* mutant grew the entire diameter of the plate (Figure 2-6). The *cpxA* and *cpxR* mutants exhibited similar survival to the wild-type strain and grew only half the diameter of the plate (Figure 2-6). Thus, neither deletion of *cpxA* nor *cpxR* phenocopies the zinc-resistance of the *cpxP* mutant.

We examined *zntA-lux* activity in each background to investigate the impact of Cpx mutations on zinc trafficking. In the wild-type strain, *zntA-lux* activity increases with increasing amounts of zinc until cell death, as previously observed (Figure 2-6). Luminescence remains high in the *cpxP* mutant past the concentration of zinc that is lethal to the wild-type strain (Figure 2-6). In the *cpxA* and *cpxR* mutants, however, luminescence remains low at all concentrations where growth is observed, even at lethal concentrations of zinc (Figure 2-6). Expression of *cpxP* is very low in the absence of *cpxR* (Figure 2-3), however deletion of *cpxR* does not result in the same zinc resistance or high expression of *zntA* as the *cpxP* mutant (Figure 2-6).

2.3.8. Cpx activation in EPEC confers zinc resistance

Because our data suggest that *cpxP* is involved in altering zinc traffficking *E. coli* K-12 (Figure 2-5), we anticipated that *cpxP* would play a similar role in EPEC. To test our hypothesis, we grew wild-type and *cpxP:kn* mutant EPEC on LB agar containing a gradient of zinc. Curiously, insertional inactivation of *cpxP* does not result in the same zinc resistance phenotype as in K-12 (Figure 2-7A). However, activation of the Cpx pathway by over-expression of *nlpE* or expression of the constitutively active *cpxA24* allele (R. Guest, unpublished observations) results in zinc-resistance (Figure 2-7B). Thus, although inactivation of *cpxP* by itself does not yield zinc-related growth phenotypes, activation of the entire Cpx response does. These data, coupled

with the observation that deletion of *cpxA* or *cpxR* alters the expression of *zntA* (Figure 2-6) suggest that, in addition to *cpxP*, other Cpx-regulated genes play roles in metal homeostasis and/or resistance.

2.4. Discussion

2.4.1. *cpxP* transcription is elevated in response to metal chelation

Iron chelation induces the Cpx response in *V. cholerae* (1) and the *E. coli* Cpx response has been similarly linked to excess zinc and copper. The expression of Cpx-regulated chaperones including *cpxP* is elevated in response to excess zinc and copper in *E. coli* and *S.* Typhimurium (13, 20, 31, 48). We confirmed here that *cpxP* expression is regulated by metals. Metal chelation leads to elevated *cpxP'-'lacZ* expression while the addition of high levels of zinc inhibits *cpxP* expression when its expression is elevated by certain inducing cues (Figure 2-1). However, the CpxA sensor kinase is not required for either of these events in *E. coli* nor is the chaperone CpxP (Figure 2-3). Since CpxR is required for transcription of *cpxP* in *E. coli*, it was not possible to decisively conclude that metal chelation requires *cpxR* to activate *cpxP* expression (Figure 2-3). We also did not replicate the findings that excess zinc activates the Cpx response as shown previously for other strains of *E. coli* and for *Salmonella* (20, 31, 48). The previous studies in *E. coli* were performed in chemostats containing minimal medium and the relative transcript abundance was measured by quantitative real-time PCR (20). Our work was performed in batch culture in rich medium using a transcriptional reporter. The discrepancies between the culture conditions and the transcriptional reporters could account for the different results in *E. coli* grown in the presence of excess zinc. Future studies should examine the effect of metal chelation and excess metals on other Cpx-regulated reporters that do not strictly require CpxR for expression.

Nonetheless, we can envision several models for the mechanism by which metal chelation and excess zinc regulate *cpxP* expression. Zinc may directly modify CpxR to change its regulatory activity, however no canonical zinc-binding sites are found in the CpxR sequence. Though we were unable to identify another transcription factor that can activate *cpxP* expression in response to chelation, it is possible that another metal-responsive transcription factor cooperates with CpxR to activate *cpxP* expression during growth in metal-limited medium. In *V. cholerae,* deletion of the ferric uptake regulator Fur results in de-repression of *cpxP* (N. Acosta, unpublished observations), suggesting that Fur normally represses the expression of *cpxP* in this organism under iron-replete conditions. Deletion of *zur*, however, did not affect basal *cpxP'-'lacZ* expression in *E. coli* and an intact copy of *zur* was not required for the activation of the Cpx pathway by *nlpE* (Figure 2-4C). Zinc-bound Zur could potentially bind the *cpxP* promoter and repress expression in excess zinc, preventing CpxR-mediated activation of *cpxP* expression. An important addition to these data would be the examination of *cpxP* expression in the absence of *zur* in metal-limited and –replete media to determine whether Zur affects *cpxP* expression under differing metal conditions. Therefore, elevated *cpxP* expression is responsive to zinc in a manner that is dependent on the transcriptional regulator Zur but whether Zur acts directly on the *cpxP* promoter is unclear.

2.4.2. CpxP alters zinc trafficking and expression of CpxP may be toxic in the presence of zinc

The addition of zinc to cells induced by metal limitation, *nlpE,* or *bfpA* over-expression decreased *cpxP'-'lacZ* expression (Figure 2-2), suggesting that zinc can reverse the activation by these signals. However, zinc was unable to suppress the activation by growth in alkaline pH or entry into stationary phase. Alkaline pH induces the Cpx pathway in a *cpxA*-dependent manner

(12) and deprotonates imidazole groups on histidine residues. Potentially, proteins that reversibly bind zinc at histidine residues and are capable of limiting *cpxP* expression may be unable to bind zinc at elevated pH, even when zinc is present in excess. Thus, alkaline pH might render zinc unable to bind to proteins that are required to produce the observed decrease in *cpxP'-'lacZ* expression. The biochemical cue sensed by the Cpx system upon entry into stationary phase is unknown but is proposed to be an accumulated metabolite that requires Cpx-mediated changes for turnover (46). Our data suggest that zinc does not interfere with the ability of the growth-related inducing cue to activate Cpx (Figure 2-2). Intriguingly, the Cpx system was not activated by growth in sub-inhibitory concentrations (10-15μM) of the zinc-specific chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) (data not shown), suggesting that the Cpx system does not sense zinc chelation *per se*. Zinc strongly decreases *cpxP'-'lacZ* expression when elevated in the presence of chelation and other cues, possibly because expression of *cpxP* is toxic at high levels of zinc.

In agreement with this hypothesis, deletion of *cpxP* renders cells resistant to zinc (Figure 2-5). Additionally, mutations in *cpxA* and *cpxR* decrease the expression of the zinc efflux pump *zntA*, even at lethal concentrations of zinc (Figure 2-6). Though the level of *cpxP* transcription is exceedingly low in the absence of *cpxR*, the *cpxR* mutant does not phenocopy the *cpxP* mutant. These data support the hypothesis that additional CpxR-regulated genes can influence zinc-trafficking in *E. coli* K-12. Similarly, *zntA-lux* expression is low in the *cpxA* mutant, perhaps due to high levels of *cpxP* expression, which we have shown decrease *zntA-lux* expression (Figure 2-5). Taken together, these results suggest that the Cpx response alters zinc trafficking in the cell through CpxP and other unidentified regulon members in the presence of excess zinc.

2.4.3. The Cpx response impacts zinc resistance in laboratory and pathogenic strains of *E. coli* but in different fashions

Though deletion of *cpxP* renders cells zinc-resistant in the laboratory strain of *E. coli* (Figure 2-5), insertional inactivation of *cpxP* in EPEC does not result in zinc-resistance (Figure 2-7A). However, over-expression of *nlpE* to activate the Cpx response renders cells zinc-resistant (Figure 2-7B), suggesting that an increase in expression of other Cpx-regulated factors can alter zinc resistance in EPEC. We have not confirmed that the zinc-resistance of *nlpE*-expressing strain depends on increased zinc efflux as it does in the laboratory strain of *E. coli* in a *cpxP* mutant background (Figure 2-5). However, it is possible that other mechanisms of zinc resistance may be at work in EPEC. The Cpx system regulates a number of proteins of unknown function and some of these proteins may be able to enhance or assist in the transport of solutes across the cytoplasmic or outer membranes, as proposed for small proteins (42). In *B. subtilis*, low-molecular weight thiols reversibly bind an estimated 67% of the cellular zinc pool in response to toxic zinc shock, providing a cache of zinc that can be accessed during periods of starvation (25). Activation of the Cpx pathway in *E. coli* increases the expression of cysteine biosynthesis genes (36), perhaps contributing to high cellular sulfur flux and increased thiol synthesis that could bind up excess zinc. Measurements of intracellular zinc flux in the different Cpx mutant backgrounds can distinguish between these possibilities: up-regulation of zinc efflux will result in decreased accumulation of zinc in EPEC where the Cpx response is activated while increased zinc sequestration will leave zinc flux unaffected in this background as compared to that of the wild-type strain. These studies are currently in progress.

2.4.4. The Cpx pathway is involved with different metals in different organisms

Chelation of iron activates the Cpx pathway in *V. cholerae* (1) whereas the Cpx pathway is connected to zinc in *E. coli*. This difference may reflect the different environmental niches of the two organisms. *V. cholerae* can exist as a free-living marine organism whereas the natural reservoir for *E. coli* is thought to be the lower intestines of its mammalian hosts (49). Interestingly, no homologue for high-affinity zinc uptake has been identified to date in *V. cholerae*, perhaps because *V. cholerae* is not under selective pressure to obtain zinc in its niche.

2.4.5. Conclusion

We have shown that expression of *cpxP,* one of the most strongly Cpx-regulated genes identified in any organism to date, is activated by metal limitation in *E. coli* and that the addition of exogenous zinc can suppress *cpxP* activation of some but not all Cpx inducing cues. The ability to maintain metal homeostasis is critical to enteric pathogens and thus our data suggest that the Cpx pathway may positively affect virulence by facilitating adaptation to changing metal conditions.

2.5. Tables

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

Strain name	Genotype or description	Reference
ALN194	E2348/69 *cpxP:kn*	(44)
ALN239	E2348/69 pCA24N	(36)
DB12	TR50 pTrc99A	(5)
E2348/69	Prototypical EPEC strain	(21)
JLW62	TR50 pLD404	This study
JLW64	TR50 pKDS301	This study
JLW65	TR50 pBR322	This study
JLW117	E2348/69 pCA-*nlpE*	This study
JLW392	TR50 Δ*cpxP*	This study
JLW400	JLW392 pTrc99A	This study
JLW404	JLW392 pCpxP	This study
JLW851	TR50 pJW15-*zntA*p	This study
JLW852	JLW392 pJW15-*zntA*p	This study
JLW858	JLW392 pJW15-*zntA*p pTrc99A	This study
JLW859	JLW392 pJW15-*zntA*p pCpxP	This study
JLW970a	TR50 Δ*baeR*	This study
JLW982	RM53 pJW15-*zntA*p	This study
JLW983	JSW1 pJW15-*zntA*p	This study
JSW1	TR50 Δ*cpxR*	This study
MC4100	F- *araD139*Δ(*argF-lac*)U169 *rpsL150*(Str^R) *relA1 flbB5301 decC1 ptsF25 rbsR*	(6)
RM53	TR50 Δ*cpxA*	This study
SV28	ALN194 pTrc99A	This study
SV29	ALN194 pCpxP	This study
TR50	MC4100 *cpxP'-'lacZ*	(34)
TR71	MC4100 *rpoHp3'-'lacZ*	This study
Plasmids	**Description**	**Source or reference**
pBR322	High-copy IPTG-inducible vector, Amp^R	New England BioLabs
pLD404	High-copy constitutive expression of *nlpE*	(40)
pTrc99A	High-copy IPTG-inducible vector, Amp^R	Pharmacia
pKDS301	High-copy IPTG-inducible expression of *bfpA*	(28)
pCpxP	High-copy IPTG-inducible expression of *cpxP*	(35)
pJW15	Vector bearing a promoterless *luxCDABE* fusion	(32)
pJW15-*zntA*p	Vector bearing 525bp upstream of *zntA* and the first two codons of the open reading frame fused to *luxCDABE*	This study
pCA24N	High-copy IPTG-inducible vector, Cam^R	(17)
pCA-*nlpE*	High-copy IPTG-inducible over-expression of *nlpE*	(17)

2.6. Figures

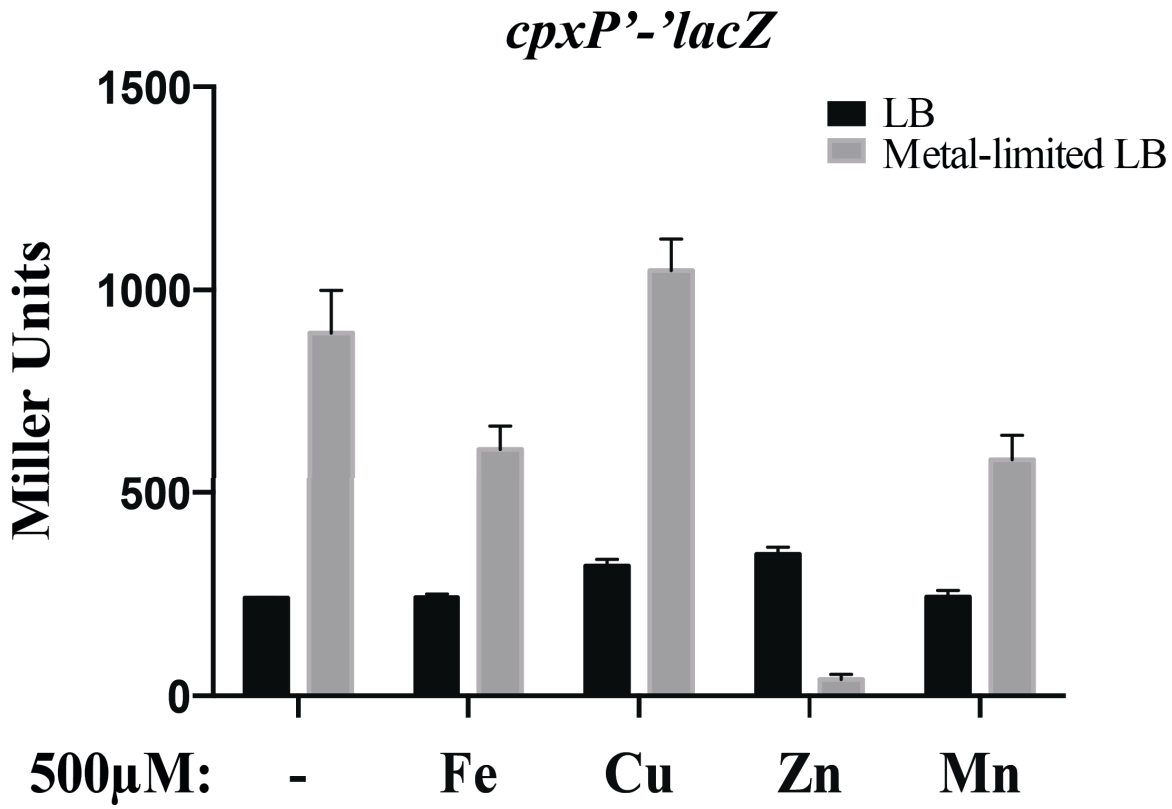
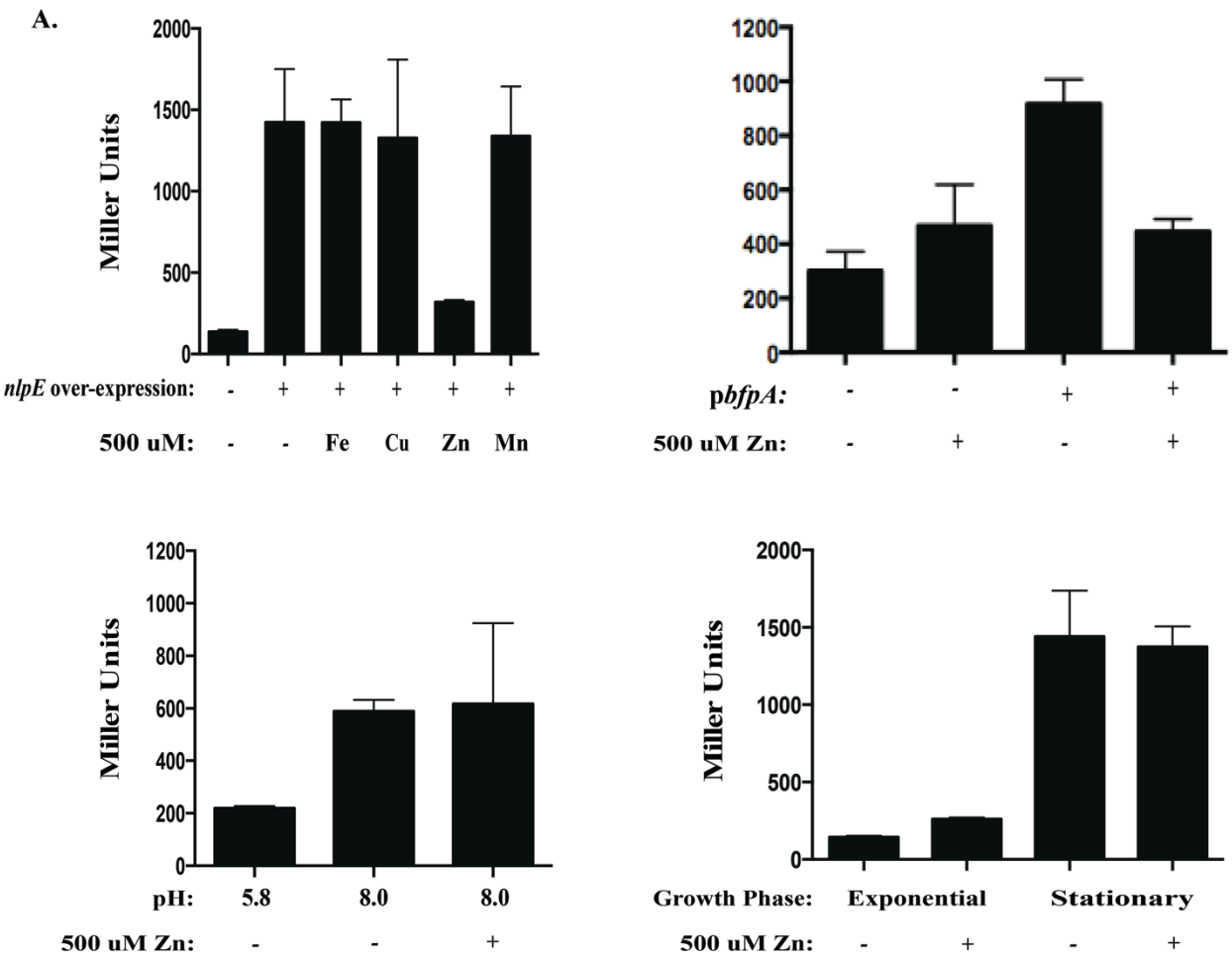


Figure 2-1: Metal chelation induces the Cpx envelope stress response and addition of exogenous zinc suppresses the activation.

Average β-galactosidase activity of TR50 grown in untreated (dark bars) or metal-limited LB (grey bars) in the presence of metal salts (indicated above) or MilliQ (-). The average β-galactosidase activity and standard deviation of three technical replicates per sample is represented by each bar and error bar, respectively. A representative result of two independent experiments is shown.


A.
Miller Units
2000
1500
1000
500
0
nlpE over-expression: - + + + + +
500 uM: - - Fe Cu Zn Mn
1200
1000
800
600
400
200
0
pbfpA: - - + +
500 uM Zn: - + - +
Miller Units
1200
1000
800
600
400
200
0
pH: 5.8 8.0 8.0
500 uM Zn: - - +
Miller Units
2000
1500
1000
500
0
Growth Phase: Exponential Stationary
500 uM Zn: - + - +

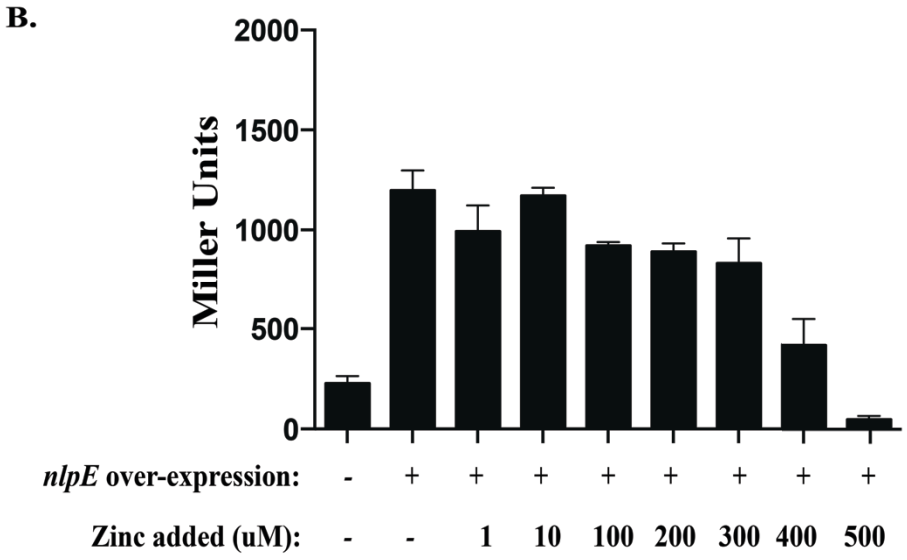

B.
Miller Units
2000
1500
1000
500
0
nlpE over-expression: - + + + + + + + +
Zinc added (uM): - - 1 10 100 200 300 400 500

Figure 2-2: Addition of exogenous zinc suppresses activation of the Cpx pathway by *nlpE* and *bfpA* over-expression but not alkaline pH or entry into stationary phase.

A. The addition of zinc reverses the activation of the *cpxP'-'lacZ* reporter by some but not all Cpx inducing cues. β-galactosidase activity of TR50 over-expressing *nlpE* (JLW62) or the vector control (JLW65), *bfpA* (JLW64) or the vector control (DB12), grown in 100mM sodium-phosphate buffered LB pH 8.0 or 5.8 (final concentration), and harvested in exponential (2h) or at the entry to stationary phase (4h) in the presence or absence of added metal salts at the concentration indicated. **B.** β-galactosidase activity of TR50 in the presence of 0, 1, 10, 100, 200, 300, 400, or 500μM zinc sulfate (final concentration) was added. The average β-galactosidase activity and standard deviation of three technical replicates for each sample is represented by each bar and error bar, respectively. A representative result of two independent experiments is given for each graph.

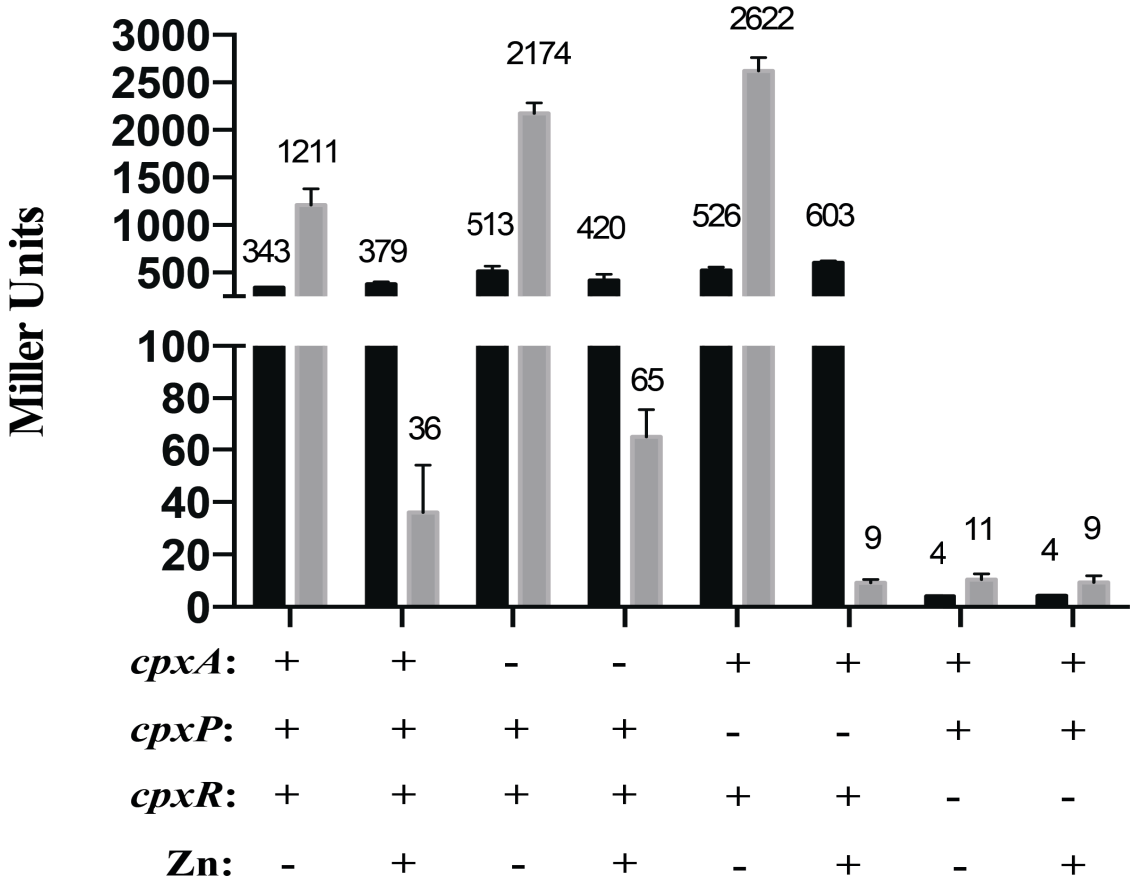


Figure 2-3: Activation of the Cpx pathway by metal limitation depends on *cpxR* but not *cpxA* or *cpxP*.

β-galactosidase activity of wild-type (TR50), *cpxA* (RM53), *cpxP* (JLW392), or *cpxR* (JSW1) mutant cells grown in untreated (dark bars) or metal-limited LB (grey bars). 500μM zinc sulfate (final concentration) was added to samples where indicated. The average β-galactosidase activity is given above each bar and the error bars represent the standard deviation of three technical replicates per samples. A representative result of three independent experiments is given.

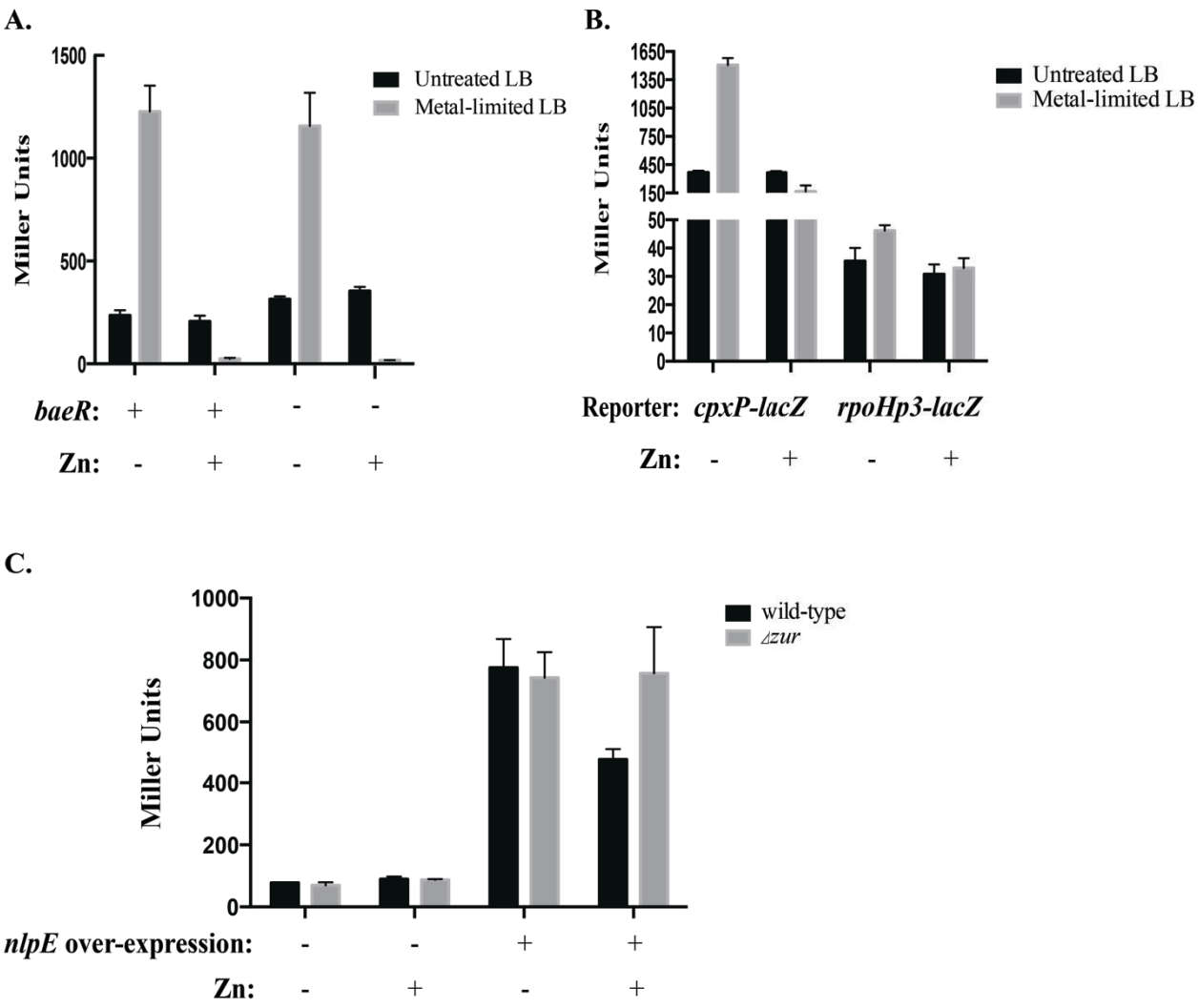


Figure 2-4: The role of zinc-responsive transcription factors on activating the Cpx pathway in response to cellular metal status.

A. *Left.* The Cpx pathway does not depend on *baeR* or activation of σ^E to respond to metal limitation or excess zinc. β-galactosidase activity of wild-type (TR50) and *baeR* (JLW970a) in metal-limited (light grey bars) and untreated LB (dark grey bars). *Right.* Activity of Cpx- (TR50) and σ^E-dependent (TR71) reporters in response to metal limitation (light grey bars) and the addition of zinc (dark bars). **B.** The *cpxP'-'lacZ* reporter requires the zinc uptake regulator Zur to respond to zinc. β-galactosidase activity of wild-type and *zur* mutant over-expressing pCA-*nlpE*

(JSW2, JLW913) or the vector control (JLW7, JLW912). Zinc sulfate was added to cultures at the final concentrations indicated above. For A and B, the average β-galactosidase activity and standard deviation of three technical replicates per sample are represented by each bar and corresponding error bar, respectively. A representative result of two independent experiments is shown here.

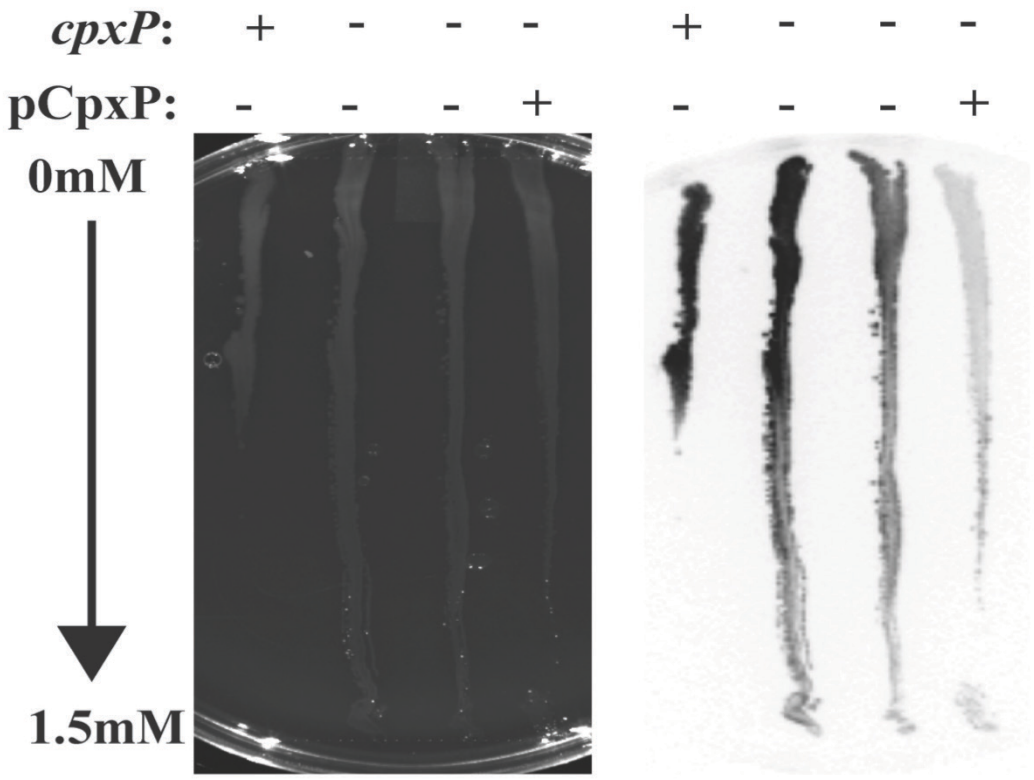


Figure 2-5: The *cpxP* mutant is resistant to zinc and over-expression of *cpxP* decreases zinc efflux protein expression.

Survival (left panel) and expression of a *zntA-lux* reporter (right panel) in a wild-type (JLW851), *cpxP* mutant (JLW852), *cpxP* mutant bearing the vector control pTrc99A (JLW858), and the complemented *cpxP* mutant bearing the pCpxP plasmid (JLW859) on LB agar containing a linear gradient of zinc sulfate (maximum concentration of 1.5 mM). *zntA-lux* expression was measured by imaging the luminescence produced using the Chemi-Doc Imager (Bio-Rad). A representative result of three independent experiments is shown above.

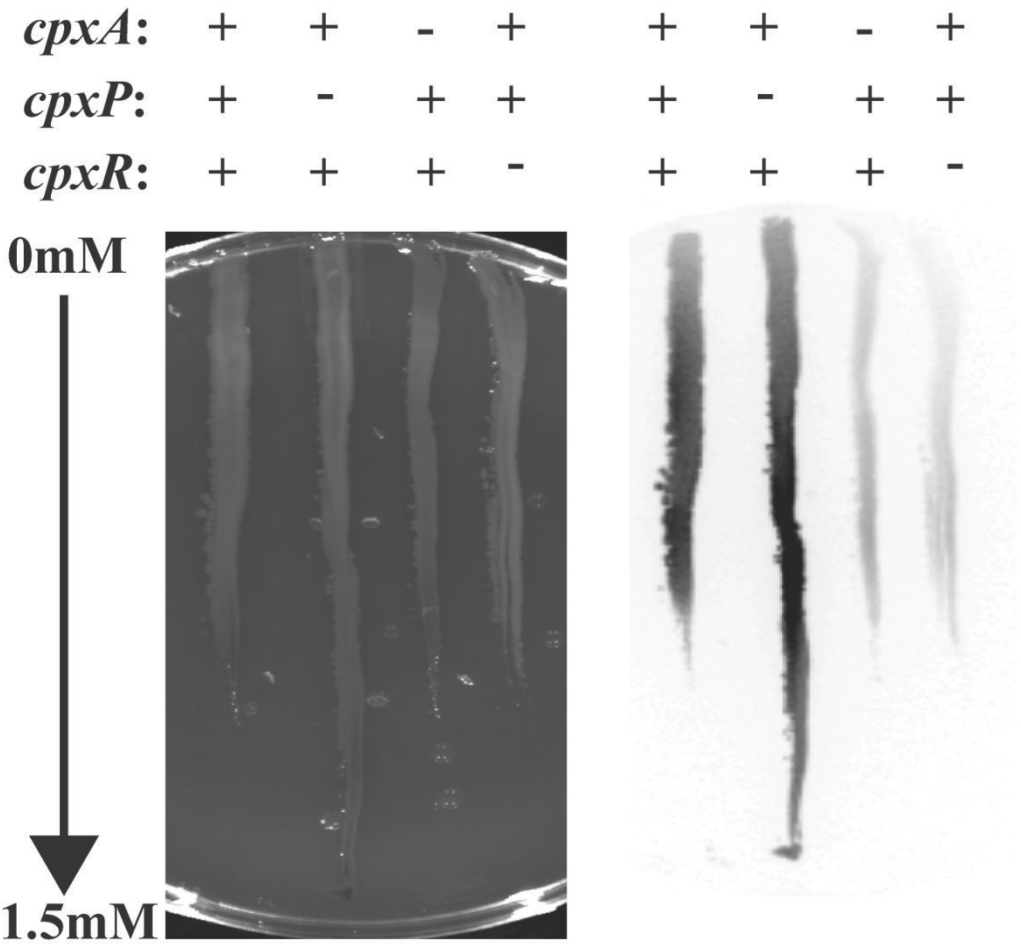


Figure 2-6: Mutations in multiple components of the Cpx TCS impact zinc efflux.

Survival (left panel) and *zntA-lux* expression (right panel) of wild-type (JLW851), *cpxP* (JLW852), *cpxA* (JLW982), and *cpxR* mutant (JLW983) on LB agar containing a gradient of zinc sulfate (maximum concentration of 1.5 mM). Expression of the *zntA-lux* reporter was assessed by imaging the luminescence produced using the Chemi-Doc Imager (Bio-Rad). A representative result of two independent experiments is shown here.

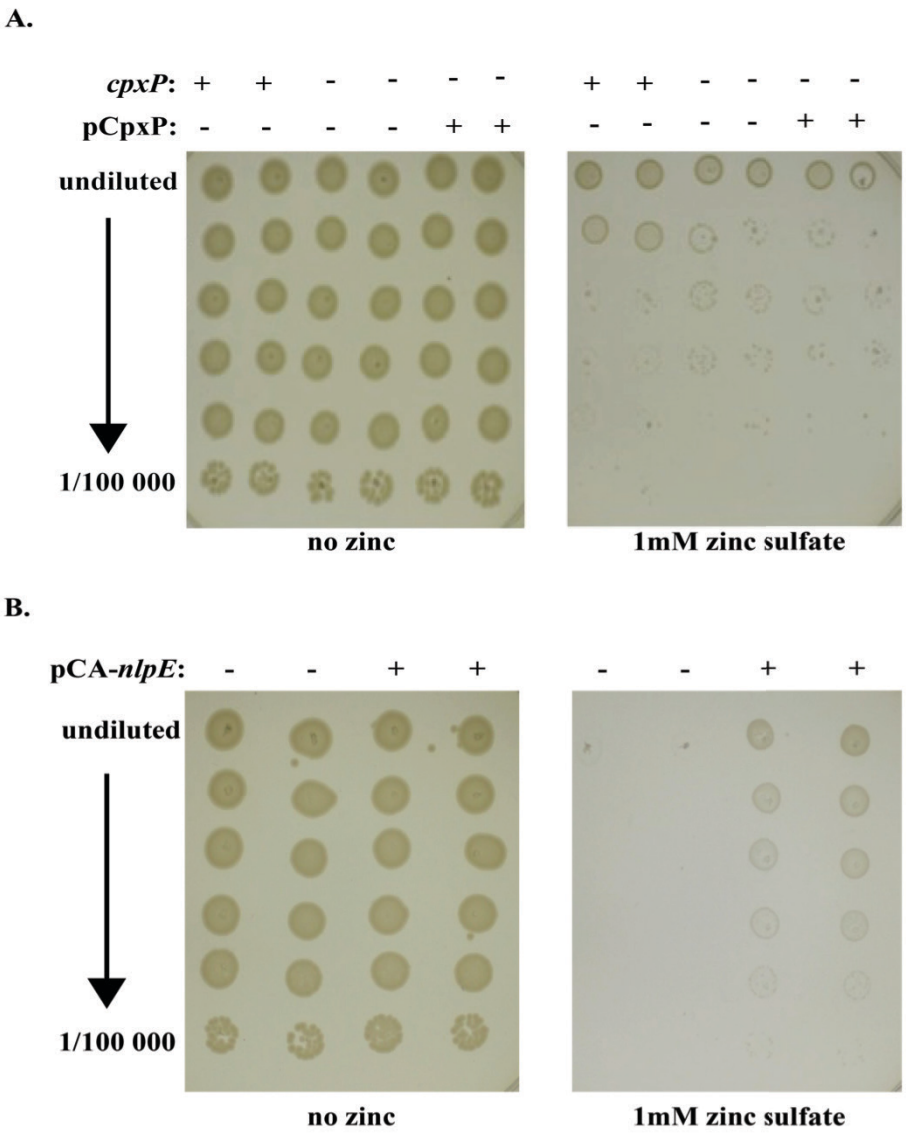


Figure 2-7: Activation of the Cpx pathway in EPEC leads to zinc resistance.

A. Insertional inactivation of *cpxP* does not alter the resistance of EPEC to zinc. The survival of wild-type (E2348/69), *cpxP:kn* (SV28), and the complemented mutant (SV29) EPEC or **B**. EPEC over-expressing *nlpE* (JLW117) or the vector control (ALN239) on LB agar containing 1mM zinc sulfate (final concentration) is shown above. Representative results for two independent experiments for A and B are shown above.

2.7. References

1. **Acosta N., S. Pukatzki, and T. L. Raivio.** 2014. The *Vibrio cholerae* Cpx envelope stress response senses and mediates adaptation to low iron. J Bacteriol . **197(2)**: 262-76.

2. **Becker K. W., and E. P. Skaar.** 2014. Metal limitation and toxicity at the interface between host and pathogen. FEMS Microbiol Rev. **38(6)**: 1235-49.

3. **Bina X. R., D. Provenzano, N. Nguyen, and J. E. Bina.** 2008. *Vibrio cholerae* RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. Infect Immun **76**:3595-3605.

4. **Brocklehurst K. R., J. L. Hobman, B. Lawley, L. Blank, S. J. Marshall, N. L. Brown, and A. P. Morby.** 1999. ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. Mol Microbiol **31**:893-902.

5. **Buelow D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J Bacteriol **187**:6622-6630.

6. **Casadaban M. J., and S. N. Cohen.** 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 **76**:4530-4533.

7. **Cerasi M., S. Ammendola, and A. Battistoni.** 2013. Competition for zinc binding in the host-pathogen interaction. Front Cell Infect Microbiol **3**:108.

8. **Cheung J., C. A. Bingman, M. Reyngold, W. A. Hendrickson, and C. D. Waldburger.** 2008. Crystal structure of a functional dimer of the PhoQ sensor domain. J Biol Chem **283**:13762-13770.

9. **Danese P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli.* J Bacteriol **180**:5875-5884.

10. **Danese P. N., W. B. Snyder, C. L. Cosma, L. J. Davis, and T. J. Silhavy.** 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. Genes Dev **9**:387-398.

11. **Debnath I., J. P. Norton, A. E. Barber, E. M. Ott, B. K. Dhakal, R. R. Kulesus, and M. A. Mulvey.** 2013. The Cpx stress response system potentiates the fitness and virulence of uropathogenic *Escherichia coli*. Infect Immun **81**:1450-1459.

12. **DiGiuseppe P. A., and T. J. Silhavy.** 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol **185**:2432-2440.

13. **Graham A. I., G. Sanguinetti, N. Bramall, C. W. McLeod, and R. K. Poole.** 2012. Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of the bacterial response to zinc reveals transient behaviour of the Fur and SoxS regulators. Microbiology **158**:284-292.

14. **Hood M. I., and E. P. Skaar.** 2012. Nutritional immunity: transition metals at the pathogen-host interface. Nat Rev Microbiol **10**:525-537.

15. **Jones C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren.** 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J **16**:6394-6406.

16. **Kershaw C. J., N. L. Brown, C. Constantinidou, M. D. Patel, and J. L. Hobman.** 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. Microbiology **151**:1187-1198.

17. **Kitagawa M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori.** 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**:291-299.

18. **Lasaro M., Z. Liu, R. Bishar, K. Kelly, S. Chattopadhyay, S. Paul, E. Sokurenko, J. Zhu, and M. Goulian.** 2014. *Escherichia coli* isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts. J Bacteriol **196**:1723-1732.

19. **Leblanc S. K., C. W. Oates, and T. L. Raivio.** 2011. Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. J Bacteriol **193**:3367-3375.

20. **Lee L. J., J. A. Barrett, and R. K. Poole.** 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J Bacteriol **187**:1124-1134.

21. **Levine M., D. Nalin, R. Hornick, E. Bergquist, D. Waterman, C. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. The Lancet **311**:1119-1122.

22. **MacRitchie D. M., J. D. Ward, A. Z. Nevesinjac, and T. L. Raivio.** 2008. Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic *Escherichia coli*. Infect Immun **76**:1465-1475.

23. **MacRitchie D. M., N. Acosta, and T. L. Raivio.** 2012. DegP is involved in Cpx-mediated posttranscriptional regulation of the type III secretion apparatus in enteropathogenic *Escherichia coli*. Infect Immun **80**:1766-1772.

24. **Ma Z., M. J. Faulkner, and J. D. Helmann.** 2012. Origins of specificity and cross-talk in metal ion sensing by *Bacillus subtilis* Fur. Mol Microbiol **86**:1144-1155.

25. **Ma Z., P. Chandrangsu, T. C. Helmann, A. Romsang, A. Gaballa, and J. D. Helmann.** 2014. Bacillithiol is a major buffer of the labile zinc pool in *Bacillus subtilis*. Mol Microbiol .

26. **Mellies J. L., K. Thomas, M. Turvey, N. R. Evans, J. Crane, E. Boedeker, and G. C. Benison.** 2012. Zinc-induced envelope stress diminishes type III secretion in enteropathogenic *Escherichia coli*. BMC Microbiol **12**:123.

27. **Nevesinjac A. Z., and T. L. Raivio.** 2005. The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic *Escherichia coli*. J Bacteriol **187**:672-686.

28. **Nevesinjac A. Z., and T. L. Raivio.** 2005. The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic *Escherichia coli*. J Bacteriol **187**:672-686.

29. **Outten F. W., D. L. Huffman, J. A. Hale, and T. V. O'Halloran.** 2001. The Independent *cue* and *cus* Systems Confer Copper Tolerance during Aerobic and Anaerobic Growth in *Escherichia coli*. J Biol Chem **276**:30670-30677.

30. **Patzer S. I., and K. Hantke.** 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in Escherichia coli. Mol Microbiol **28**:1199-1210.

31. **Pontel L. B., N. L. Scampoli, S. Porwollik, S. K. Checa, M. McClelland, and F. C. Soncini.** 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**:1659-1669.

32. **Price N. L., and T. L. Raivio.** 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol **191**:1798-1815.

33. **Raffa R. G., and T. L. Raivio.** 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol Microbiol **45**:1599-1611.

34. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

35. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

36. **Raivio T. L., S. K. Leblanc, and N. L. Price.** 2013. The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. J Bacteriol **195**:2755-2767.

37. **Rosner J. L., and R. G. Martin.** 2013. Reduction of cellular stress by TolC-dependent efflux pumps in *Escherichia coli* indicated by BaeSR and CpxARP activation of spy in efflux mutants. J Bacteriol **195**:1042-1050.

38. **Slamti L., and M. K. Waldor.** 2009. Genetic analysis of activation of the *Vibrio cholerae* Cpx pathway. J Bacteriol **191**:5044-5056.

39. **Slauch J. M., and T. J. Silhavy.** 1991. Genetic fusions as experimental tools. Methods Enzymol **204**:213-248.

40. **Snyder W. B., L. J. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy.** 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J Bacteriol **177**:4216-4223.

41. **Sobota J. M., and J. A. Imlay.** 2011. Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. Proc Natl Acad Sci U S A **108**:5402-5407.

42. **Storz G., Y. I. Wolf, and K. S. Ramamurthi.** 2014. Small proteins can no longer be ignored. Annu Rev Biochem **83**:753-777.

43. **Vance C. K., and A. F. Miller.** 2001. Novel insights into the basis for *Escherichia coli* superoxide dismutase's metal ion specificity from Mn-substituted FeSOD and its very high E(m). Biochemistry **40**:13079-13087.

44. **Vogt S. L., A. Z. Nevesinjac, R. M. Humphries, M. S. Donnenberg, G. D. Armstrong, and T. L. Raivio.** 2010. The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. Mol Microbiol **76**:1095-1110.

45. **Wang D., and C. A. Fierke.** 2013. The BaeSR regulon is involved in defense against zinc toxicity in *E. coli.* Metallomics **5**:372-383.

46. **Wolfe A. J., N. Parikh, B. P. Lima, and B. Zemaitaitis.** 2008. Signal integration by the two-component signal transduction response regulator CpxR. J Bacteriol **190**:2314-2322.

47. **Yamamoto K., and A. Ishihama.** 2005. Transcriptional response of *Escherichia coli* to external copper. Mol Microbiol **56**:215-227.

48. **Yamamoto K., H. Ogasawara, and A. Ishihama.** 2008. Involvement of multiple transcription factors for metal-induced *spy* gene expression in *Escherichia coli*. J Biotechnol **133**:196-200.

49. **Yang, H. H., Morrow, J. B., Grasso, D., Vinopal, R. T., and B. F. Smets.** 2006. Intestinal versus external growth conditions change the surficial properties in a collection of environmental *Escherichia coli* isolates. Environ Sci Technol **40(22)**: 6976-82.

Chapter 3: Mutational analysis of the zinc-binding chaperone-inhibitor CpxP in *Escherichia coli*[1]

[1] $CpxPR_{60}Q$ was cloned into pGEX-6P-1 and purified by Gina Thede

3.1. Introduction

Bacterial signal-sensing is crucial for adaptation and survival. Two-component systems (TCS) are found in all domains of life and are a common way for bacteria to sense environmental changes and elicit physiological responses to adapt to those changes. The Cpx TCS in *Escherichia coli* senses signals related to protein-folding stress in the Gram-negative envelope, including alkaline pH, the over-expression of the lipoprotein *nlpE*, and heterologous expression of pilin subunits in the absence of their cognate chaperones (10, 15, 24). The inner membrane sensor kinase CpxA senses signals and transduces the signal to the cytoplasmic response regulator CpxR through a conserved phosphotransfer (20). Phosphorylated CpxR up-regulates the expression of over 100 genes, including the chaperones *cpxP* and *spy* (17, 22) .

CpxP is a periplasmic protein that inhibits the activation of the Cpx pathway, likely by interacting directly with the periplasmic sensing domain of CpxA and preventing activation of the kinase (5, 28) . CpxP also exhibits weak chaperone activity *in vitro* as compared to the paralogous chaperone Spy (19). Homologues of CpxP are identified by the conserved LTXXQ motifs, though the function of the motifs is unknown. Spy is closely related to CpxP, sharing 29% sequence identity and a similar 3-D structure (19, 25, 31) but, unlike CpxP, has no known signal transduction role in *E. coli* (19). Both CpxP and Spy possess two LTXXQ motifs, one at the N-terminus with divergent XX residues and one at the C-terminus with an absolutely conserved sequence. The N- and C-terminal LTXXQ sequences of different protomers of the dimer interact via extensive hydrogen bonding contacts between the glutamine residues, thus keeping the dimer structure intact (25). The variable XX residues of each motif face the solvent and are therefore available for protein-protein interactions.

The structures of ZraP from *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and CnrX from *Cupriavidus metallidurans* also share homology with CpxP without sharing significant sequence identity (25). ZraP is a periplasmic protein that binds zinc as a decamer and over-expression of ZraP inhibits the ZraSR TCS in a manner analogous to CpxP and the CpxAR TCS (1). CnrX, a membrane-bound protein, binds nickel and cobalt and interacts with the membrane-bound anti-sigma factor CnrY to facilitate the release of the sigma factor CnrH into the cytoplasm. Release of CnrH leads to the transcription of cobalt and nickel efflux genes required to resist heavy metal toxicity (27). Thus, with the exception of Spy, the known structural homologues of CpxP are periplasmic metal-binding proteins that regulate cellular signal-transduction.

The expression of *cpxP* and *spy* is up-regulated by excess zinc and copper in *E. coli* (7, 12, 13, 29, 30). The addition of zinc or copper to LB medium induces the expression of Cpx-regulated chaperones and proteases in *S.* Typhimurium (16). In contrast, we have shown that metal chelation activates the expression of *cpxP* and that this activation is reversed by the addition of excess zinc (Figure 2-1). Furthermore, deletion of *cpxP* leads to zinc resistance in the laboratory strain of *E. coli* K-12 (Figure 2-6), perhaps suggesting that elevated *cpxP* expression is toxic to the cell in the presence of high levels of zinc (Chapter 2).

We wished to identify the residues that contribute to the zinc-associated functions of CpxP. We confirmed that CpxP binds zinc and sought to mutate the residues responsible for zinc-binding. Mutation of the putative zinc-binding residue E_{79} resulted in only a modest effect on inhibitor function and no effect on chaperone activity or the zinc-resistance of a *cpxP* mutant. We also examined the N-terminal LTXXQ motifs of CpxP and Spy because they are conserved in CpxP homologues and because the variable XX residues face the solvent and are available for

potential protein-protein interactions. We show that alanine substitutions of the variable XX residues have no effect on the inhibitor or zinc-associated functions of CpxP but positively affect chaperone activity. In contrast, replacement of the N-terminal XX residues of Spy with those of CpxP negatively affected the signaling and chaperone activities of Spy. Over-expression of *spy* could restore zinc sensitivity to the *cpxP* mutant but mutations in the XX residues of Spy abolished the ability of Spy over-expression to complement this phenotype. We propose a model where the putative zinc-binding residue E_{79} primes CpxP to leave CpxA by communicating the oligomer or zinc-binding status of CpxP to the residues that mediate inhibition. In contrast, we provide evidence for a substrate-binding role of the variable residues in the N-terminal LTXXQ motif that does not affect the function of the proximal residues that mediate inhibition.

3.2. Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 3-1. Mutant bacterial strains were generated by standard genetic techniques. Mutant plasmids were generated using either the Quikchange Lightning Site-directed Mutagenesis Kit (Agilent) or a two-step PCR method (11) as indicated in Table 3-2. For two-step PCR, the first reaction amplified two fragments, each containing the mutation of interest and a restriction site. The first reaction was prepared as per the manufacturer's recommendations for recombinant Taq polymerase (Invitrogen) and the PCR cycling was carried out as follows: 30 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute, followed by 4 minutes at 72°C. Reactions were purified using the QIAquick PCR purification kit according to the manufacturer's instructions (QIAgen). 0.5μL of each fragment was added as template for the second reaction, which was performed as follows: 35 cycles of 95°C for 30 seconds, 50°C for 45 seconds, and 72°C for 90 seconds, followed by 10 minutes at 72°C. The second PCR product

was assessed by gel electrophoresis and the product was purified using the QIAquick PCR Purification kit (QIAgen). Molecular cloning was performed by standard genetic techniques.

Growth conditions and media. Unless otherwise specified, all strains were grown in Luria-Bertani (LB) broth or on LB agar at 37°C with aeration (225 rpm). 10 μM or 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) was added to LB agar as indicated. Antibiotics were added where appropriate at the following concentrations: 50 μg/mL kanamycin and 100 μg/mL ampicillin (all Sigma).

β-galactosidase assays. Single isolated colonies were inoculated into 2 mL LB broth plus antibiotics in 5 mL glass plating tubes and grown overnight at 37°C with aeration, in quintuplicate. The next morning, overnight cultures were diluted 1:50 into 2 mL fresh LB plus antibiotics in 5 mL glass plating tubes and grown for 3.5 h at 37°C with aeration by fixing a test tube rack to the platform of an orbital shaker set at 225 rpm. 0.1mM IPTG (final concentration) was added and cultures were returned to the incubator for an additional hour. Cells were harvested and β-galactosidase activity was assayed by the microtitre plate assay, as described previously (23).

Protein purification. Proteins were purified as described previously (25) with the following modifications: cell pellets were frozen and thawed before sonication to increase the efficiency of lysis and cleavage of the GST tag was performed on ice in a room kept at 4°C for 24-32 hours. Cleavage progress was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie blue to detect protein species.

Far-UV circular dichroism (Far-UV CD). Protein samples were diluted to 0.270 mg/mL in 50 mM sodium phosphate buffer pH 5.8 containing 250 mM NaCl in a thermostated fused silica cell with a path length of 0.05 cm. Far-UV CD experiments were performed on a Jasco

J720 spectropolarimeter from 250-190 nm at room temperature. Spectra represent an average of 3 scans per sample.

Luciferase refolding assays. Luciferase refolding assays were performed by modifying the protocols of Chen *et al.* (2011) (4) and the Molecular Probes ATP Determination Kit (Invitrogen). Briefly, 1X reaction buffer was prepared (for just over 1mL of reaction buffer): 550 µL of MilliQ water, 440 µL of 1X reaction buffer prepared from the 20X stock solution in the ATP determination kit (500 mM tricine buffer pH 7.8, 100 mM $MgSO_4$, 2 mM EDTA, 2 mM sodium azide, final concentrations), 10 µL 0.1M DTT, 1 µL 5 mM ATP, and 5 µL of 3 mg/mL D-luciferin. The appropriate amount of reaction buffer was aliquoted into a black 96-well microtitre plate and incubated at 45°C in a Wallac^2 1420 plate reader (Perkin Elmer) for 20 minutes to pre-warm the reaction. 10nM purified chaperone proteins (final concentration) were diluted in buffer and 5nM firefly luciferase was added (final concentration) to every reaction into a final reaction volume of 200 µL just before the microplate was inserted into the plate reader for measurements. One well contained only 200µL reaction buffer (buffer control). Luminescence was monitored every 30 seconds for 20 minutes. The time point at which luciferase exhibited 0-10% of the initial activity and that the positive control (Spy-treated) reactions exhibited higher luminescence than the CpxP-containing reactions from each assay was considered for analysis (approximately 10 minutes for each assay). Three independent experiments were performed and the results of two assays are given in Figure 2-1.

Inductively-coupled plasma mass spectrometry (ICP-MS). 30 µg of purified protein samples were diluted in 1 mL 3.5% HNO_3 (Sigma) in an acid-washed 1.5 mL eppendorf tube and boiled at 95°C for 30 minutes in the presence or absence of 1 mM EDTA. Samples were spun at 16 100 *g* for 20 minutes to pellet insoluble material. The supernatant was filtered through a 0.2

μm pore filtration device (Millipore) into an acid-washed 15 mL conical tube to ensure that no insoluble debris will interfere with the instruments. The filtrate was diluted to 2 mL with 3.5% HNO_3. The entire 2 mL sample was analyzed for ion content on a Nu Plasma 1 Multi-Collector Inductively Coupled Plasma Mass Spectrometer at the Canadian Center for Isotopic Microanalysis (CCIM).

Calculation of zinc molecules per CpxP molecule was performed by calculating the molecules of zinc present in a 2 mL sample containing 4.4 ppm (((4.4 mg/L x 2mL)/65.38 g/mol) x 6.02×10^{23} molecules per mol = 8.1028×10^{16} molecules) and the molecules of CpxP present in a 30 μg sample (((30 μg/17 000 kDa or g/mol)) x 6.02×10^{23} molecules per mol = 1.062×10^{15} molecules). The number of zinc molecules divided by the number of CpxP molecules yields a ratio of 76.4 zinc molecules per CpxP molecules.

Gradient zinc assays. Gradient zinc plates were prepared as follows: two flasks of equal volumes of LB agar were prepared and sterilized as per the manufacturer's instructions (Sigma). Agar was cooled to 55-65°C and 0.1mM IPTG and 20μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were added aseptically (both Sigma, final concentrations). 20 mL of LB agar containing IPTG and X-gal was poured on a slant from a maximum height of 8 mm and dried for 1-1.5 h at room temperature. 2 mM zinc sulfate (final concentration) was prepared fresh and added to the other flask of agar in addition to IPTG and X-gal. Dried plates were placed on a level surface and 20 mL of zinc-containing agar was poured over the dried LB agar to create a smooth surface. Plates were dried 16-20 hours at room temperature. The next day, sterile inoculating loops were used to streak aliquots of overnight cultures of each strain across the surface of the gradient plates. Plates were dried upright for approximately 30 minutes at room temperature then turned upside down and incubated at 37°C overnight. Each strain was assayed

in biological duplicate in two independent experiments. Plates were imaged on the Start-It Colony Counter imager (Fisher) and luminescence was detected using the Chemi-Doc Imaging system (Bio-Rad). Experiments were performed at least twice and representative results are shown.

Zinc plate assays. Single isolated colonies were inoculated into 2 mL LB broth in a 5 mL glass plating tube in duplicate and grown overnight at 37°C with aeration by fixing a test tube rack to the platform of an orbital shaker set at 225 rpm. The next morning, the cultures were standardized to the lowest A_{600} and 5 μL aliquots were spotted on LB agar containing 10 μM IPTG, 20 μg/mL X-gal, with or without 1 mM $ZnSO_4$. Plates were dried upright at room temperature then incubated at 37°C overnight. Plates were imaged using the Start-It Colony Counter imager (Fisher).

3.3. Results

3.3.1. Purified CpxP binds zinc

Previously, we showed that CpxP has a potential role in cellular zinc trafficking in the laboratory strain of *E. coli* (Figure 2-5) and we hypothesized that CpxP might bind zinc to alter cellular zinc trafficking. To test our hypothesis, we purified CpxP lacking the periplasmic targeting sequence ($CpxP_{20\text{-}166}$) fused to glutathione-*S*-transferase (GST) from the cytoplasm under non-denaturing conditions as described previously (25), then cleaved off the GST tag and isolated CpxP-containing fractions by size-exclusion chromatography. We then performed inductively-coupled plasma mass spectrometry (ICP-MS) on the purified protein samples. ICP-MS allows for the unambiguous identification and quantification of metal cation species present in an aqueous sample. In 30 μg samples of purified CpxP, an average of 4.31 ppm zinc was detected (Table 3-3) or approximately 76.4 zinc ions per CpxP molecule. In contrast, only 0.355

ppm zinc was detected in CpxP protein treated with 1mM EDTA or 6.2 zinc ions per CpxP monomer (Table 3-3). We measured the amounts of over 20 metal ion species in our purified CpxP protein samples but only the levels of zinc were dramatically increased between protein-containing samples and controls containing only buffer or the EDTA-treated protein-containing samples (Table 3-3). Therefore, we conclude that CpxP binds zinc, though we were unable to determine the stoichiometry of zinc-binding due to high background and the sensitivity of ICP-MS.

3.3.2. Mutation of CpxP and Spy

The ICP-MS results suggested that levels of zinc were elevated in CpxP-containing samples and we sought to determine the physiological relevance of these finidngs. Therefore, we next examined the CpxP structure for potential zinc binding sites (PDB ID: 3QZC). However, there are no cysteines in CpxP and no histidine-rich sites in the protein, suggesting that CpxP does not bind zinc using canonical zinc-binding motifs. We previously noted multiple potential zinc-binding sites in our crystal structure of CpxP that could mediate reversible zinc-binding (25). However, a high concentration of zinc was present in the crystallization buffer and the physiological relevance of zinc ions in the crystal structure was not previously determined (25). One half-site consisting of the residues E_{79} from one protomer and H_{136} from the other protomer is symmetrical in the dimer structure (Figure 3-1) (25), suggesting it may have biological relevance. We introduced alanine residues at E_{79} and H_{136} into *cpxP*, cloned each of the mutant genes into a vector to express CpxP fused to GST, and purified the proteins as described for wild-type CpxP (25).

We also examined the conserved N-terminal LTXXQ motif for the contribution of the variable XX residues to protein function (Figure 2-1). We reasoned that the variable XX residues

in the conserved motif used to identify CpxP homologues might contribute to one of the functions of CpxP and its metal-binding paralogues. CpxP shares the most structural similarity to Spy, sharing a root mean square deviation (rmsd) of 1.4Å over 91 superposed α-carbons (25). Therefore, we introduced alanine substitutions into the variable residues in the N-terminal LTXXQ motif of CpxP (CpxPAA) and replaced the N-terminal XX residues of Spy with those of CpxP (SpyEH) (Figure 3-1). These mutations would allow us to test whether the identity of the N-terminal XX residues of CpxP are necessary or sufficient for a given function because Spy does not possess the same signaling nor chaperone activities as CpxP.

Previously, we identified the *cpxP*R_{60}Q mutation in a screen for mutations in CpxP that ablate the inhibitory function of CpxP (2) (Figure 3-1). We cloned the *cpxP*AA, *spy*EH, and R_{60}Q mutations into a vector encoding an N-terminal fusion to GST and purified these proteins as well. We confirmed that CpxPE_{79}A and CpxPH_{136}A were expressed by Western blot (Figure 3-2A) and that there were no gross structural changes in the CpxPAA and SpyEH mutant proteins by performing far-UV CD (Figure 3-2B).

3.3.3. Residues in the N-terminal LTXXQ motif affect the chaperone activities of CpxP and Spy

We first asked which, if any, residues in CpxP and Spy were required for the wild-type chaperone activity of CpxP and Spy. In order to do this, we developed a chaperone assay in which luminescence was measured from firefly luciferase that was thermally denatured at 45°C in the presence or absence of purified chaperone proteins in a molar ratio of 1:2 luciferase to chaperones. In the presence of strong chaperones, luciferase will retain more luminescence than in the presence of weaker or no chaperones. We monitored luminescence over 20 minutes and analyzed the time point at which 0 to 10% of the initial luciferase activity was retained and that

reactions treated with the strong chaperone Spy exhibited higher luminescence than reactions treated with the weaker chaperone CpxP. Luciferase denatured in the presence of CpxP or Spy retained an average of 1.8- and 6-fold more luminescence than luciferase denatured in the absence of chaperones (Figure 3-3). These observations agree with published reports that Spy is a more robust chaperone than CpxP *in vitro* (19). Similar amounts of luminescence were recovered from luciferase denatured in the presence of CpxPE_{79}A or CpxPH_{136}A as luciferase treated with wild-type CpxP (Figure 3-3), suggesting the residues that bind a zinc ion in the crystal structure do not impact chaperone activity. CpxPR_{60}Q, a mutant previously shown to ablate the activity of CpxP to inhibit CpxA (2) resulted in an average increase of 4.2-fold luminescence recovered in our assay (Figure 3-3). Similarly, the CpxPAA mutant protein resulted in 5-fold more luminescence recovered as compared to luciferase denatured without chaperones (Figure 3-3). Therefore, the R_{60}Q and E_{53}A/H_{54}A mutations that lie in a conserved N-terminal α-helix positively impact the chaperone activity of CpxP. In the presence of SpyEH, 2-fold less luminescence was recovered from thermally denatured luciferase than in the presence of wild-type Spy (Figure 3-3), suggesting that the identity of the variable XX residues in both CpxP and Spy impact the chaperone activities of the proteins. Thus, mutation of the putative zinc-binding residues of CpxP do not measurably impact chaperone activity in our assay but mutations in the conserved N-terminal α-helix positively affect the chaperone activity of CpxP and negatively affect the chaperone activity of Spy *in vitro*.

3.3.4. Predicted zinc-binding residues and R_{60} proximal to the N-terminal LTXXQ motif are required for robust inhibitor activity of CpxP

To examine whether the amino acids involved in zinc-binding in the crystal structure of CpxP alter the signaling activities of CpxP, we introduced the E_{79}A, R_{60}Q, and E_{53}A/H_{54}A

mutations into the high-copy IPTG-inducible pCpxP plasmid (21) and the $D_{59}E/A_{60}H$ mutation into the pSpy plasmid with the same plasmid backbone as pCpxP (2) and over-expressed the wild-type and mutant proteins in reporter strains bearing a Cpx-regulated *cpxP'-'lacZ* reporter. In a background lacking a copy of the inhibitor *cpxP*, over-expression of wild-type *cpxP* reduced *cpxP'-'lacZ* activity approximately 3-fold, consistent with previous reports (Figure 3-4A). Over-expression of *cpxP*E_{79}A, however, resulted in only 1.8-fold inhibition of *cpxP* expression as compared to the isogenic vector control strain (Figure 3-4A). Thus, mutation of the putative zinc-binding residue E_{79} negatively affects inhibitor activity. Over-expression of *cpxP*R_{60}Q resulted in similar activity as the vector control strain (Figure 3-4A), confirming previous observations that residue R_{60} participates in inhibition (2, 31) . Over-expression of *cpxP*AA decreased *cpxP'-'lacZ* activity to a similar extent as wild-type *cpxP*, suggesting that CpxPAA retains full inhibitory activity (Figure 3-4A). Attempts to clone the pCpxPH_{136}A mutation were unsuccessful prior to the submission of this thesis and this mutation was not included in this analysis.

We wondered whether changing the N-terminal LTXXQ motif in Spy to that of CpxP might endow it with the ability to act as an inhibitor of CpxA, especially in light of our observation that the SpyEH mutant protein behaved more like CpxP in chaperone assays (Figure 3-3). It has been shown that Spy does not function as an inhibitor of the Cpx response in otherwise wild-type bacteria (2) and we replicated that result here (Figure 3-4A). However, in the absence of *cpxP*, we found that *spy* over-expression led to a decrease in *cpxP'-'lacZ* expression almost identical to that seen when CpxP was over-expressed (Figure 3-4A). Further, this inhibitory effect was due specifically to Spy, since over-expression of other Cpx-regulated periplasmic protein-folding and/or degrading factors (DegP, PpiA, DsbA) did not confer a similar decrease in a *cpxP* mutant background (Figure 3-4B). Accordingly, we measured the ability of the SpyEH mutant to inhibit

cpxP'-'lacZ expression in a *cpxP* mutant background in order to determine whether these residues impacted the signaling function of Spy. Surprisingly, unlike CpxP, mutation of the N-terminal LTXXQ motif of Spy resulted in a complete loss of inhibition of *cpxP'-'lacZ* expression upon over-expression (Figure 3-4A). Therefore, the variable XX residues of CpxP are dispensable for inhibition of the Cpx response but required for inhibition of the Cpx response by Spy.

3.3.5. Over-expression of *cpxP* mutations impacts the zinc-resistance of the *cpxP* mutant

We observed that deletion of *cpxP* leads to zinc resistance in *E. coli* K-12 and its deletion or over-expression alters the expression of a reporter for the expression of the zinc efflux protein *zntA* (Figure 2-5). To begin to understand how CpxP affects zinc, we sought to determine if any of the *cpxP* mutations we introduced affected these zinc phenotypes. Accordingly, we examined the survival of the *cpxP* mutant over-expressing each of the mutations on LB agar containing a gradient of zinc sulfate. The over-expression of wild-type CpxP, Spy, CpxPE_{79}A, and CpxPAA restored zinc-sensitivity to the *cpxP* mutant (Figure 3-5). Therefore, all of the proteins that retain some ability to inhibit the Cpx pathway can also impact zinc resistance when over-expressed in the *cpxP* mutant. Conversely, mutations that fail to inhibit the Cpx pathway also cannot restore zinc sensitivity (compare Figures 3-4A and 3-5). Specifically, over-expression of CpxPR_{60}Q or SpyEH does not complement the zinc resistance of the *cpxP* mutant (Figure 3-5). These results suggest that the ability of CpxP to restore zinc sensitivity might depend on the ability of CpxP to signal to the Cpx response and, further, the zinc-resistance of the *cpxP* deletion mutant may depend on the induction of the Cpx response. This conclusion is supported by the observation that deletion of *cpxA* or *cpxR* does not lead to zinc resistance despite the fact that levels of CpxP

expression are low under these conditions (5, 21). Also, the expression of the zinc efflux protein *zntA* is lowered in *cpxA* and *cpxR* mutants but elevated in the *cpxP* mutant (Figure 2-6).

3.3.6. Deletion of *cpxP* or *spy* but not *zraP* leads to zinc resistance

Previously, we showed that the expression of *cpxP* is decreased in the presence of excess zinc (Figure 2-1) and that deletion of *cpxP* results in zinc-resistance (Figure 2-5), suggesting that the expression of CpxP might be toxic in the presence of high levels of zinc. Specifically, the over-expression of *cpxP* decreases the expression of the high-affinity zinc efflux protein *zntA* (Figure 2-5). We have also shown that the over-expression of Spy can restore zinc sensitivity to the *cpxP* mutant (Figure 3-5), suggesting that Spy might also have a similar physiological role to CpxP with respect to zinc trafficking. Therefore, we tested whether deletion of the Cpx-regulated chaperone *spy* could also mediate zinc resistance by spotting aliquots of wild-type, *cpxP*, and *spy* mutant cultures standardized to the lowest A_{600} on LB agar containing toxic levels of zinc. On zinc-containing agar, the wild-type strain grew poorly at all dilutions while the *cpxP* deletion mutant grew well at all dilutions tested (Figure 3-6). Similarly, the *spy* mutant grew at all dilutions tested (Figure 3-6). Therefore, the *spy* mutant is also zinc-resistant. Next, we wondered whether other CpxP homologues might mediate similar zinc resistance. Accordingly, we deleted the zinc-binding paralogue *zraP* and compared the survival of the *zraP* mutant to the *cpxP* and *spy* mutants on zinc-containing agar. In the presence of toxic levels of zinc, the *zraP* mutant grew poorly at all dilutions (Figure 3-6), suggesting that deletion of *zraP* cannot mediate the same zinc resistance as mutations in the Cpx-regulated chaperones *cpxP* and *spy*. We also examined the growth of the double *cpxP spy* and *cpxP zraP* double mutants as well as the *cpxP spy zraP* triple mutants spotted on agar containing zinc and observed similar survival to the

single *cpxP* and *spy* single mutants. Therefore, loss of the Cpx-regulated chaperones *cpxP* and *spy* results in zinc resistance on solid medium but not the zinc-binding paralogue *zraP*.

3.4. Discussion

3.4.1. The inhibitor-chaperone CpxP binds zinc

The ATP-independent chaperone CpxP is paralogous to metal-binding proteins that regulate bacterial signal-transduction. We have provided evidence that CpxP binds zinc (Table 2-3). We predicted that E_{79} and H_{136} of CpxP would form a half-site for zinc-binding based on our crystal structure (Figure 3-1). We introduced alanine substitutions into each of these residues and tested the impact of the mutations on the chaperone, inhibitor, and zinc-associated functions of CpxP. Mutation of the putative zinc-binding residues had no effect on the chaperone function of CpxP (Figure 3-3). Similarly, over-expression of *cpxP*E_{79}A could restore zinc sensitivity to the *cpxP* deletion mutant as well as wild-type CpxP (Figure 3-5) and had only a modest effect on the inhibitory activity of the protein (Figure 3-4). Therefore, if the E_{79}A mutation does affect zinc-binding, then zinc-binding has little impact on the known functions of CpxP. Alternatively, E_{79} and H_{136} may not be required for zinc-binding by CpxP. A number of other potential zinc-binding sites were predicted in the crystal structure (25). Therefore, ICP-MS analysis of the E_{79}A and H_{136}A mutants must be performed to confirm a loss of zinc-binding in these mutants or further mutational analysis is needed to analyze the physiological relevance of zinc-binding by CpxP.

Another group previously identified E_{79} and H_{136} as part of the dimer interface of CpxP and mutations in these residues resulted in decreased protein stability and inhibitor activity (31). However, purification of E_{79}R and H_{136}Q and subsequent gel filtration studies showed that these mutants formed stable dimers *in vitro* (31). All mutant forms of *cpxP* used in this study resulted

in stable protein expression that did not differ from the stability of wild-type CpxP *in vivo* (Figure 3-2). Therefore, we propose that the modest decrease in the inhibitor activity of CpxP is not due to a loss of dimerization but potentially due to the loss of zinc-binding (Figure 3-4A). ICP-MS studies to confirm the loss of zinc-binding in CpxPE_{79}A and CpxPH_{136}A are currently underway.

3.4.2. Changes in the chaperone activity of CpxP do not ablate inhibitor function

One model used to explain the role of CpxP in the activation of the Cpx pathway posits that the binding of misfolded proteins to CpxP causes conformational changes in CpxP that render the protein incapable of interacting with the CpxA sensor kinase (9, 28). In this model, increased chaperone function results in a decreased ability to inhibit the Cpx response. This work provides evidence that mutations that increase chaperone activity do not negatively impact the inhibitor activity of CpxP. The CpxPAA mutant exhibited strong chaperone and inhibitor activities (compare Figures 3-3 and 3-4A). Conversely, the purified CpxPR_{60}Q mutant protein was a much better chaperone than wild-type CpxP but could not mediate inhibition of the Cpx pathway (compare Figures 3-3 and 3-4A). These data support a model where distinct residues mediate the inhibitor and chaperone activities of CpxP and fit well with the hypothesis that misfolded proteins bind CpxP at sites separate from the regions that interact with CpxA (9, 28). The positively charged residues R_{56}, R_{60}, and R_{67} are required for full inhibition of the Cpx pathway and are proposed to interact with a negatively-charged cleft in the periplasmic domain of CpxA (28, 31) . We propose that the distal E_{79} residue could communicate to the helix containing the aforementioned arginine residues through subtle rearrangements that travel along the unstructured loop between the α2 and α1 helices, thereby affecting the inhibition of the Cpx pathway at a separate site from the proposed interaction region and accounting for the mild effect

of this mutation on the inhibitor function of CpxP (Figure 3-4A). We note, however, that we have not identified mutations that ablate the chaperone activity of CpxP and, therefore, have not determined whether the chaperone activity of CpxP is required for inhibition.

Alternatively, E_{79} may participate in the dimerization of the protein (31). CnrX encodes a transmembrane protein with a periplasmic domain that binds nickel and enhances the transcription of nickel and cobalt efflux genes by interacting with the anti-sigma factor CnrY and stimulating the release of CnrH to the cytoplasm (8, 26). The crystal structure of the soluble domain of CnrX from *C. metallidurans* CH34, a paralogue of CpxP from *E. coli*, suggests that the apo-form of the enzyme is nearly identical to the nickel- or cobalt-bound forms, suggesting that metal-binding does not cause large conformational changes in the protein (27). However, the CnrX-dependent transcription of the *cnrCBA* efflux pump genes is stimulated by nickel and cobalt *in vivo* (26). Therefore, selective metal-binding to CnrX does not significantly alter protein structure but impacts the ability of the protein to stimulate the transcription of nickel and cobalt efflux genes. We do not know whether E_{79} participates in zinc-binding or oligomerization, but alterations to this part of the protein may affect signaling through small interhelical movements within CpxP to alter its interaction with CpxA.

3.4.3. The variable XX residues of the N-terminal LTXXQ motifs of CpxP and Spy contribute to the chaperone activities of the proteins

We examined the contribution of the variable XX residues in the N-terminal LTXXQ motif to CpxP function by introducing alanine residues in both of the XX residues and replacing the cognate residues in Spy with the E_{53} and H_{54} residues of CpxP. While CpxPAA was a better chaperone than wild-type CpxP (Figure 3-3), SpyEH exhibited decreased chaperone activity relative to wild-type Spy. The variable XX residues of the N-terminal LTXXQ motifs of CpxP

and Spy, therefore, impact the chaperone activities of the proteins. A study of gain-of-function mutations in Spy identified a number of residues along the edges of the concave surface of Spy as potentially important for substrate binding, including A_{60} (18). The N-terminal XX residues of CpxP may also dock substrates and the increased hydrophobicity of CpxPAA might elevate the affinity of CpxP for misfolded substrates at this site. Similarly, glutamine residues are enriched in aggregative proteins such as prions and amyloids (14). The more hydrophobic nature of the glutamine substitution in CpxPR_{60}Q may therefore also contribute to the enhanced chaperone activity of CpxP by increasing the affinity of the protein for exposed hydrophobic surfaces. E_{53}, H_{54}, and R_{60} lie in close proximity on a conserved N-terminal α-helix. Whereas R_{60} is required for inhibitor activity, the identity of the residues mutated in CpxPAA - E_{53} and H_{54} - is not required for the inhibition of the Cpx pathway (Figure 3-4A), suggesting that residues that impact chaperone activity are close to the residues that mediate the interaction between CpxA and CpxP. Yet, the enhanced chaperone activity of CpxPAA does not interfere with the ability of the protein to inhibit the Cpx response (compare Figures 3-3 and 3-4A), suggesting that misfolded proteins may not directly disrupt the CpxA-CpxP interaction interface. Alternatively, the XX residues of CpxP regulate the chaperone activity of CpxP. Introduction of these residues into the cognate positions in Spy decreased the chaperone activity of Spy (Figure 3-3). This observation suggests that the identity of these residues might be responsible for the low chaperone activity of CpxP, perhaps by affecting higher order oligomer formation or by ensuring that CpxP binds and refolds only specific substrates.

3.4.4. Spy inhibits the Cpx pathway in the absence of *cpxP*

Spy is a strong periplasmic chaperone and substitutions of the D_{59} and A_{60} residues with those of the weaker chaperone CpxP negatively impact chaperone activity (Figure 3-3).

Unexpectedly, over-expression of *spy* decreases Cpx activity in the absence of *cpxP* in a manner that depended on the identity of the variable XX residues of the N-terminal LTXXQ motif (Figure 3-4A). Therefore, the ability of Spy to inhibit the Cpx pathway in the absence of CpxP depends on the robust and specific chaperone activity of the protein, and this aspect of Spy function is unique amongst Cpx-regulated folding factors as no other periplasmic chaperone tested was able to mediate the same decrease in Cpx activity in the presence or absence of *cpxP* (Figure 3-4B). Possibly, the robust chaperone activity of Spy when over-expressed is able to efficiently remove misfolded periplasmic substrates to keep Cpx activity low even in the absence of *cpxP*. The observation that other protein-folding factors are unable to mediate the same decrease in Cpx activity may reflect the relatively weaker chaperone activity of other protein-folding factors or the inability of these factors to remove specific substrates that activate the Cpx pathway. Additionally, Spy may be able to interact with the periplasmic sensing domain of CpxA in the absence of CpxP. Notably, the identity of the variable XX residues of CpxP are not sufficient to endow Spy with the ability to inihibit the Cpx pathway because over-expression of this mutant had no effect on Cpx pathway activity in the absence of *cpxP* (Figure 3-4A).

3.4.5. Mutations in CpxP that ablate the inhibitor function also negatively impact the zinc-associated function of the protein

Previously, we showed that the *cpxP* deletion mutant was resistant to zinc and promoted increased expression of the zinc efflux protein *zntA* in the presence of toxic levels of zinc (Figure 2-5). Over-expression of *cpxP* restored zinc sensitivity and strongly decreased *zntA* expression. (Figure 2-5). Here, we show that the over-expression of wild-type CpxP and any CpxP mutant that retains the ability to inhibit the Cpx pathway can also restore zinc sensitivity to the *cpxP* mutant (Figure 3-5). Over-expression of the gain-of-function chaperone CpxPR_{60}Q and the loss-

of-function chaperone SpyEH failed to restore zinc sensitivity to the *cpxP* mutant. These observations suggest that the zinc-associated function of CpxP is determined by the ability of CpxP to interact with the sensor kinase CpxA. Therefore, the levels of Cpx regulon expression may determine zinc resistance, such that elevated levels of Cpx activity in the absence of *cpxP* promote zinc efflux. Interestingly, only deletion of the Cpx-regulated chaperones *cpxP* and *spy* mediated zinc efflux while deletion of the zinc-binding ATP-independent chaperone *zraP* did not (Figure 3-6). Thus, deletion of CpxP paralogues does not uniformly increase zinc resistance but altering the expression of Cpx regulon members does. These observations lend credence to our previous assertion that the Cpx pathway alters zinc trafficking (Chapter 2), though we do not know the mechanism leading to these phenotypes.

3.4.6. A model for the regulation of the inhibitor and chaperone activities of CpxP

The results of our mutational analysis support a model where strong chaperone activity is compatible with both strong and weak inhibitor activity (Figure 3-7). DiGiuseppe and Silhavy (2003) proposed that envelope stress inactivates CpxP to prevent it from acting as an inhibitor of the Cpx pathway based on the finding that when CpxP is tethered to the inner membrane, it cannot prevent induction of the Cpx pathway by alkaline pH (6). However, we found that CpxPAA is both a strong chaperone and inhibitor, suggesting that the strong chaperone activity of CpxP does not 'inactivate' the inhibitor activity of the protein. The presence of misfolded proteins might stimulate CpxP to leave CpxA but CpxP remains competent to bind CpxA (Figure 3-7). As misfolded proteins are re-folded or degraded, more CpxP is free to re-associate with the periplasmic sensing domain of CpxA, thus restoring pathway activity to basal levels as the stress subsides. The balance between the inhibitor and chaperone functions of CpxP is therefore

determined by the presence of substrates, though we still do not understand how substrates remove CpxP from CpxA.

3.4.7. Conclusion

The inhibitor-chaperone CpxP is homologous to metal-binding signal transduction proteins that are identified the conserved LTXXQ motif (25). This work has shown that the variable residues in the N-terminal LTXXQ motif impact the chaperone activities of CpxP and Spy while the residues that mediate the interaction with CpxA impact the zinc-related function of CpxP. We have also established that CpxP binds zinc. Therefore, the metal-binding and signal integration properties are conserved in the prototypical member of the CpxP-like family of proteins. These proteins may integrate metal-related signals to modulate signal transduction events that mediate responses to extracytoplasmic stress.

3.4.8. Acknowledgements

We are grateful to Ross Edwards and Jun Lu for assistance with protein purification and circular dichroism, respectively. We would also like to thank Guangcheng Chen in the Canadian Center for Isotopic Microanalysis (CCIM) in the Department of Earth and Atmospheric Sciences at the University of Alberta for assistance with ICP-MS.

3.5. Tables

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

Strain or plasmid name	Genotype or description	Source or reference
BL21DE3	*fhuA2 lon ompT gal (λ DE3) dcm ΔhsdS*	New England BioLabs
DB12	TR50 pTrc99A	(2)
DB58	TR50 pDegP	(2)
DB59	TR50 pDsbA	(2)
DB60	TR50 pPpiA	(2)
DB61	TR50 pSpy	(2)
JLW8	TR50 pCpxP	This study
JLW392	TR50 Δ*cpxP*	This study
JLW400	JLW392 pTrc99A	This study
JLW401	JLW392 pSpy	This study
JLW403	JLW392 pSpyEH	This study
JLW404	JLW392 pCpxP	This study
JLW406	JLW392 pCpxPAA	This study
JLW581	BL21DE3 pGEX-6P-1-*cpxP*AA	This study
JLW626	BL21DE3 pGEX-6P-1	This study
JLW632	BL21DE3 pGEX-6P-1-$cpxP_{20\text{-}166}$	This study
JLW633	BL21DE3 pGEX-6P-1-$spy_{23\text{-}161}$	This study
JLW634	BL21DE3 pGEX-6P-1-*spy*EH	This study
JLW713	TR50 pCpxPR_{60}Q	This study
JLW738	TR50 pCpxPAA	This study
JLW739	TR50 pSpyEH	This study
JLW937	TR50 Δ*zraP*	This study
JLW941	JLW392 Δ*spy*	This study
JLW943	JLW392 Δ*zraP*	This study
JLW945	TR50 Δ*spy*	This study
JLW952	JLW941 Δ*zraP*	This study
JLW955	TR50 pCpxPE_{79}A	This study
JLW985	JLW392 pCpxPR_{60}Q	This study
JLW956	JLW392 pCpxPE_{79}A	This study
JSW1	TR50 Δ*cpxR*	This study
MC4100	F^- *araD139* Δ(*argF-lac*)U169 *rpsL150*(Str^r) *relA1 flbB5301 decC1 ptsF25 rbsR*	(3)
RM53	TR50 Δ*cpxA*	This study
TR50	MC4100 *cpxP'-'lacZ* at λRS88	(20)
Plasmids	**Description**	**Source or Reference**
pGEX-6P1	High-copy IPTG-inducible vector encoding N-terminal fusion to glutathione-S-transferase (GST)	GE Life Sciences

pGEX-6P1-*cpxP*$_{20\text{-}166}$	Vector encoding a cytoplasmic GST-CpxP fusion for protein purification	Gina Thede, unpublished
pGEX-6P-1-*cpxP*AA	Vector encoding a cytoplasmic GST-CpxPAA fusion for protein purification, made by subcloning *cpxPAA*$_{20\text{-}166}$ from pCpxPAA	This study
pGEX-6P-1-*cpxP*E$_{79}$A	Vector encoding a cytoplasmic GST-CpxPE$_{79}$A fusion for protein purification, made by a two-step PCR method	This study
pGEX-6P-1-*cpxP*H$_{136}$A	Vector encoding a cytoplasmic GST-CpxPH$_{136}$A fusion for protein purification, made by a two-step PCR method	This study
pGEX-6P-1-*spy*$_{23\text{-}161}$	Vector encoding a cytoplasmic GST-Spy fusion for protein purification, made by subcloning from pSpy	This study
pGEX-6P-1-*spyEH*	Vector encoding a cytoplasmic GST-SpyEH fusion for protein purification, made by subcloning from pSpyEH	This study
pTrc99A	High-copy IPTG-inducible vector for *cpxP, spy, degP, dsbA,* and *ppiA* over-expression	Pharmacia
pCpxP	Over-expression of *cpxP*	(21)
pCpxPAA	Made by introducing the E$_{53}$A and H$_{54}$A mutations sequentially into pCpxP using the Quikchange Lightning Kit (Agilent)	This study
pCpxPR$_{60}$Q	Made by introducing R$_{60}$Q into pCpxP using Quikchange Lighting (Agilent)	This study
pCpxPE$_{79}$A	Made by introducing E$_{79}$A mutation into pCpxP using a two-step PCR method	This study
pSpy	Over-expression of *spy*	(2)
pSpyEH	Made by introducing D$_{59}$E and A$_{60}$H mutations into pSpy using Quikchange Lightning (Agilent)	This study
pDegP	Over-expression of *degP*	(2)
pDsbA	Over-expression of *dsbA*	(2)
pPpiA	Over-expression of *ppiA*	(2)

Table 3-2: Primers used in this study

Name	Sequence (5' to 3')	Use/Source
pTrc99Aseq-fw	TGCAGGTCGTAAATCACTGC	Forward primer for sequencing of pTrc99A derivatives
pTrc99Aseq-rv	CTGGCAGTTCCCTACTCTCG	Reverse primer for sequencing of pTrc99A derivatives
cpxPE53A-for	CGGCATAAGTTTAACCGCACATCAG CGTCAGCAGA	Site-directed mutagenesis of *cpxP*, designed by Agilent Quikchange primer design
cpxPE53A-rv	TCTGCTGAACGCTGATGTGCGGTTA AACTTATGCCG	Site-directed mutagenesis of *cpxP*, designed by Agilent Quikchange primer design
cpxPH54A-fw	TCGACGGCATAAGTTTAACCGAAGC TCAGCGTCAGCAGAT	Site-directed mutagenesis of *cpxP*, designed by Agilent Quikchange primer design
cpxPH54A-rv	ATCTGCTGACGCTGAGCTTCGGTTA AACTTATGCCGTCGA	Site-directed mutagenesis of *cpxP*, designed by Agilent Quikchange primer design
cpxPE79A-fw	TAATGTTAGCGAACTGGCGACAATG CATCGCCTTG	Forward primer for site-directed mutagenesis of *cpxP* in a two-step PCR
cpxPE79A-rv	CAAGGCGATGCATTGTCGCCAGTTC GCTAACATTA	Reverse primer for site-directed mutagenesis of *cpxP* in a two-step PCR
cpxPH136A-fw	CCATTCGTTGTTGAGCTTTCGTGTTT AAAACCGCTTGCTGCTC	Forward primer for site-directed mutagenesis of *cpxP* in a two-step PCR
cpxPH136A-rv	GAGCAGCAAGCGGTTTTAAACGAGA AAGCTCAACAACGAATGG	Reverse primer for site-directed mutagenesis of *cpxP* in a two-step PCR
spyD59E-fw2	AAGACCTGAACCTGACCGAGGCGCA GAAAC	Forward primer for site-directed mutagenesis of *spy* using Agilent Quikchange kit
spyD59E-rv2	GTTTCTGCGCCTCGGTCAGGTTCAG GTCTT	Reverse primer for site-directed mutagenesis of *spy* using Agilent Quikchange Kit
SDMspyA60	ACCTGAACCTGACCGACCACCAGAA	Forward primer for site-

H-for	ACAGCAGATCCG	directed mutagenesis of *spy* using Agilent Quikchange kit
SDMspyA60H-rev	CGGATCTGCTGTTTCTGGTGGTCGGTCAGGTTCAGGT	Reverse primer for site-directed mutagenesis of *spy* using Agilent Quikchange Kit
pGEXcpxP20-fw	GGGATCCCACGCTGCTGAAGTC	Forward primer for sub-cloning from $cpxP_{20\text{-}166}$ into pGEX-6P-1
pGEXcpxP166-rv	CGAATTCCTACTGGGAACGTGA	Reverse primer f or sub-cloning from $cpxP_{20\text{-}166}$ into pGEX-6P-1
pGEXspy23-fw	GGGATCCGCCGCAGACACCACTACC	Forward primer for sub-cloning from $spy_{23\text{-}161}$ into pGEX-6P-1
pGEXspy-rv	CGAATTCTTATTCAGCAGTTGC	Reverse primer for sub-cloning from $spy_{23\text{-}161}$ into pGEX-6P-1
EcoRIcpxP-fw	CAGAATTCATGCGCATAGTTACCG	Forward primer for cloning *cpxP* into pTrc99A
cpxPBamHI-rv	AAGGATCCCTACTGGGAACGTGAGT	Reverse primer for cloning *cpxP* into pTrc99A

Table 3-3: Metal ion content of purified chaperone proteins

Sample	Zn^{2+} (ppm)	Fe^{2+} (ppm)	Cu^{2+} (ppm)
Buffer	0.0207	<DL	0.0201
CpxP	4.40	0.280	0.0615
CpxP + 1mM EDTA	0.355	0.237	0.585

Sample	Li	B	Na	Mg	Al	P	K	Ca	Ti
Buffer	<DL	0.128	60	0.0292	0.0761	0.159	0.0971	0.406	0.00044
CpxP	0.0026	0.581	43	0.656	0.608	0.693	0.0634	12.4	0.00424
CpxP + 1mM EDTA	0.00083	0.467	<DL	0.250	0.318	0.606	0.570	6.73	0.00343

Sample	V	Cr	Ga	As	Rb	Sr	Y	Zr
Buffer	<DL	0.00297	<DL	<DL	<DL	0.00145	<DL	0.00043
CpxP	0.00236	0.002	0.00017	0.00018	0.00058	0.0236	0.004	0.00310
CpxP + 1mM EDTA	0.00020	0.00533	0	0.00014	0.00039	0.0199	0.00135	0.000172

Sample	Nb	Mo	Pd	Ag	Cd
Buffer	<DL	0.00030	<DL	0.00035	0.00064
CpxP	0.00053	0.001	0.00061	0.00021	0.009
CpxP + 1mM EDTA	0.00041	0.00103	0.00207	<DL	0.00985

Metal ion content represents an average of two samples collected in the same experiment, with the exception of EDTA-treated CpxP for which only one sample was tested, as determined by ICP-MS of filtrates from purified CpxP-containing samples. '<DL' denotes below the detection limit for a given ion.

3.6. Figures

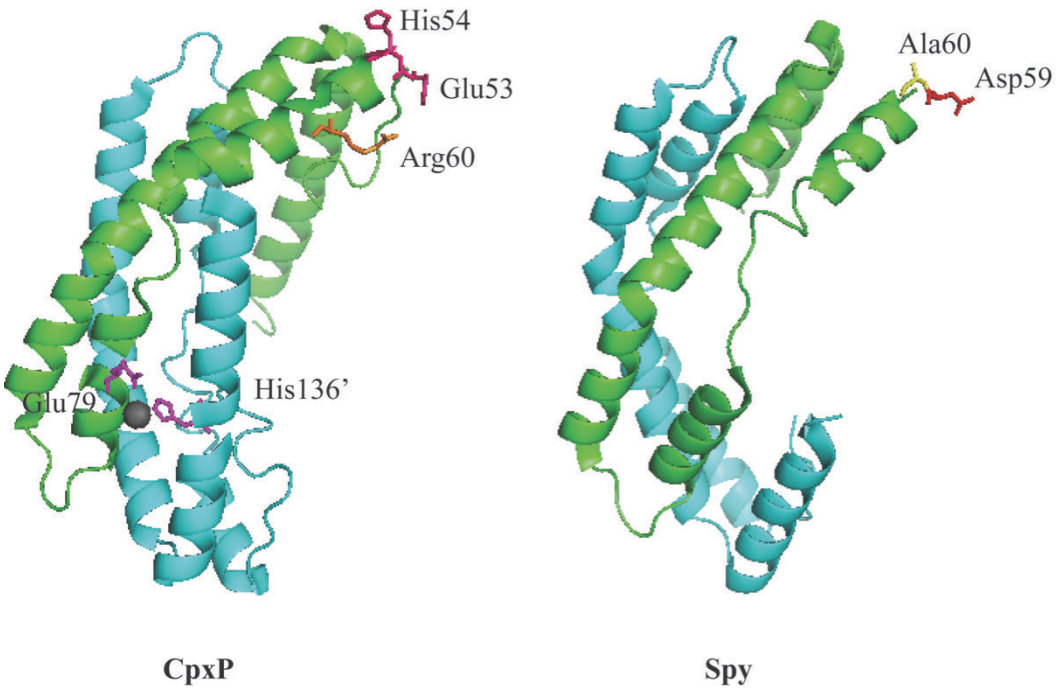


Figure 3-1: Mutational analysis of CpxP and Spy.

Cartoon structures of the CpxP (left) and Spy (right) dimers with the residues that were mutated in this study shown as stick structures, colored, and labelled. The putative zinc-binding site at E_{79} and H_{136} contains a zinc-bound in the structure solved by Thede *et al.* (2011) (25) indicated by a grey sphere. One protomer of each dimer is labeled in green and the other in blue. The amino acid numbering starts from the first methionine of the translated sequence and not the mature protein. The CpxP and Spy structures were obtained from the Protein Structures Database (PDB: 3QZC) and modified in Pymol (http://pymol.org).

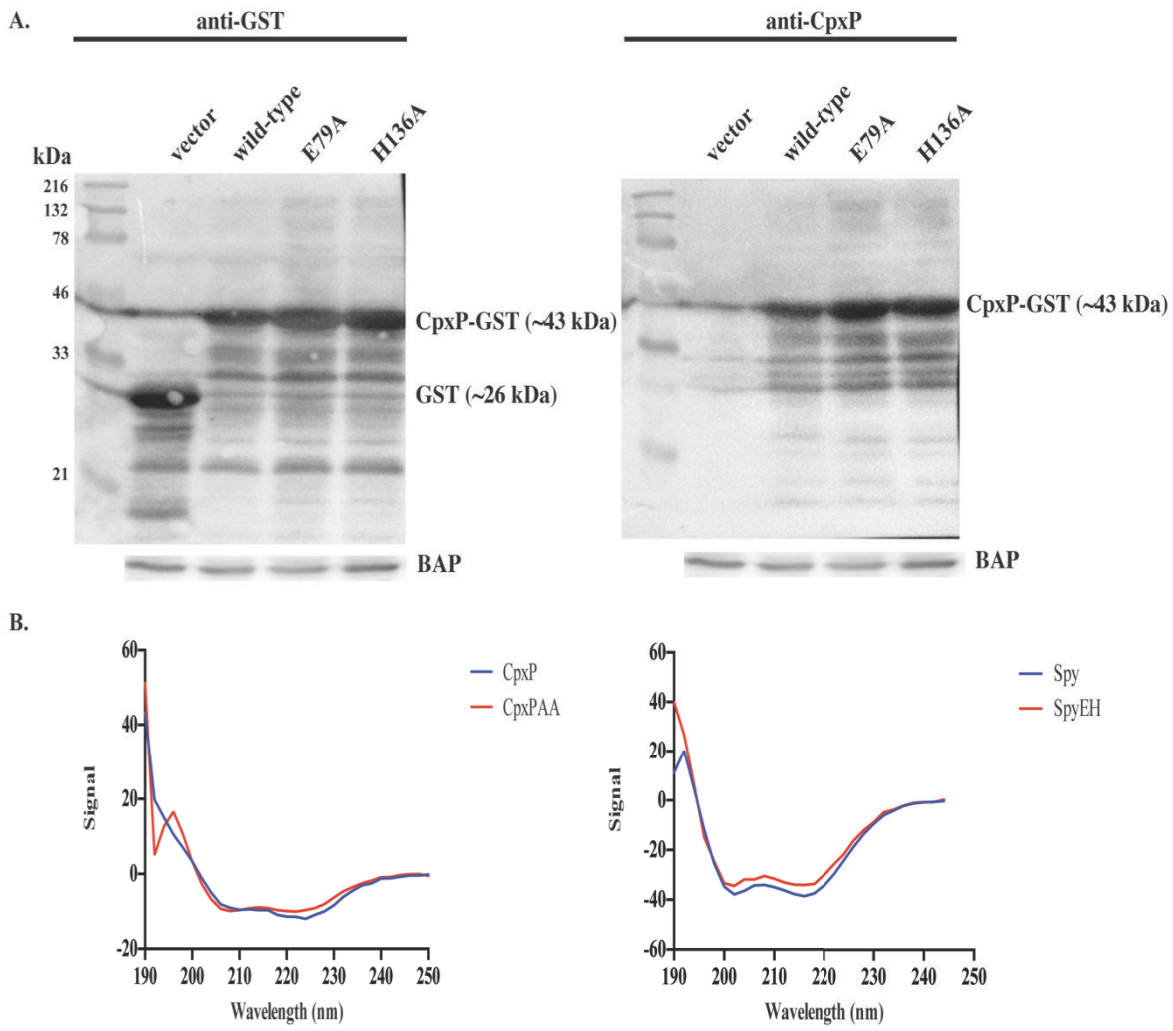
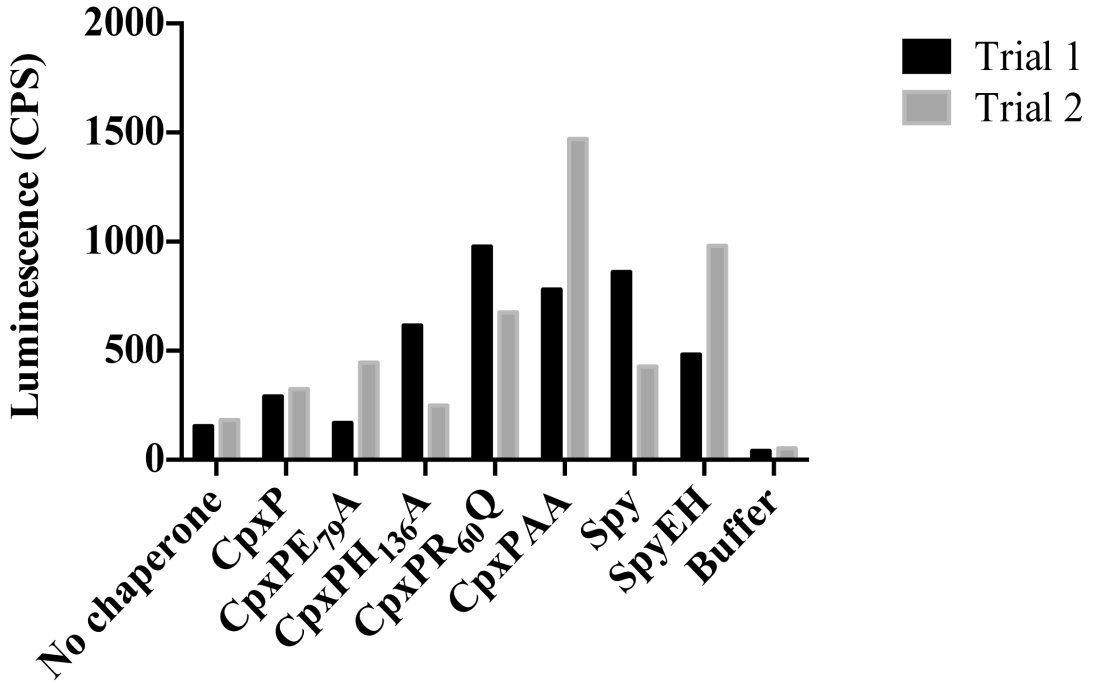


Figure 3-2: Mutations in the putative zinc-binding site of CpxP and variable XX residues of CpxP and Spy do not affect stability or secondary protein structure.

A. Western blots to detect the expression of CpxP-GST in BL21DE3 over-expressing CpxP-GST (JLW632), CpxPE_{79}A-GST (JLW930), CpxPH_{136}A-GST (JLW931), or the vector control (JLW626). The positions of CpxP-GST and GST proteins as predicted by molecular weight are indicated on the left of each blot. The expression of bacterial alkaline phosphatase (BAP) is not expected to be affected by the conditions tested and serves as a loading control. **B.** Far-UV circular dichroism was performed on purified protein samples to assess the secondary structure

of wild-type and mutant proteins. The spectra shown above are an average of three scans per sample.



Sample	Normalized luminescence Trial 1	Trial 2	Average ± Standard Deviation
CpxP	1.89	1.77	1.83 ± 0.08
CpxPE$_{79}$A	1.10	2.44	1.77 ± 0.95
CpxPH$_{136}$A	3.99	1.36	2.68 ± 1.86
CpxPR$_{60}$Q	3.13	5.36	4.24 ± 1.57
CpxPAA	6.35	8.03	5.02 ± 1.88
Spy	5.06	7.14	6.55 ± 2.10
SpyEH	5.59	2.33	3.96 ± 2.30

Figure 3-3: The variable residues of the N-terminal LTXXQ motifs affect the chaperone activities of CpxP and Spy.

To investigate the chaperone activity of wild-type and mutant CpxP and Spy proteins, 5 nM firefly luciferase (final concentration) was denatured at 45°C in the presence or absence of 10 nM chaperone proteins (final concentration). The bars in the bar graph represent the raw luminescence of each sample (top) while the table contains the luminescence of each chaperone-treated sample normalized to the luminescence recovered from luciferase denatured without chaperone proteins (bottom). The buffer control contains only reaction buffer and no luciferase. The luminescence values from two independent experiments are given (Trials 1 and 2, indicated above).

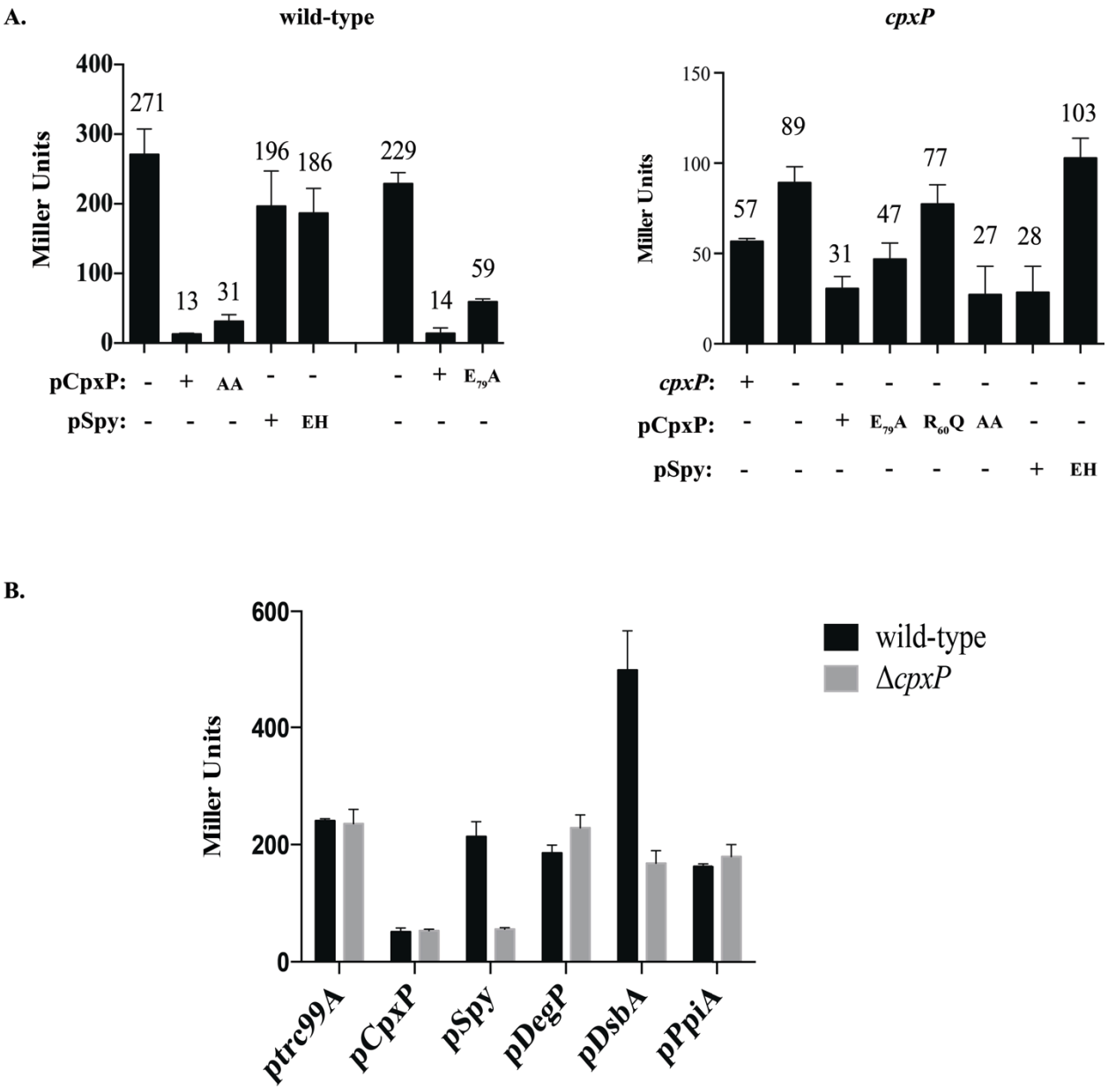


Figure 3-4: Mutations in the zinc-binding residues of CpxP affect inhibition but mutations in the N-terminal LTXXQ motif do not affect signaling.

A. β-galactosidase activity of wild-type *E. coli* (TR50, *left*), or the *cpxP* mutant (JLW392, *right*) over-expressing pCpxP (JLW404), pCpxPE_{79}A (JLW956), pCpxPR_{60}Q (JLW985), pCpxPAA (JLW406), pSpy (JLW401), pSpyEH (JLW403), or the vector control (JLW400). The presence

or absence of chromosomal *cpxP* and the alleles over-expressed from the *trc* promoter in pTrcc99A are given below the graphs. The average β-galactosidase activity of each sample is indicated above each bar. **B.** β-galactosidase activity of wild-type or *cpxP* mutant bacteria over-expressing *cpxP* (JLW8), *spy* (DB61), *degP* (DB58), *dsbA* (DB59), or *ppiA* (DB60). The average β-galactosidase activity and standard deviations of three technical replicates per sample are represented by each bar and error bar, respectively. Representative results of at least two independent experiments for each graph are given above.

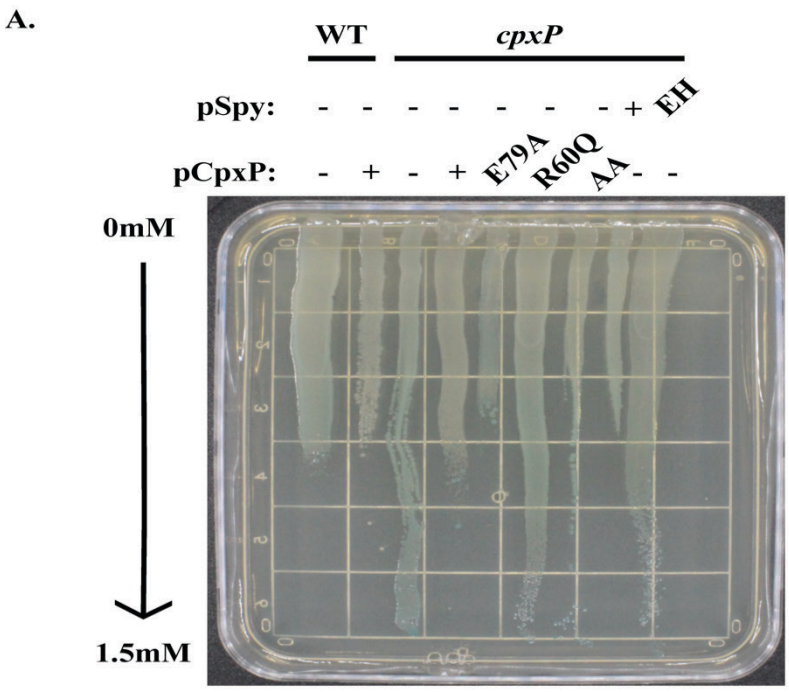


Figure 3-5: Deletion of *cpxP* results in increased zinc efflux and zinc resistance.

Survival and β-galactosidase activity of wild-type or *cpxP* mutant *E. coli* over-expressing *cpxP, spy,* or their mutant derivatives on LB agar containing a gradient of zinc sulfate (maximum concentration of 1.5 mM) and 20 μg/mL X-gal (final concentration). Strains, from left to right: DB12, JLW8, JLW400, JLW404, JLW956, JLW985, JLW406, JLW401, JLW403. A representative result from two independent experiments is shown above.

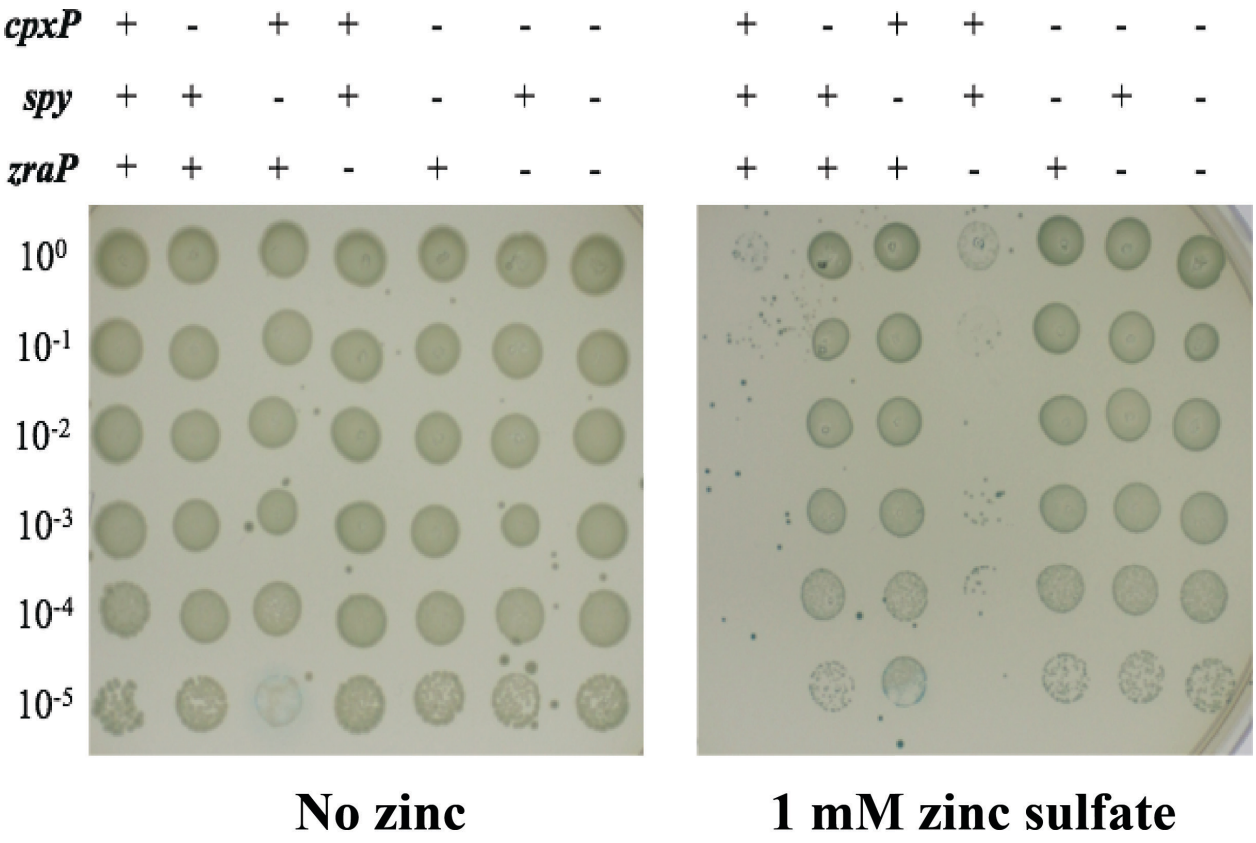


Figure 3-6: Deletion of *cpxP* and *spy* but not *zraP* results in zinc-resistance.

Survival of wild-type (TR50), *cpxP* (JLW392), *spy* (JLW945), and *zraP* (JLW937) single, double *cpxP spy* (JLW941) and *cpxP zraP* (943), and triple *cpxP spy zraP* (JLW952) mutants on LB agar without (left) or with 1mM zinc sulfate (final concentration, right). Representative results of two independent experiments are shown above.

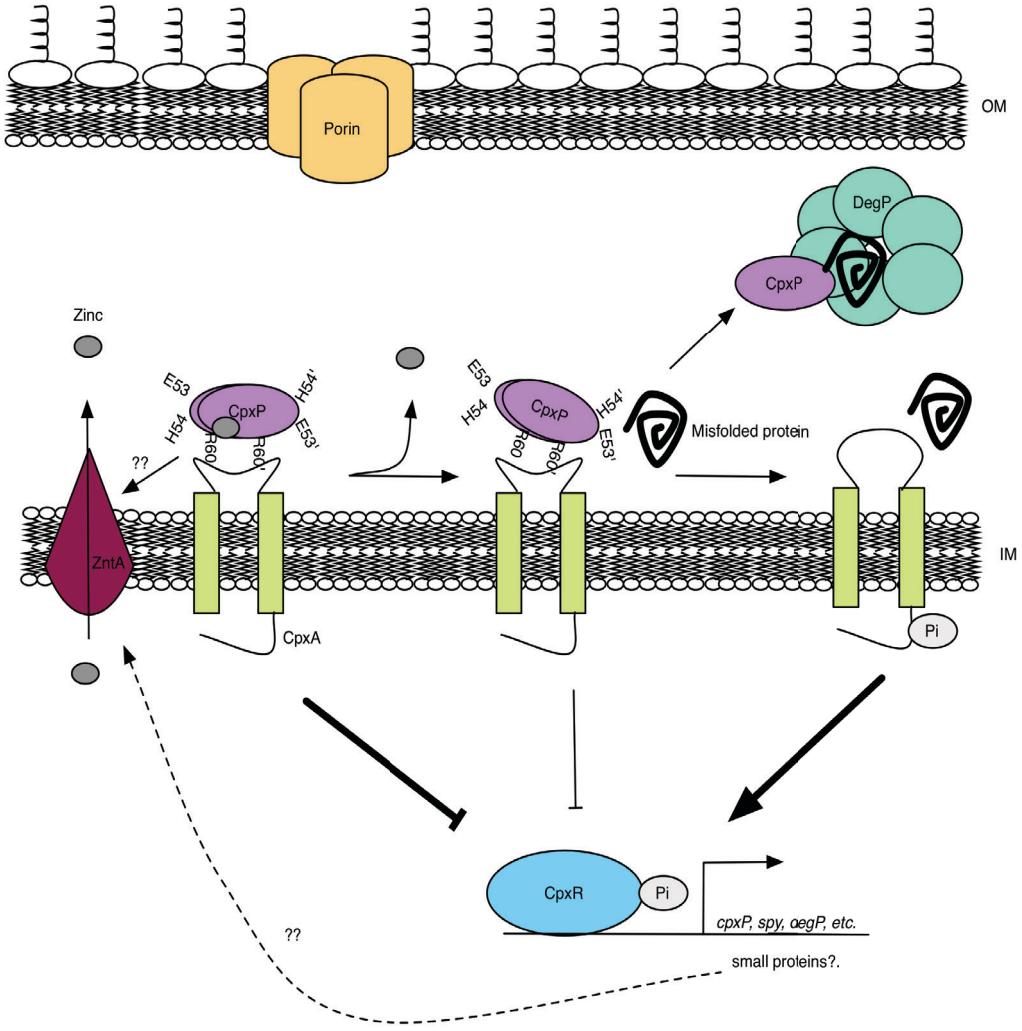


Figure 3-7: A model for the role of CpxP and zinc in Cpx activation.

CpxP interacts with the periplasmic sensing domain of CpxA, likely via the arginine residues that line the edges of the concave surface of CpxP (2, 31). CpxP also inhibits zinc efflux through an uncharacterized mechanism that likely depends on elevated Cpx regulon expression. Loss of the zinc ion primes CpxP to leave CpxA and the presence of misfolded proteins titrates CpxP away from CpxA. CpxP and misfolded proteins are degraded by DegP, freeing CpxA to sense signals and activate the Cpx response. CpxP remains competent to bind CpxA as the levels of misfolded substrate decreases. 'OM' indicates outer membrane, 'IM' inner membrane.

3.7. References

1. **Appia-Ayme C., A. Hall, E. Patrick, S. Rajadurai, T. A. Clarke, and G. Rowley.** 2012. ZraP is a periplasmic molecular chaperone and a repressor of the zinc-responsive two-component regulator ZraSR. Biochem J **442**:85-93.

2. **Buelow D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J Bacteriol **187**:6622-6630.

3. **Casadaban M. J., and S. N. Cohen.** 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 **76**:4530-4533.

4. **Chen N. G., K. Gregory, Y. Sun, and V. Golovlev.** 2011. Transient model of thermal deactivation of enzymes. Biochim Biophys Acta **1814**:1318-1324.

5. **Danese P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli.* J Bacteriol **180**:5875-5884.

6. **DiGiuseppe P. A., and T. J. Silhavy.** 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol **185**:2432-2440.

7. **Graham A. I., G. Sanguinetti, N. Bramall, C. W. McLeod, and R. K. Poole.** 2012. Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of the bacterial response to zinc reveals transient behaviour of the Fur and SoxS regulators. Microbiology **158**:284-292.

8. **Grass G., C. Grosse, and D. H. Nies.** 2000. Regulation of the cnr cobalt and nickel resistance determinant from *Ralstonia* sp. strain CH34. J Bacteriol **182**:1390-1398.

9. **Isaac D. D., J. S. Pinkner, S. J. Hultgren, and T. J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc Natl Acad Sci U S A **102**:17775-17779.

10. **Jones C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren.** 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J **16**:6394-6406.

11. **Kammann M., J. Laufs, J. Schell, and B. Gronenborn.** 1989. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). Nucleic Acids Res **17**:5404.

12. **Kershaw C. J., N. L. Brown, C. Constantinidou, M. D. Patel, and J. L. Hobman.** 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. Microbiology **151**:1187-1198.

13. **Lee L. J., J. A. Barrett, and R. K. Poole.** 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J Bacteriol **187**:1124-1134.

14. **Michelitsch M. D., and J. S. Weissman.** 2000. A census of glutamine/asparagine-rich regions: Implications for their conserved function and the prediction of novel prions. Proc Natl Acad Sci U S A **97**:11910-11915.

15. **Nakayama S., and H. Watanabe.** 1995. Involvement of *cpxA*, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of *Shigella sonnei virF* gene. J Bacteriol **177**:5062-5069.

16. **Pontel L. B., N. L. Scampoli, S. Porwollik, S. K. Checa, M. McClelland, and F. C. Soncini.** 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**:1659-1669.

17. **Price N. L., and T. L. Raivio.** 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol **191**:1798-1815.

18. **Quan S., L. Wang, E. V. Petrotchenko, K. A. Makepeace, S. Horowitz, J. Yang, Y. Zhang, C. H. Borchers, and J. C. Bardwell.** 2014. Super Spy variants implicate flexibility in chaperone action. Elife **3**:e01584.

19. **Quan S., P. Koldewey, T. Tapley, N. Kirsch, K. M. Ruane, J. Pfizenmaier, R. Shi, S. Hofmann, L. Foit, G. Ren, U. Jakob, Z. Xu, M. Cygler, and J. C. Bardwell.** 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat Struct Mol Biol **18**:262-269.

20. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

21. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

22. **Raivio T. L., S. K. Leblanc, and N. L. Price.** 2013. The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. J Bacteriol **195**:2755-2767.

23. **Slauch J. M., and T. J. Silhavy.** 1991. Genetic fusions as experimental tools. Methods Enzymol **204**:213-248.

24. **Snyder W. B., L. J. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy.** 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J Bacteriol **177**:4216-4223.

25. **Thede G. L., D. C. Arthur, R. A. Edwards, D. R. Buelow, J. L. Wong, T. L. Raivio, and J. N. Glover.** 2011. Structure of the periplasmic stress response protein CpxP. J Bacteriol **193**:2149-2157.

26. **Tibazarwa C., S. Wuertz, M. Mergeay, L. Wyns, and D. van Der Lelie.** 2000. Regulation of the *cnr* cobalt and nickel resistance determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34. J Bacteriol **182**:1399-1409.

27. **Trepreau J., E. Girard, A. P. Maillard, E. de Rosny, I. Petit-Haertlein, R. Kahn, and J. Covès.** 2011. Structural basis for metal sensing by CnrX. J Mol Biol **408**:766-779.

28. **Tschauner K., P. Hörnschemeyer, V. S. Müller, and S. Hunke.** 2014. Dynamic Interaction between the CpxA Sensor Kinase and the Periplasmic Accessory Protein CpxP Mediates Signal Recognition in *E. coli*. PLoS One **9**:e107383.

29. **Yamamoto K., and A. Ishihama.** 2005. Transcriptional response of *Escherichia coli* to external copper. Mol Microbiol **56**:215-227.

30. **Yamamoto K., H. Ogasawara, and A. Ishihama.** 2008. Involvement of multiple transcription factors for metal-induced *spy* gene expression in *Escherichia coli*. J Biotechnol **133**:196-200.

31. **Zhou X., R. Keller, R. Volkmer, N. Krauss, P. Scheerer, and S. Hunke.** 2011. Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. J Biol Chem **286**:9805-9814.

Chapter 4: NuoF and EfeB are novel *cpxP*-dependent inhibitors of the Cpx two-component system in *Escherichia coli* [2]

[2] Junshu Wang screened half of the 176 ASKA candidates on MacConkey agar and identified the candidate inhibitors shown on the right side of Figure 4-2A. Randi Guest constructed RG158 (E2348/69 *nuo*), the parent strain for bacterial strains used in Figure 4-5B.

4.1. Introduction

Bacterial signal sensing occurs through a complex regulatory network of sensory, effector, auxiliary, and connector proteins. Two-component signal transduction systems (TCS) are abundant in nature and are a common way bacteria sense signals and effect cellular changes to adapt. The Cpx TCS senses and responds to envelope protein-misfolding in Gram-negative bacteria through the sensor kinase CpxA and the response regulator CpxR. Upon sensing an envelope stress signal such as the over-expression of the lipoprotein *nlpE*, heterologous expression of P-pilin subunits PapE and PapG from uropathogenic *E. coli* (UPEC), or growth in alkaline pH, the inner membrane kinase CpxA transduces the signal to the cytoplasmic response regulator CpxR through a conserved phosphotransfer (23). Phosphorylated CpxR activates the expression of genes involved in maintaining envelope protein-folding homeostasis, including *cpxP* (7, 8) .

CpxP is a small periplasmic protein that inhibits the Cpx response by interacting with the periplasmic sensing domain of CpxA (31, 36) . Misfolded pilins are thought to allosterically inactivate the ability of CpxP to inhibit CpxA signal-sensing through a conformational change (31), suggesting that the chaperone activity of CpxP influences its inhibitor activity. Chaperone-activated CpxP then ushers bound protein clients to the periplasmic chaperone-protease DegP for degradation (13).

Though CpxP inhibits the Cpx pathway, deletion of *cpxP* is not sufficient to de-repress pathway activity, suggesting that other inhibitors of the Cpx pathway may exist (25). We hypothesized that the Cpx system regulates other envelope-localized proteins that could function analogously to CpxP by decreasing Cpx pathway activity in a CpxA-dependent manner. Therefore, we performed a genetic screen for Cpx-regulated envelope-localized genes that inhibit

Cpx activity upon over-expression and identified *nuoF* and *efeB* as novel inhibitors of the Cpx pathway. NuoF encodes the catalytic soluble cytoplasmic component of the NADH dehydrogenase I complex that binds and oxidizes NADH (2, 35) and EfeB encodes a heme peroxidase that facilitates iron import by the EfeU permease that is cryptic in *E. coli* K-12 (5). We show that *nuoF* over-expression specifically inhibits the Cpx pathway in a *cpxA*- and *cpxP*-dependent manner that does not require the 13 other members of the Nuo complex. Similarly, *efeB* over-expression inhibits the Cpx pathway in a *cpxA*- and *cpxP*-dependent manner that does not require expression of the permease *efeU*. This work identifies the first *cpxP*-dependent signals that suggest new roles for the Cpx system in sensing and responding to metabolism and metals.

4.2. Materials and Methods

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 4-1 and were constructed by standard genetic techniques. Deletion of the 14-member *nuo* operon was completed by Randi Guest and performed by allelic exchange as described by previously (34).

Growth media and conditions. Unless otherwise indicated, bacteria were grown in Luria-Bertani (LB) broth with antibiotics at the following concentrations: 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and 50 μg/mL kanamycin (all from Sigma) at 37°C with aeration (225 rpm). MacConkey agar was prepared as per manufacturer's instructions (Difco) and 0.1 mM IPTG (final concentration, Invitrogen) was added after sterilizing.

Genetic screen for novel inhibitors. Envelope-localized genes were identified by comparing the unambiguous gene identity codes (JWXXX) from the microarray described in Raivio *et al.* (2013) (26) with the cellular localization listed on Genobase (http://ecoli.aist-

nara.ac.jp). Genes with known or predicted localization in membranes or the periplasm were identified and plasmids from the ASKA library (15) were extracted using the Sigma GenElute Plasmid Prep Kit, as per the manufacturer's instructions. Plasmids were transformed into TR50, plated on solid LB agar with chloramphenicol, and incubated at 37°C overnight. The next morning, four single isolated colonies of each transformant were streaked onto MacConkey agar plates along with the isogenic vector control strain and the positive control pCA-*ylbF*. MacConkey agar was chosen for this screen because the *cpxP'-'lacZ* reporter has high activity that is easily scored as bright red growth on MacConkey agar and lower activity is also easily scored as lighter pink or white growth. *ylbF* is a cytoplasmic gene of unknown function that inhibits the Cpx pathway on solid agar only (J. Wang, unpublished observations). Plasmids that produced a lighter colour on MacConkey agar than the isogenic vector control were identified and the original plasmid preparation was re-transformed into TR50. The inhibition phenotype was confirmed once more on MacConkey agar. Transformants that consistently produced a lighter color than the vector control on MacConkey agar were frozen in glycerol and stored at -80°C until screening on solid agar was complete. After the initial screening, candidates were struck out on to solid LB agar and β-galactosidase assays were performed from MacConkey agar to quantify inhibitory activity, as described below.

β-galactosidase assays. To quantify β-galactosidase activity of strains bearing candidate inhibitor plasmids, single isolated colonies were streaked from LB agar on to a MacConkey agar plate divided into eight sectors such that bacterial growth was confluent over the entire sector. Strains that could not produce confluent growth on MacConkey even in the absence of added IPTG were not tested further. Plates were incubated 37°C for 16-20 h. The next morning, a sterile wooden dowel was used to swipe bacteria from the sector in a manner that vertically

bisected growth and bacteria were inoculated into 2 mL LB in quintuplicate and vortexed briefly to resuspend cells. Cells were washed twice with 2 mL LB to remove residual dyes then resuspended in 2 mL 1X Z-buffer. One swipe of a sector with a wooden dowel generally gave an A_{600} of 0.4-0.6 in 1X Z-buffer. β-galactosidase assays were completed as described previously (29). The average β-galactosidase activity and standard deviation of three samples normalized to growth (A_{600}) is plotted in the Figures.

In assays to test for Cpx-dependence and in subsequent analyses, single isolated colonies were inoculated into 2 mL LB plus the appropriate antibiotics and grown at 37°C with aeration 16-20 h. Overnight cultures were diluted 1:50 into fresh LB plus antibiotics and grown 4.5 h at 37°C. 0.1 mM IPTG (final concentration) was added to each culture and cultures were returned to the incubator for an additional hour of growth. Cpx pathway activity was assayed by the microtitre plate assay, as described previously (29) where 50 µL of cell lysate from each sample was diluted in 150 µL of 1X Z-buffer for β-galactosidase analysis. The average β-galactosidase activity and standard deviation of three samples normalized to growth (A_{600}) is given.

Luminescence assays. Luminescence was measured as described in previously (31). Briefly, single isolated colonies were inoculated into 2 mL LB broth with the appropriate antibiotics and grown 37°C overnight with aeration. The next morning, overnight cultures were diluted 1:100 into fresh LB and grown one hour at 37°C with aeration. 200 µL aliquots were transferred to a black, clear-bottomed 96-well microtitre plate (Corning) (uninduced controls). 0.1 mM IPTG was added to the remaining cultures and vortexed briefly to mix. Another 200 µL from each culture was aliquoted to the microtitre plate (induced samples). The microtitre plate was fixed to a rack and grown at 37°C in a shaking incubator at 225 rpm for 6-8 h. Luminescence and A_{600} readings were taken at 0, 2, 4, and 6 h after induction and the average

luminescence normalized to growth (CPS/A_{600}) and standard deviation of triplicate samples was plotted versus time.

Growth curves. Cultures for growth curves were prepared exactly as described for luminescence assays (above) but 200 μL aliquots were transferred to clear 96-well microtitre plates (Corning) and the A_{600}, not luminescence, was monitored over 7-8 h of growth with readings every hour.

4.3. Results

4.3.1. Over-expression of *nuoF* and *efeB* inhibits the Cpx pathway in a *cpxA*-dependent manner

Previously, we performed a microarray analysis of genes differentially regulated by *nlpE* over-expression in the lab strain *E. coli* MC4100 and the enteropathogenic strain E2348/69 (EPEC) in complex medium (LB) and defined medium (DMEM) (26). To identify envelope-localized genes affected in any condition, we cross-referenced all of the genes with greater than 2-fold changes in expression according to our microarray data with the predicted localizations on the *E. coli* database Genobase (http://ecoli.aist-nara.ac.jp) (21). We selected genes with known or predicted localization in membranes or the periplasm, regardless of whether activation of the Cpx response resulted in an increase or decrease in expression of the transcripts. The expression of 176 candidate genes were affected in at least one condition in at least one strain, had predicted or confirmed envelope-localization, and could be found in the ASKA library, a library of IPTG-inducible plasmids expressing nearly every gene in *E. coli* K-12 (15). We transformed the plasmids into a strain of *E. coli* bearing a *cpxP'-'lacZ* transcriptional reporter (TR50) and examined transformant growth on MacConkey agar containing IPTG to induce protein expression. Transformants that were lighter in color than the isogenic vector control strain on

MacConkey agar exhibited decreased *cpxP'-'lacZ* expression and were selected as candidate inhibitors (Figure 4-1). From this initial screen, we identified 25 plasmids that consistently resulted in lighter red colonies than the vector control strain on MacConkey agar. We then quantified the β-galactosidase activity of each strain and looked for plasmids that resulted in a 2-fold or greater decrease in *cpxP'-'lacZ* activity upon IPTG-induction to over-express the cloned gene (Figure 4-2A). Six candidate plasmids with greater than two-fold effects on *cpxP'-'lacZ* expression that did not also affect growth were identified. Over-expression of the genes encoding a murein amidase (*amiA*), thiosulfate-binding protein (*sbp*), membrane tension-sensing conductance channel (*yjeP*/*mscM*), an inner membrane stress-responsive protein (*pspA*), the NADH-binding component of the Nuo Complex I (*nuoF*), and the heme peroxidase (*efeB*) strongly inhibited Cpx activity. Over-expression of *nuoA*, *gltA*, and *ytfK*, encoding a membrane-embedded subunit of the Nuo complex, citrate synthase, and a protein of unknown function, respectively, resulted in poor growth on MacConkey agar and these plasmids were not analysed further.

We sought to find gene products that inhibited the Cpx pathway analogously to CpxP, which implies a dependence on both the response regulator *cpxR* and the sensor kinase *cpxA*. Accordingly, we next tested whether inhibition of *cpxP'-'lacZ* expression was dependent on *cpxR*. CpxR is the only known regulator of *cpxP* expression and the activity of the *cpxP'-'lacZ* reporter is almost entirely dependent on the presence of *cpxR* (8). Therefore, we could not reliably assay inhibition of the *cpxP'-'lacZ* reporter in the absence of *cpxR*. To circumvent this problem, we employed another Cpx-regulated reporter gene, *degP'-'lacZ,* which has significant activity in the absence of *cpxR* due to the activity of the alternative sigma factor σ^E (9). Surprisingly, only two of the six candidate inhibitors required *cpxR* for inhibition of the Cpx

pathway, *nuoF* and *efeB* (Figure 4-2B). The two plasmids bearing the genes *nuoF* and *efeB* were transformed into a *cpxA* mutant reporter strain and assayed for activity both directly from MacConkey and when grown in liquid. In both experiments, over-expression of both *nuoF* and *efeB* resulted in decreased reporter expression, although the strongest effect was observed in liquid (Figure 4-2C). In a wild-type background, over-expression of *nuoF* decreased *cpxP-lacZ* activity approximately 24-fold while over-expression of *efeB* decreased Cpx activity approximately 30-fold. In contrast, in a *cpxA* mutant background, over-expression of either gene resulted in only a 1.2-fold decrease, suggesting that *cpxA* is required for *nuoF*- and *efeB*-mediated inhibition. We sequenced the inserts of each plasmid and confirmed the identity of the *nuoF* and *efeB* constructs (data not shown). Based on these data, we conclude that NuoF and EfeB are novel *cpxR*- and *cpxA*-dependent inhibitors of the Cpx pathway when over-expressed.

4.3.2. *nuoF* and *efeB* are *cpxP*-dependent inhibitors

Deletion of *cpxP* is not sufficient to fully de-repress the Cpx pathway (25) and we hypothesized that deletion of *nuoF* or *efeB* or both would remove the remaining inhibition of Cpx activity in a *cpxP* mutant. To test this, we created strains carrying a *cpxP'-'lacZ* transcriptional reporter gene and all possible single, double, or triple mutant combinations of *cpxP, nuoF*, and *efeB* mutations and measured β-galactosidase expression in these strains. Consistent with an inhibitory role, deletion of *nuoF* activated the Cpx pathway to a similar extent as the *cpxP* mutant, approximately 1.6-fold (Figure 4-3A). In contrast, deletion of *efeB* had no effect on Cpx pathway activity (Figure 4-3A). The double and triple mutants had activities similar to the single *cpxP* or *nuoF* mutants, suggesting that the effects of deleting all three inhibitors on Cpx pathway activity are not additive. Therefore, *nuoF*, *efeB*, and *cpxP* likely work in the same pathway to inhibit Cpx pathway upon over-expression.

Since the effect of deleting both *cpxP* and *nuoF* was not additive, these inhibitors may work in a single pathway. To test whether the new inhibitors NuoF and EfeB worked independently of CpxP, we over-expressed *nuoF* and *efeB* in wild-type and *cpxP* mutant strains and measured expression from the *cpxP'-'lacZ* reporter gene. Indeed, inhibition by *nuoF* and *efeB* was at least partially-dependent on intact *cpxP* (Figure 4-3B). While over-expression of either *nuoF* or *efeB* in a wild-type background resulted in an approximately 30-fold decrease in Cpx activity, over-expression in a *cpxP* background resulted in only a 1.6-fold decrease. The signals generated by *nuoF* and *efeB*, therefore, are *cpxP*-dependent and, to our knowledge, are the only *cpxP*-dependent signals known.

nuoF requires *cpxP* for inhibition and we sought to determine whether *cpxP* also requires *nuoF* to inhibit the Cpx pathway. We transformed single and double *cpxP* and *nuoF* mutants with vectors over-expressing *cpxP*. Over-expression of *cpxP* decreased Cpx activity to a similar extent in both wild-type and *nuoF* mutant backgrounds (Figure 4-3C), suggesting that *cpxP* does not require *nuoF* to inhibit the Cpx pathway, even though *nuoF* requires *cpxP*.

4.3.4. *nuoF* and *efeB* are not required for acid-mediated repression of Cpx activity

CpxP is an alkaline-inducible gene and its expression is strongly inhibited by acidic pH (8). However, growth in acidic pH highly induces the expression of both *efeB* and *nuoF* (5, 19) . Therefore, we hypothesized that the increase in NuoF and EfeB levels at low pH might contribute to Cpx repression. Accordingly, we grew up cultures of wild-type, *nuoF,* and *efeB* mutants in sodium phosphate-buffered LB at pH 5.8 or pH 7.0 and examined *cpxP'-'lacZ* activity in each strain. Deletion of neither *nuoF* nor *efeB* affected Cpx activity at low pH (Figure 4-4). Therefore, though both *nuoF* and *efeB* are highly expressed at low pH, neither inhibitor is necessary for low pH-mediated changes to Cpx activity.

4.3.4. *nuoF* does not require a functional *nuo* complex to inhibit the Cpx pathway.

To further characterize the nature of the *nuoF* inhibitory signal, we tested whether *nuoF* requires a functional NADH dehydrogenase I complex (Nuo) to inhibit the Cpx pathway. *nuoF* encodes the NADH-binding subunit of Nuo or NADH dehydrogenase complex I, a 14-member complex that aids in establishing proton motive force and oxidizing NADH during aerobic and anaerobic respiration (4, 35). We wondered whether other members of the Nuo complex could exert a similar effect on Cpx pathway activity. NuoF interacts with two other soluble components of the Nuo complex, NuoE and NuoG, while NuoM is a non-conserved membrane-embedded member of the complex (3, 11) . While over-expression of *nuoF* strongly decreased Cpx activity, over-expression of its proposed interacting partners *nuoE* or *nuoG* did not inhibit Cpx activity, nor did a membrane-embedded component, *nuoM* (Figure 4-5A). Thus, amongst the genes that interact with NuoF in Complex I, *nuoF* specifically inhibits Cpx activity upon over-expression.

Because NuoF is the catalytic component of Nuo, we hypothesized that over-expression of *nuoF* changes the NADH oxidase activity of the Nuo complex and these changes are sensed by Cpx. To test our hypothesis, we used an enteropathogenic *E. coli* (EPEC) mutant in which all fourteen *nuo* genes have been deleted. We reasoned that *nuoF* should lose the ability to inhibit the Cpx response in the absence of a functional complex if the Cpx pathway senses altered NADH oxidase activity. We monitored luminescence of a *cpxP-lux* reporter in the EPEC *nuo* mutant over-expressing *nuoF* and observed that *nuoF* over-expression strongly decreased luminescence in both wild-type and *nuo* mutant backgrounds (Figure 4-5B). Thus, *nuoF* does not require a functional NADH dehydrogenase I complex to inhibit the Cpx pathway and Cpx is not sensing altered Complex I activity upon *nuoF* over-expression.

4.3.5. Deletion of *nuoF* but not *efeB* aids cells in surviving Cpx-related stresses

Our analyses have suggested that over-expression of *nuoF* and *efeB* represses Cpx pathway activity through CpxP and CpxA. However, the Cpx pathway positively regulates *cpxP* expression and over-expression of *cpxP* negatively-regulates Cpx activity in a negative feedback loop thought to assist in attenuation of the response after stress has subsided (25). In contrast, expression of both *nuoF* and *efeB* is strongly down-regulated upon Cpx activation (26) and over-expression of either gene strongly down-regulates Cpx activity (Figure 4-2C). We wondered whether the strong negative regulation of Cpx inhibitors aids survival during *nlpE* over-expression, a Cpx-related stress (30). To answer this question, we over-expressed *nlpE* in wild-type, *cpxP*, *nuoF*, and *efeB* single mutants and monitored growth over 8 hours. We expected that deletion of *nuoF* and *efeB*, which are strongly down-regulated by the Cpx response upon activation by *nlpE* (26), would result in increased growth because the expression of these genes might be detrimental to cell survival under these conditions. Surprisingly, over-expression of *nlpE* strongly arrested growth over the entire time tested (Figure 4-6) and this growth arrest has not been observed previously. We do not know the basis for *nlpE*-mediated toxicity in our assay. Nevertheless, the strong toxicity of high-level *nlpE* expression allowed us to observe clear growth differences between the wild-type and mutant strains. Deletion of *cpxP* and *nuoF* increased survival in the presence of *nlpE* over-expression while deletion of *efeB* had no effect on survival. The *nuoF* mutant grew better than wild-type at all time points assayed whereas the *cpxP* mutant obtained a significant growth advantage between 4 and 5 hours post-induction. The *efeB* mutation had no effect on growth at any time point assayed, however, expression of *efeB* is not detectable above pH 5 (5), suggesting that *efeB* deletion would not have an effect under the conditions tested. However, over-expression of EfeB still decreased *cpxP'-'lacZ* expression at

neutral pH (Figure 4-2C), suggesting that EfeB can affect activation of the Cpx response when it is expressed at high levels.

4.4. Discussion

4.4.1. NuoF and EfeB are novel inhibitors of the Cpx pathway

A genetic screen for novel inhibitors of the Cpx pathway identified two *cpxA-, cpxR-,* and *cpxP*-dependent inhibitors: *nuoF* and *efeB*. Whereas *nuoF* encodes the cytoplasmic NADH-binding subunit of Nuo, *efeB* encodes the periplasmic heme peroxidase for the cryptic EfeUOB heme transporter in *E. coli* K-12 (5, 35) . Over-expression of *nuoF* in the cytoplasm generates a *nuo*-independent signal that is sensed by the periplasmic auxiliary regulator CpxP. At this time, we do not know the nature of the signal generated by *nuoF* over-expression, however, we believe the NADH-binding activity of *nuoF* may play a role in signalling. Mutagenesis studies targeting a conserved glutamate required for NADH-binding are currently underway (10).

At present, we favor a model where the NADH-binding activity of *nuoF* alters the NADH to NAD^+ ratio in *E. coli* and generates a metabolic signal that is sensed by CpxP. Though highly speculative, the idea that metabolism is sensed by Cpx is not novel. The accumulation of a specific metabolite is thought to induce the Cpx pathway in mutants lacking the outer membrane efflux pump protein *tolC*, though the specific metabolite remains unidentified (27). Unless NuoF associates with other electron transport complexes with cofactors of the appropriate redox potentials, NuoF will bind NADH and fail to regenerate NAD^+, an important cellular energy currency for metabolism. Possibly, the inability to recycle NAD^+ will prevent the synthesis or export of a metabolite that induces Cpx activity.

EfeB also requires CpxP and likely does not require the functional components of the EfeUOB complex to inhibit Cpx. The EfeU heme permease is not expressed in *E. coli* K-12 due to a frameshift mutation but both the accessory proteins EfeO and EfeB are expected to be expressed (5). Furthermore, the CpxR- and Fur-mediated repression of the *efeUOB* promoter is maintained in *E. coli* K-12 where *efeU* is cryptic (5, 28). EfeB is thought to facilitate the import of iron into the cytoplasm in a similar manner to the yeast homologue Fet3p, where iron is extracted from heme and transferred to the EfeU homologue Ftr1p (5). EfeB is part of the dye decolorizing peroxidase family (DyP family) and is uniquely bifunctional, with both deferrochelatase and peroxidase activities (17). We were unable to identify any *efeB*-related phenotypes with regard to Cpx pathway activity in our study, but the expression of EfeB is highest at pH values below 6 and the majority of our assays were performed at pH 7.0. Over-expression of *efeB*, however, mediates Cpx repression at neutral pH. Targeted mutagenesis of the residues required for each of the functions of EfeB will help us determine whether the known enzymatic activities of EfeB are required for Cpx signaling.

Previous work in our lab has established a potential role for the Cpx system in sensing and responding to changing cellular metal status (1) (Chapters 2 and 3 of this thesis). The guaiacol peroxidase activity of EfeB is particularly intriguing given that guaiacol is a precursor of catechol, a principal component of catecholate siderophores such as enterobactin. In the periplasm, apo-enterobactin is thought to facilitate the production of toxic cuprous ion by reducing cupric ion (12, 14). However, the multicopper oxidase CueO oxidizes enterobactin to prevent the reduction of copper and prevent oxidative damage of periplasmic components by cuprous ion (12, 14) . Potentially, EfeB can mediate oxidation of similar catecholate compounds and decrease Cpx activity by remediating periplasmic metal stress. Current work in our lab has

revealed that deletion of the isochorismate synthase *entC* that catalyzes the first biosynthetic step in the synthesis of enterobactin decreases the elevated Cpx activity in the *tolC* mutant grown in iron-deplete minimal medium (Court, *E.,* unpublished observations). Apo-enterobactin accumulates in the periplasm of *E. coli* grown in iron-deplete minimal medium and causes growth arrest and aberrant cell morphology (33). Therefore, it would be interesting to examine the ability of EfeB over-expression to suppress the Cpx activation observed in the *tolC* mutant grown in iron-deplete minimal medium to see if the function of EfeB is indeed connected to the changes in metal homeostasis mediated by apo-enterobactin in the periplasm.

4.4.2. How do cells maintain Cpx inhibition in the absence of inhibitors?

Only CpxR is known to regulate the basal activity of *cpxP* in *E. coli,* yet we identified six Cpx-regulated genes that strongly inhibit Cpx activity, even in the absence of *cpxR.* Over-expression of the muramidase *amiA*, the thiosulfate solute-binding protein *sbp,* the mechanosensitive ion channel *yjeP* also known as *mscM*, and the inner-membrane associated protein *pspA* had *cpxR*-independent effects on Cpx activity. Possibly, the over-expression of these genes feeds into other signaling pathways that affect the expression of Cpx-regulated reporter genes. For example, the sigma factor σ^E regulates some genes that are also regulated by CpxR, such as *degP*, *ompC*, and *ompF* (24). Both BaeR and CpxR mediate the activation of *spy,* a gene encoding a periplasmic chaperone, in response to some overlapping and some distinct cues (22). Therefore*,* other transcriptional regulators might be changing the expression of Cpx-regulon members through distinct signaling mechanisms that do not require CpxR.

In this study, we set out to find inhibitors that might mediate the Cpx inhibition that remains in the absence of *cpxP*. We showed that NuoF behaves similarly to CpxP in that its over-expression down-regulates the Cpx response, while deletion of *nuoF* leads to activation. Similarly, over-

expression of EfeB also inhibited Cpx-mediated gene expression. Interestingly, the triple *cpxP nuoF efeB* mutant did not have significantly different activity from that of the *cpxP* or *nuoF* single mutants and the Cpx response was not further activated. There may be proteins that are not Cpx-regulated that inhibit the Cpx pathway in the absence of *cpxP* that could not be identified within the parameters of our screen. Alternatively, CpxA may maintain a conformation that could mediate Cpx inhibition in the absence of stress without the aid of other proteins. The ArcB sensor kinase is kept in an active state by growth in anaerobic conditions that maintain the reduced state of cysteine residues proximal to the membrane. ArcB phosphorylates the response regulator ArcA, leading to the expression of genes that aid anaerobic growth. In the presence of oxygen, quinones oxidize the cysteine residues, creating a disulfide bond that inactivates the ArcB kinase, likely by increasing the rigidity of helical movements and preventing autophosphorylation and signal transduction to ArcA (18). Perhaps residues in the periplasmic sensing domain of CpxA could serve a similar purpose by sensing specific periplasmic conditions and only allowing conformational changes in CpxA in the presence of those stressors. Removal of CpxP or other inhibitors may be involved in the step-wise activation of CpxA to ensure that Cpx pathway activity is activated only in the presence of accumulated envelope stress.

NuoF and EfeB are novel inhibitors of the Cpx response in *E. coli* K-12 that inhibit the Cpx pathway through CpxP. Though the mechanism is unclear, we propose that *nuoF* and *efeB* generate cellular signals that are integrated by the auxiliary regulator *cpxP* to modulate signal-sensing during envelope stress. NuoF may represent a novel input for metabolic stresses into the Cpx pathway whereas EfeB may integrate metal-related or periplasmic redox stress to the Cpx pathway, thus expanding the potential roles for the Cpx response.

4.5. Tables

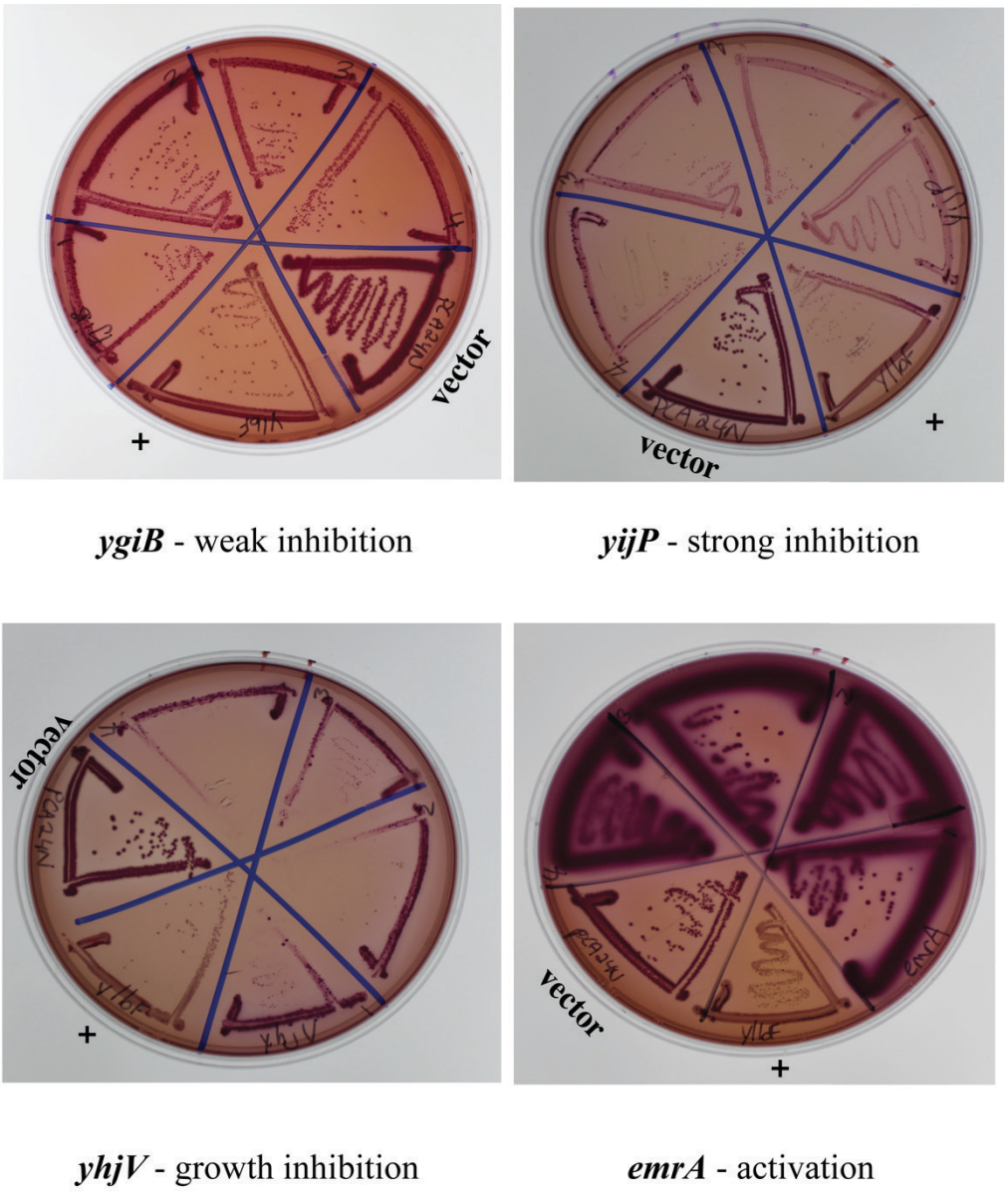
Table 4-1: Bacterial strains and plasmids used in this study

Strain Name	Genotype or Description	Reference
E2348/69	Prototypical enteropathogenic *E. coli* strain bearing the EAF plasmid	(16)
JLW7	TR50 pCA24N	This study
JLW392	TR50 Δ*cpxP*	This study
JLW400	JLW392 pTrc99A	This study
JLW404	JLW392 pCpxP	This study
JLW606	TR49 pCA24N	This study
JLW607	TR49 pCA-*ylbF*	This study
JLW609	TR50 pCA-*ylbF*	This study
JLW671	TR50 pCA-*emrD*	This study
JLW641	TR50 pCA-*sbp*	This study
JLW660	TR50 pCA-*amiA*	This study
JLW662	TR50 pCA-*ytfK*	This study
JLW621	TR50 pCA-*nuoA*	This study
JLW623	TR50 pCA-*nuoJ*	This study
JLW637	TR50 pCA-*ygaH*	This study
JLW643	TR50 pCA-*yhjV*	This study
JLW640	TR50 pCA-*yijP*	This study
JLW635	TR50 pCA-*yjeP*	This study
JLW642	TR50 pCA-*hflC*	This study
JLW620	TR50 pCA-*yojL*	This study
JLW638	TR50 pCA-*ygdQ*	This study
JLW639	TR50 pCA-*ygiB*	This study
JLW636	TR50 pCA-*ygbE*	This study
JLW651	TR49 pCA-*amiA*	This study
JLW652	TR49 pCA-*sbp*	This study
JLW653	TR49 pCA-*yjeP*	This study
JLW654	TR49 pCA-*pspA*	This study
JLW655	TR49 pCA-*nuoF*	This study
JLW656	TR49 pCA-*efeB*	This study
JLW677	JLW392 pCA24N	This study
JLW698	RM53 pCA24N	This study
JLW770	TR50 pCA-*efeB*	This study
JLW771	TR50 pCA-*nuoF*	This study
JLW773	TR69 pCA24N	This study
JLW774	TR69 pCA-*ylbF*	This study
JLW775	TR69 pCA-*amiA*	This study
JLW777	TR69 pCA-*sbp*	This study
JLW778	TR69 pCA-*yjeP*	This study
JLW780	TR69 pCA-*nuoF*	This study
JLW781	TR69 pCA-*pspA*	This study

JLW782	TR69 pCA-*efeB*	This study
JLW785	TR52 pCA24N	This study
JLW786	TR52 pCA-*ylbF*	This study
JLW787	TR52 pCA-*amiA*	This study
JLW788	TR52 pCA-*sbp*	This study
JLW790	TR52 pCA-*yjeP*	This study
JLW791	TR52 pCA-*pspA*	This study
JLW792	TR52 pCA-*efeB*	This study
JLW793	TR52 pCA-*nuoF*	This study
JLW857a	TR50 Δ*efeB*	This study
JLW864b	TR50 Δ*nuoF*	This study
JLW867	JLW864b pCA24N	This study
JLW870	JLW864b *cpxP*	This study
JLW872	JLW864b pCA-*nlpE*	This study
JLW877	RM53 pCA-*nuoF*	This study
JLW878	RM53 pCA-*efeB*	This study
JLW881	JLW857a *nuoF:kn*	This study
JLW882b	JLW857a *cpxP*	This study
JLW883	JLW392 Δ*nuoF ΔefeB*	This study
JLW889	JLW392 pCA-*nlpE*	This study
JLW890	JLW392 pCA-*nuoF*	This study
JLW895	JLW857b pCA24N	This study
JLW896	JLW857b pCA-*nlpE*	This study
JLW897	JLW870 pCA24N	This study
JLW898	JLW870 pCA-*nlpE*	This study
JLW897	JLW870 pCA24N	This study
JLW901	NLP94 pCA24N	This study
JLW902	NLP94 pCA-*nuoF*	This study
JLW903	RG158 pCA-*nuoF*	This study
JLW904	RG158 pCA24N	This study
JLW905	JLW864b pTrc99A	This study
JLW906	JLW864b pCpxP	This study
JLW907	JLW870 pTrc99A	This study
JLW908	JLW870 pCpxP	This study
JLW909	JLW870 pCA-*nuoF*	This study
JLW967	TR50 pCA-*nuoM*	This study
JLW968	TR50 pCA-*nuoE*	This study
JLW971	TR50 pCA-*nuoG*	This study
JSW2	TR50 pCA-*nlpE*	This study
JSW3	TR50 pCA-*dacC*	This study
JSW4	TR50 pCA-*ycbB*	This study
JSW5	TR50 pCA-*yebA*	This study
JSW6	TR50 pCA-*gltA*	This study
JSW7	TR50 pCA-*tsx*	This study
JSW8	TR50 pCA-*sdhC*	This study
JSW9	TR50 pCA-*htpX*	This study

MC4100	F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150*(Str^r) *relA1 flbB5301 decC1 ptsF25 rbsR*	(6)
NLP94	E2348/69 pJW25	(20)
RG158	NLP94 *nuoABCDEFGHIJKLM*	This study
RM53	TR50 *cpxA*	This study
TR49	TR50 *degP'-'lacZ* at λRS88	This study
TR50	MC4100 *cpxP'-'lacZ* at λRS88	(23)
TR69	TR49 *cpxR::spc*	This study
Plasmids	**Description**	**Source or Reference**
pCA24N	High-copy IPTG-inducible vector, Cam^R	(15)
pTrc99A	High-copy IPTG-inducible vector, Amp^R	Pharmacia
pCpxP	Vector for high-copy expression of *cpxP*, Amp^R	(25)

4.6. Figures



ygiB - weak inhibition

yijP - strong inhibition

yhjV - growth inhibition

emrA - activation

Figure 4-1: A genetic screen for novel inhibitors of the Cpx pathway identified a number of different regulators of Cpx activity.

β-galactosidase activity of TR50 over-expressing *ygiB* (JLW639), *yijP* (JLW640), *yhjV* (JLW643), and *emrA* (JLW622). Plasmids bearing Cpx-regulated genes that encode envelope-

localized proteins were transformed into TR50 and plated on LB agar plus chloramphenicol. Single isolated colonies were struck on to MacConkey agar and examined for growth and β-galactosidase (*cpxP'-'lacZ*) activity. Examples of strong and weak inhibitors, growth inhibitors, and activators are given above. The position of the vector control is labeled as 'vector'. The positive control strain over-expressing *ylbF* is marked with a '+' sign. Strong and weak inhibitors were selected for further analysis. The survival and β-galactosidase activity of each strain over-expressing a candidate inhibitor were assessed twice on MacConkey agar to confirm the *lacZ* phenotype.

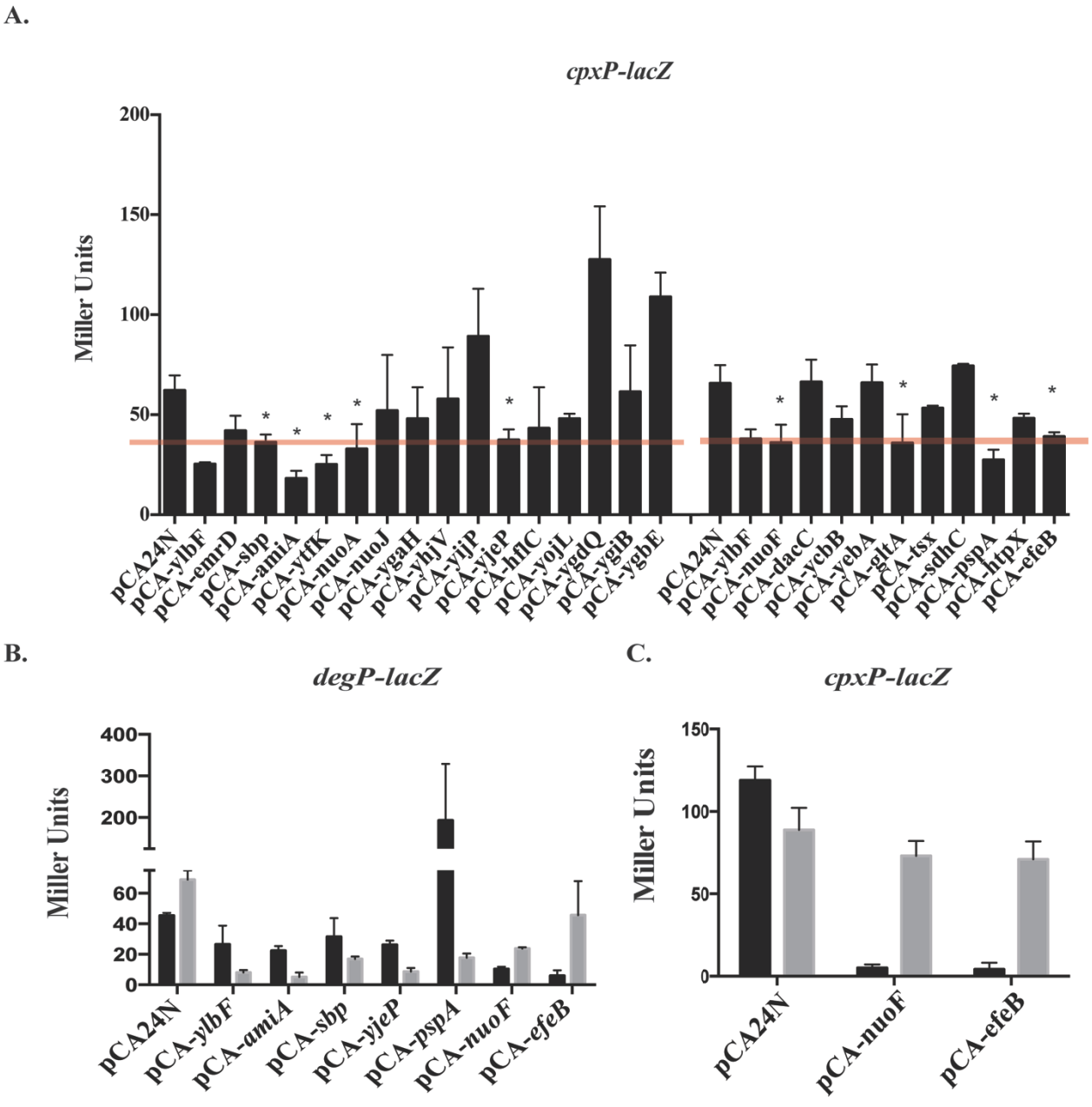


Figure 4-2: Over-expression of *nuoF* and *efeB* inhibit the Cpx TCS in a *cpxA*-dependent manner.

A. Many Cpx-regulated genes have effects on Cpx activity upon over-expression. β-galactosidase activity of TR50 over-expressing candidate inhibitor genes assayed directly off of MacConkey agar. The red line marks 50% of the activity of the vector control strain and strains

with β-galactosidase activity below the red line were selected for further study (marked with '*'). The average β-galactosidase activity and standard deviation of three technical replicates per sample is given by each bar and respective error bar above. A representative result of two independent experiments is shown. Candidate inhibitors on the left half of the graph were identified by Julia Wong while those on the right half were identified by Junshu Wang. The β-galactosidase activity of strains over-expressing candidate inhibitors was assayed by Julia Wong. **B.** Only *nuoF* and *efeB* have *cpxR*-dependent effects on Cpx activity. β-galactosidase activity of wild-type (dark bars) or *cpxR* mutant (light grey bars) TR49 over-expressing *ylbF* (JLW607, JLW774), *amiA* (JLW651, JLW775), *sbp* (JLW652, JLW777), *yjeP* (JLW653, JLW778), *pspA* (JLW654, JLW781), *nuoF* (JLW655, JLW780), *efeB* (JLW656, JLW782), or the vector control (JLW606, JLW773). **C.** *nuoF* and *efeB* are CpxA-dependent inhibitors. β-galactosidase activity of wild-type (dark bars) or *cpxA* mutant (light grey bars) over-expressing *nuoF* (JLW771, JLW877), *efeB* (JLW770, JLW878), or the vector control (JLW7, JLW698). The average β-galactosidase activity and standard deviation of three technical replicates per sample is given here. A representative result from two independent experiments is shown.

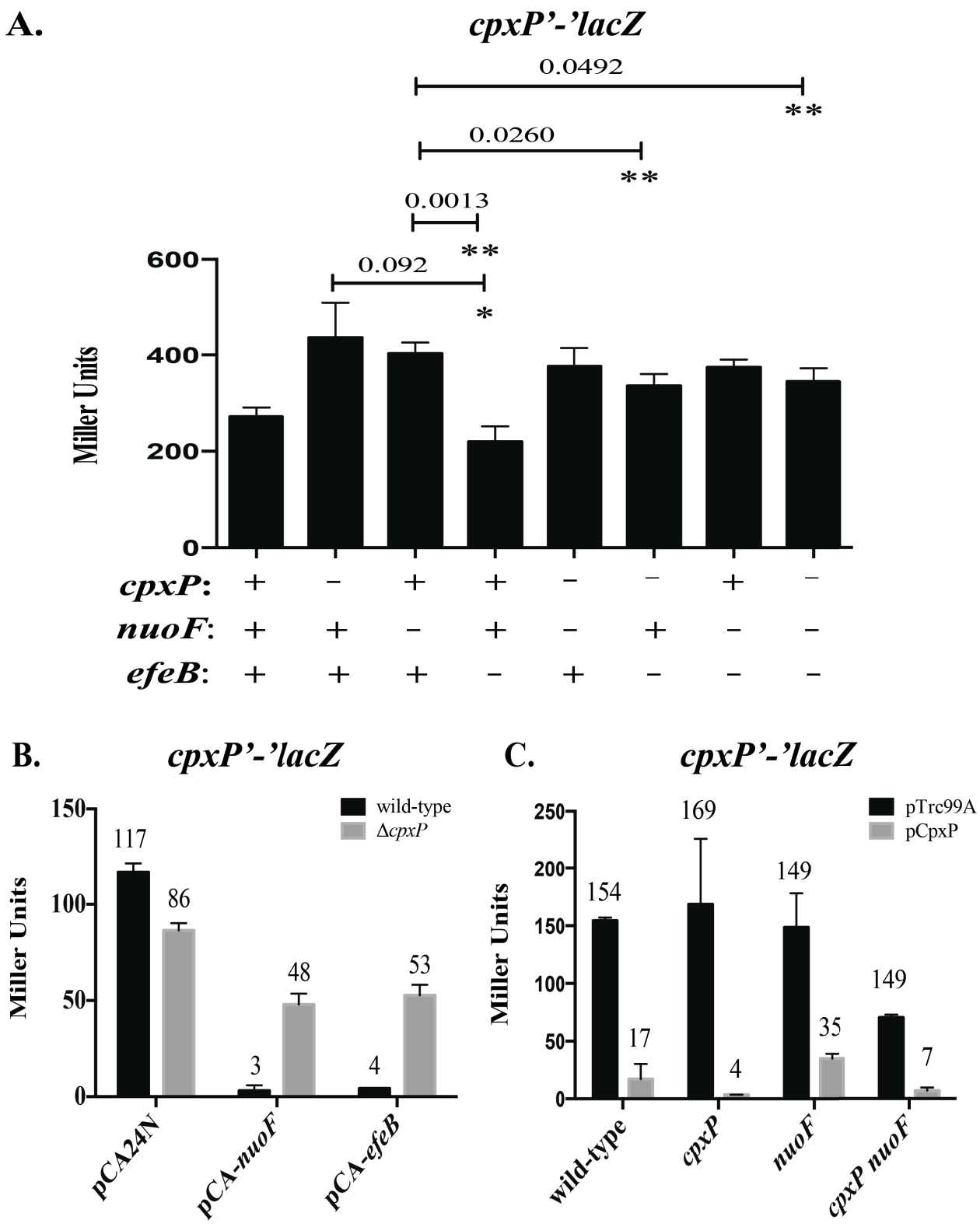

A.
cpxP'-'lacZ
0.0492
**
0.0260
**
0.0013
**
0.092
*
600
400
200
0
Miller Units
cpxP: + − + + − − + −
nuoF: + + − + − + − −
efeB: + + + − + − − −
B.
cpxP'-'lacZ
wild-type
ΔcpxP
150
100
50
0
Miller Units
117
86
3
48
4
53
pCA24N
pCA-nuoF
pCA-efeB
C.
cpxP'-'lacZ
pTrc99A
pCpxP
250
200
150
100
50
0
Miller Units
154
17
169
4
149
35
149
7
wild-type
cpxP
nuoF
cpxP nuoF

Figure 4-3: The effects of *nuoF* and *efeB* on Cpx pathway activity depend on *cpxP*.

A. Deletion of *cpxP, nuoF*, or both activates the Cpx response. β-galactosidase activity expressed from a *cpxP'-'lacZ* reporter gene was measured in wild-type (TR50), *cpxP* (JLW392), *nuoF* (JLW864b), *efeB* (JLW857a), the double *cpxP nuoF* (JLW870), *cpxP efeB* (JLW882b), *efeB nuoF:kn* (JLW881) and triple *cpxP nuoF efeB* mutants (JLW883). **B.** Inhibition of the Cpx pathway by *nuoF* and *efeB* depends on *cpxP*. β-galactosidase activity of wild-type (dark bars) and *cpxP* mutant (light grey bars) over-expressing *nuoF, efeB*, or the vector control. **C.** *cpxP* does not depend on *nuoF* to inhibit the Cpx pathway. β-galactosidase activity of wild-type, *cpxP*, *nuoF*, or *cpxP nuoF* mutant TR50 over-expressing *cpxP* (light grey bars) or the vector control (dark bars). The average β-galactosidase activity and standard deviation of three technical replicates per sample is given in the graphs above and each graph is a representative result of at least two independent experiments.

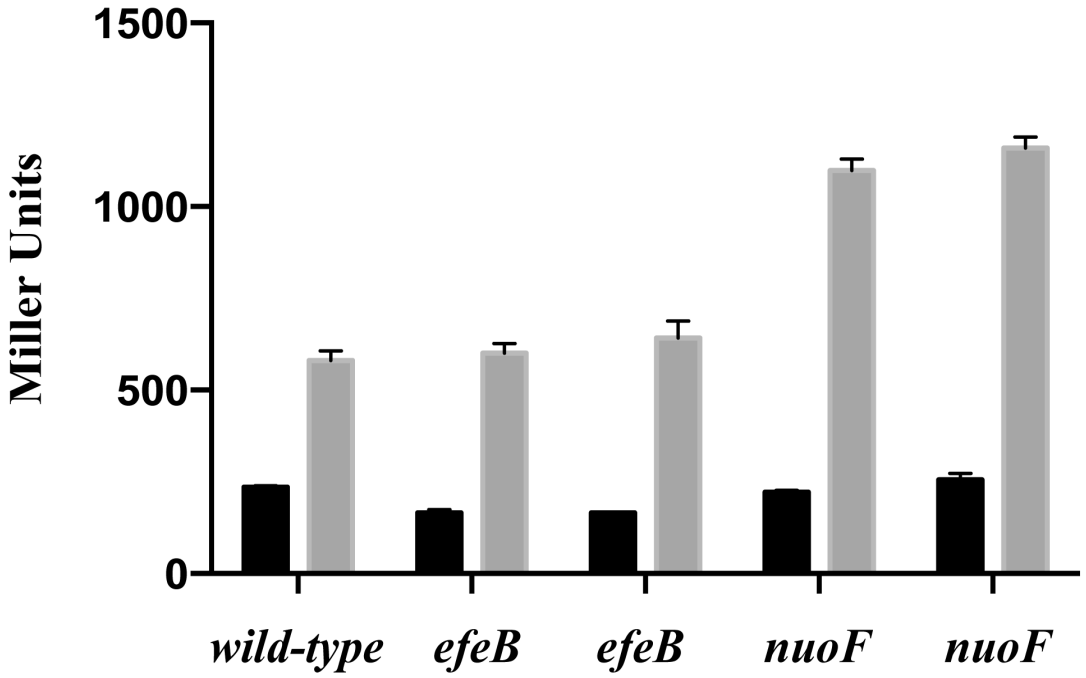


Figure 4-4: Deletion of neither *nuoF* nor *efeB* affects acid-mediated repression of the Cpx pathway.

β-galactosidase activity of wild-type (TR50), *efeB* (JLW857a), or *nuoF* mutants (JLW864b) grown in sodium phosphate-buffered LB pH 5.8 (dark bars) or 7.0 (light grey bars). The average β-galactosidase activity and standard deviation of two independently-generated mutants was assayed in triplicate and a representative result of two independent experiments is given here.

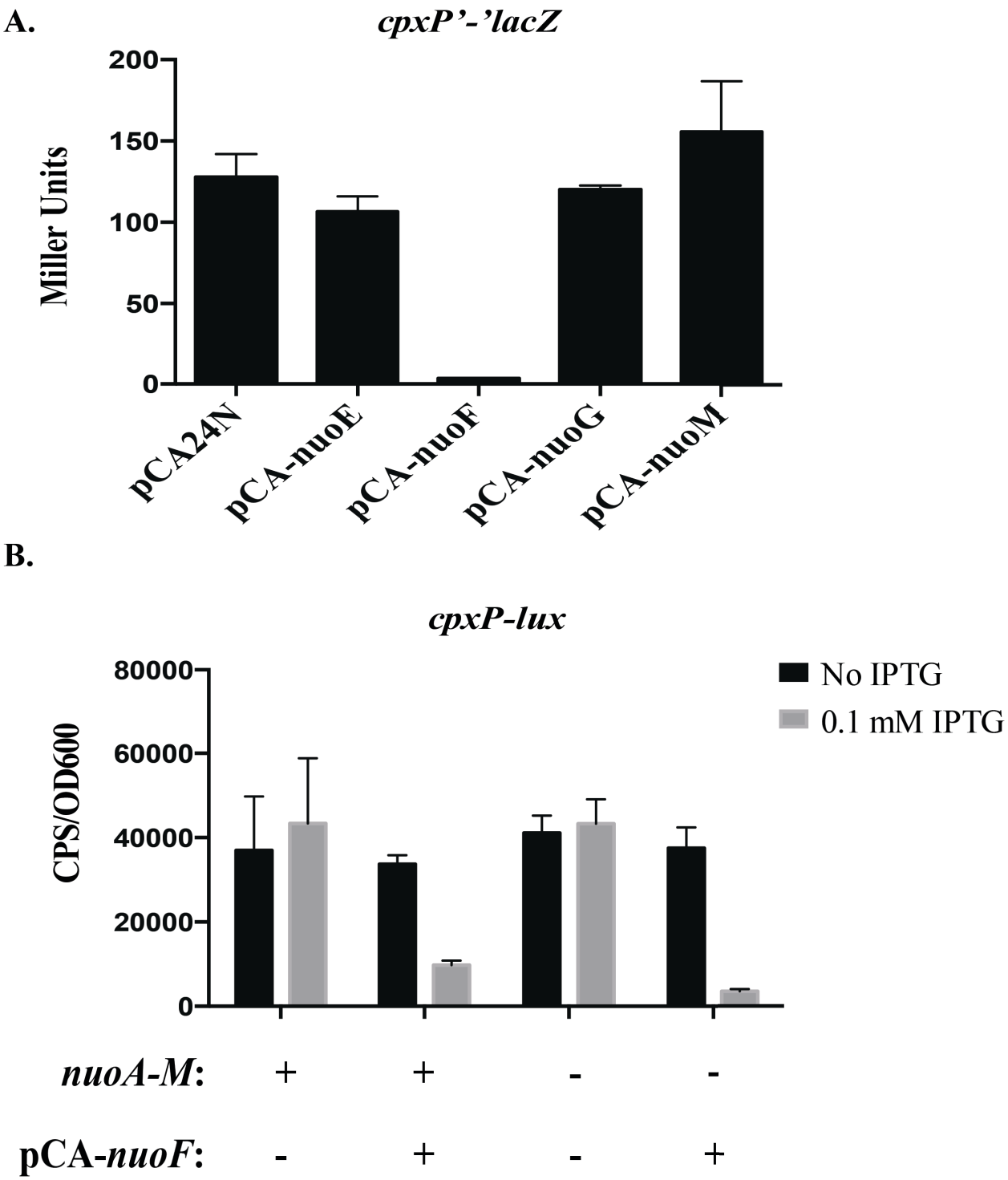

A.
cpxP'-'lacZ
Miller Units
200
150
100
50
0
pCA24N
pCA-nuoE
pCA-nuoF
pCA-nuoG
pCA-nuoM
B.
cpxP-lux
CPS/OD600
80000
60000
40000
20000
0
No IPTG
0.1 mM IPTG
nuoA-M: + + - -
pCA-nuoF: - + - +

Figure 4-5: Over-expression of *nuoF* does not require a functional NADH dehydrogenase complex I to inhibit the Cpx pathway.

A. Over-expression of other NADH-dehydrogenase complex I components does not inhibit the Cpx pathway. β-galactosidase activity expressed from a *cpxP'-'lacZ* reporter gene was measured in wild-type strains (TR50) over-expressing *nuoE* (JLW968), *nuoF* (JLW771), *nuoG* (JLW971), *nuoM* (JLW967), or the vector control (JLW7). The average β-galactosidase activity and standard deviation of three technical replicates per sample is represented by each bar and error in the graph above, respectively. **B.** Over-expression of *nuoF* inhibits the Cpx pathway in EPEC in the absence of other Complex I members. Luminescence of wild-type (E2348/69) or *nuoA-M* (RG158) over-expressing *nuoF* (JLW904) or the vector control (JLW903) in the presence (light grey bars) or absence (dark bars) of 0.1mM IPTG, 4 hours post-induction. The average luminescence and standard deviation normalized to growth (counts per second (CPS)/OD_{600}) of triplicate samples was graphed against time. A representative result of two independent experiments is shown here.

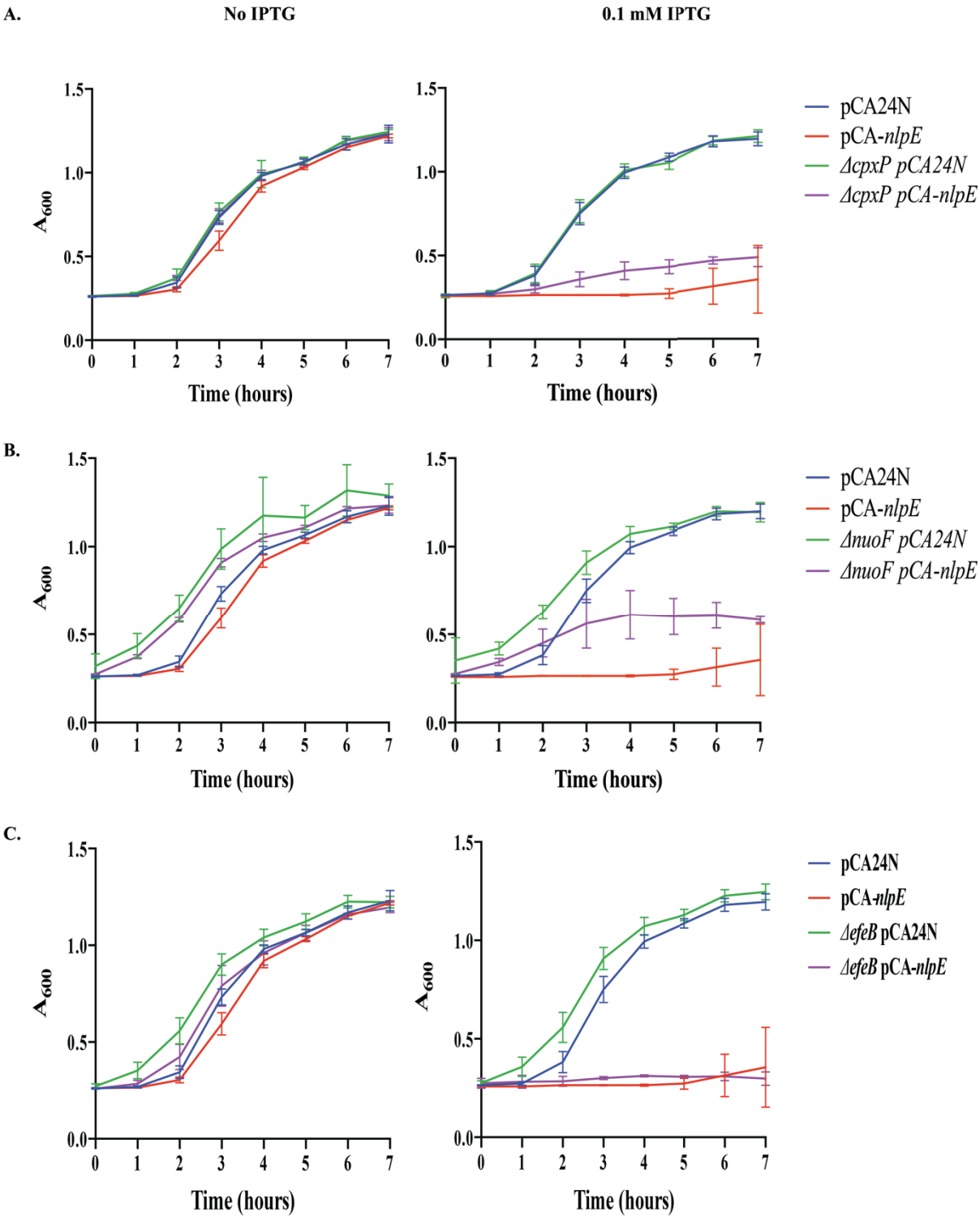

A.
No IPTG
0.1 mM IPTG
A600
Time (hours)
pCA24N
pCA-nlpE
ΔcpxP pCA24N
ΔcpxP pCA-nlpE
B.
pCA24N
pCA-nlpE
ΔnuoF pCA24N
ΔnuoF pCA-nlpE
C.
pCA24N
pCA-nlpE
ΔefeB pCA24N
ΔefeB pCA-nlpE

Figure 4-6: Deletion of either *cpxP* or *nuoF* partially rescues cells from the toxicity of over-expressing *nlpE*.

Growth of **A.** wild-type or *cpxP* mutant strains over-expressing *nlpE* (JSW2, JLW889) or the vector control (JLW7, JLW677); **B.** wild-type or *nuoF* mutant strains over-expressing *nlpE* (JSW2, JLW872) or the vector control (JLW7, JLW867); **C.** wild-type or *efeB* mutant strains over-expressing *nlpE* (JSW2, JLW895) or the vector control (JLW7, JLW896) at 37°C. Graphs on the left represent the survival of strains grown in the absence of IPTG and graphs on the right represent the survival of strains grown in the presence of 0.1 mM IPTG (final concentration). The average A_{600} and standard deviation for each triplicate set of samples is plotted against time. A representative result of three independent experiments is shown here.

4.7. References

1. **Acosta N., S. Pukatzki, and T. L. Raivio.** 2014. The *Vibrio cholerae* Cpx envelope stress response senses and mediates adaptation to low iron. J Bacteriol. **197(2)**: 262-76.

2. **Auriol C., G. Bestel-Corre, J. -B. Claude, P. Soucaille, and I. Meynial-Salles.** 2011. Stress-induced evolution of *Escherichia coli* points to original concepts in respiratory cofactor selectivity. Proceedings of the National Academy of Sciences **108**:1278-1283.

3. **Braun M., S. Bungert, and T. Friedrich.** 1998. Characterization of the Overproduced NADH Dehydrogenase Fragment of the NADH:Ubiquinone Oxidoreductase (Complex I) from *Escherichia coli*. Biochemistry **37**:1861-1867.

4. **Calhoun M. W., and R. B. Gennis.** 1993. Demonstration of separate genetic loci encoding distinct membrane-bound respiratory NADH dehydrogenases in *Escherichia coli*. J Bacteriol **175**:3013-3019.

5. **Cao J., M. R. Woodhall, J. Alvarez, M. L. Cartron, and S. C. Andrews.** 2007. EfeUOB (YcdNOB) is a tripartite, acid-induced and CpxAR-regulated, low-pH Fe2+ transporter that is cryptic in *Escherichia coli* K-12 but functional in *E. coli* O157:H7. Mol Microbiol **65**:857-875.

6. **Casadaban M. J., and S. N. Cohen.** 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 **76**:4530-4533.

7. **Cosma C. L., P. N. Danese, J. H. Carlson, T. J. Silhavy, and W. B. Snyder.** 1995. Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope-associated stresses. Mol Microbiol **18**:491-505.

8. **Danese P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*. J Bacteriol **180**:5875-5884.

9. **Danese P. N., W. B. Snyder, C. L. Cosma, L. J. Davis, and T. J. Silhavy.** 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. Genes Dev **9**:387-398.

10. **Euro L., G. Belevich, D. A. Bloch, M. I. Verkhovsky, M. Wikström, and M. Verkhovskaya.** 2009. The role of the invariant glutamate 95 in the catalytic site of Complex I from *Escherichia coli*. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1787**:68-73.

11. **Friedrich T., and D. Scheide.** 2000. The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. FEBS Lett **479**:1-5.

12. **Grass G., K. Thakali, P. E. Klebba, D. Thieme, A. Müller, G. F. Wildner, and C. Rensing.** 2004. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. J Bacteriol **186**:5826-5833.

13. **Isaac D. D., J. S. Pinkner, S. J. Hultgren, and T. J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc Natl Acad Sci U S A **102**:17775-17779.

14. **Kim C., W. W. Lorenz, J. T. Hoopes, and J. F. Dean.** 2001. Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J Bacteriol **183**:4866-4875.

15. **Kitagawa M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori.** 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**:291-299.

16. **Levine M., D. Nalin, R. Hornick, E. Bergquist, D. Waterman, C. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. The Lancet **311**:1119-1122.

17. **Liu X., Q. Du, Z. Wang, D. Zhu, Y. Huang, N. Li, T. Wei, S. Xu, and L. Gu.** 2011. Crystal structure and biochemical features of EfeB/YcdB from *Escherichia coli* O157: ASP235 plays divergent roles in different enzyme-catalyzed processes. J Biol Chem **286**:14922-14931.

18. **Malpica R., B. Franco, C. Rodriguez, O. Kwon, and D. Georgellis.** 2004. Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc Natl Acad Sci U S A **101**:13318-13323.

19. **Maurer L. M., E. Yohannes, S. S. Bondurant, M. Radmacher, and J. L. Slonczewski.** 2005. pH Regulates Genes for Flagellar Motility, Catabolism, and Oxidative Stress in *Escherichia coli* K-12. J Bacteriol **187**:304-319.

20. **Price N. L., and T. L. Raivio.** 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol **191**:1798-1815.

21. **Kihara, D.** 2011. Protein function prediction for the omics era. Dordrecht, N.Y. Springer.

22. **Raffa R. G., and T. L. Raivio.** 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol Microbiol **45**:1599-1611.

23. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

24. **Raivio T. L., and T. J. Silhavy.** 2001. Periplasmic stress and ECF sigma factors. Annual Reviews in Microbiology **55**:591-624.

25. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

26. **Raivio T. L., S. K. Leblanc, and N. L. Price.** 2013. The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. J Bacteriol **195**:2755-2767.

27. **Rosner J. L., and R. G. Martin.** 2013. Reduction of cellular stress by TolC-dependent efflux pumps in *Escherichia coli* indicated by BaeSR and CpxARP activation of *spy* in efflux mutants. J Bacteriol **195**:1042-1050.

28. **Seo S. W., D. Kim, H. Latif, E. J. O'Brien, R. Szubin, and B. O. Palsson.** 2014. Deciphering Fur transcriptional regulatory network highlights its complex role beyond iron metabolism in *Escherichia coli*. Nat Commun **5**:4910.

29. **Slauch J. M., and T. J. Silhavy.** 1991. Genetic fusions as experimental tools. Methods Enzymol **204**:213-248.

30. **Snyder W. B., L. J. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy.** 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J Bacteriol **177**:4216-4223.

31. **Tschauner K., P. Hörnschemeyer, V. S. Müller, and S. Hunke.** 2014. Dynamic Interaction between the CpxA Sensor Kinase and the Periplasmic Accessory Protein CpxP Mediates Signal Recognition in E. coli. PLoS One **9**:e107383.

32. **Wong, J. L., Vogt, S. L., and T. L. Raivio.** 2012. Using reporter genes and the *Escherichia coli* ASKA overexpression library in screens for regulators of the Gram-negative envelope stress response. Methods Mol. Biol. **966:** 337-57.

33. **Vega D. E., and K. D. Young.** 2014. Accumulation of periplasmic enterobactin impairs the growth and morphology of *Escherichia coli tolC* mutants. Mol Microbiol **91**:508-521.

34. **Vogt S. L., A. Z. Nevesinjac, R. M. Humphries, M. S. Donnenberg, G. D. Armstrong, and T. L. Raivio.** 2010. The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. Mol Microbiol **76**:1095-1110.

35. **Weidner U., S. Geier, A. Ptock, T. Friedrich, H. Leif, and H. Weiss.** 1993. The gene locus of the proton-translocating NADH: ubiquinone oxidoreductase in *Escherichia coli*. Organization of the 14 genes and relationship between the derived proteins and subunits of mitochondrial complex I. J Mol Biol **233**:109-122.

36. **Zhou X., R. Keller, R. Volkmer, N. Krauss, P. Scheerer, and S. Hunke.** 2011. Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. J Biol Chem **286**:9805-9814.

Chapter 5: General Discussion

5.1. Overview

This work sought to determine the cellular role of the inhibitor-chaperone CpxP by characterizing the amino acid residues required for function and by characterizing proteins that play a similar role as CpxP within the cell. The Cpx system plays a prominent role in the expression of virulence factors in multiple intestinal pathogens and a full understanding of how CpxP both signals to and participates in the Cpx response contributes to a broader understanding of the regulation of bacterial signal sensing and adaptation.

5.2. Bacteria live in metal-limited environments and elevated *cpxP* expression may contribute to survival under these conditions

Free metal ions are generally scarce in both aquatic and terrestrial environments due to the oxidation or sequestration of metal ions by organic material (9, 20) . Furthermore, animal hosts use a diverse suite of metal-binding proteins to sequester metal ions away from potential targets of toxicity as well as from pathogenic organisms that require these essential metals for growth and survival within the host (10). The first portion of this work addressed the role of the periplasmic protein CpxP in responding to metal-related signals. Growth in metal-limited conditions induced the expression of *cpxP* while the addition of exogenous zinc reversed this activation (Figure 2-1). This is in direct contrast with multiple studies that have suggested that *cpxP* expression is elevated in the presence of excess zinc (7, 14, 23) . One explanation for the different effects of zinc on the activation of the Cpx response may have to do with differential effects of zinc on different bacteria. The studies in *Salmonella enterica* suggest that the addition of zinc or copper can similarly increase the expression of Cpx-regulated folding factors, however, *cpxP* was not specifically identified in these studies (23). Furthermore, the σ^E regulon was induced at the same concentration of zinc at which the Cpx pathway was induced (250 or

50μM in LB or M9 minimal medium) (23) but we observed no induction of the σ^E response in the laboratory strain of *E. coli* at the same concentration of zinc that can reverse the activation of *cpxP* expression by chelation (Figure 2-4). Therefore, it is possible that zinc has differential effects on the regulation of the Cpx pathway in *Salmonella* as compared to the laboratory strain of *E. coli*. Interestingly, 200μM zinc induces the expression of *degP* in a σ^E-dependent manner in enteropathogenic *E. coli* (EPEC) (19) and also induces the Cpx response (Mellies, J., personal communication). Though we have not examined the expression of *cpxP* in response to elevated zinc concentrations in EPEC, it is possible that zinc affects the pathogenic strain of *E. coli* in a similar manner to that in *Salmonella* while the effects of zinc are different in the laboratory strain of *E. coli*.

Another study that observed an increase in *cpxP* expression in the presence of excess zinc looked at the expression of genes that changed in response to the addition of 200μM zinc to cultures grown in a chemostat (14). Their study grew the laboratory strain MG1655 in minimal medium containing glycerol as the sole carbon source in continuous culture, whereas our study examined the expression of *cpxP* under in batch culture and in rich medium. Therefore, the seemingly contradictory results may reflect the very different conditions used to test the effect of zinc on the expression of genes in *E. coli* K-12. Similarly, the studies by Graham and colleagues examined the effect of severe zinc depletion in the presence (7) and absence (8) of added zinc. Therefore, these authors measured the effects of long-time zinc starvation and the transition to zinc-replete conditions on the transcriptome of MG1655 and MC4100. The conditions in both of these studies differed significantly from the conditions tested in our experiments, since we have examined only short periods of growth in the presence of excess zinc added to already zinc-replete LB medium.

Further, all of the aforementioned studies that seem to contradict our findings measured changes in transcript levels using quantitative real-time PCR (qRT-PCR) (7, 14, 23). We have examined changes using a *cpxP'-'lacZ* reporter. qRT-PCR produces a measure of the balance between transcript synthesis and degradation while our reporter assays would only be able to measure the synthesis of *cpxP* transcripts induced by regulatory changes at the *cpxP* promoter. Therefore, authors of the previous studies may be observing post-transcriptional effects that we cannot observe using the *cpxP'-'lacZ* reporter. Regardless of whether zinc increases or decreases *cpxP* expression, our work compounds the studies that report changes in the expression of *cpxP* in different levels of zinc, providing a strong argument for the role of CpxP in surviving changes in zinc availability in *E. coli*.

5.3. The Cpx two-component system affects metal homeostasis

Growth of laboratory strain *E. coli* in medium treated with the non-specific cation chelator Chelex-100 resulted in activation of *cpxP* expression that is reversed by the addition of exogenous zinc (Figure 2-1). Though CpxP binds zinc (Table 3-3), both the sensor kinase CpxA and the inhibitor CpxP are dispensable for sensing metal chelation and the presence of zinc (Figure 2-3). The effect of zinc on *cpxP* expression, therefore, does not appear to require the upstream signaling components of the Cpx pathway. However, the zinc-responsive transcriptional regulator Zur is required for the elevated levels of *cpxP* expression to be reversed by zinc (Figure 2-4B). Therefore, expression of *cpxP* is metal-responsive and potentially links the activity of the Cpx pathway to metal homeostasis. An obvious and important future direction for this project is the determination of the signaling mechanisms that results in the activation of *cpxP* expression during growth in metal-limited environments. Microarray or RNA-sequencing (RNA-seq) analysis of wild-type, *cpxP*, and *cpxR* mutant *E. coli* grown in metal-replete and –

deplete conditions may be employed to dissect the Cpx-dependent cellular changes that accompany metal limitation.

Similarly, the mechanism by which zinc reverses activation of *cpxP* expression remains undetermined. The expression of *cpxP* may be toxic in the presence of high levels of zinc, consistent with the zinc-resistance observed in the *cpxP* deletion mutant in *E. coli* K-12 plated on zinc-containing LB agar (Figure 2-5). The addition of iron and manganese also affect Cpx activation, however not as dramatically as the addition of zinc (Figure 3-1). These observations suggest that activation of *cpxP* expression during growth in metal-limited medium may not be due to the absence of one specific metal but perhaps to a more general disruption of metal homeostasis. To analyze the changes of specific intracellular ion fluxes, inductively-coupled plasma mass spectrometry (ICP-MS) could be performed on whole cell extracts from wild-type, *cpxA*, *cpxP*, and *cpxR* mutant grown in metal-replete and –deplete conditions to identify changes in metal ion species that depend on different components of the Cpx system. These changes could tell us how the absence of different Cpx components affect intracellular metal levels and provide a more global perspective of how the Cpx system affects metal homeostasis *in vivo*.

5.4. The Cpx two-component system in laboratory strain *E. coli* and enteropathogenic *E. coli* responds to metals in different manners

In enteropathogenic *E. coli* (EPEC), activation of the Cpx pathway by *nlpE* over-expression increases zinc-resistance but insertional inactivation of *cpxP* does not (Figure 2-7). Therefore, CpxP may not play the same role in zinc trafficking in EPEC as it does in the laboratory strain of *E. coli*, but other Cpx-regulated factors may mediate similar zinc resistance in EPEC. Notably, the Cpx regulons in EPEC and *E. coli* MC4100 contain different numbers and subsets of genes (25). In EPEC, genes of the respiratory chain (*nuo* and *cyo*) are strongly down-

regulated in both LB and DMEM while the *nuo* genes are down-regulated only in DMEM in MC4100 (25). Therefore, it is possible that the regulatory networks that exist in each organism have diverged to reflect the different genomes, niches, and environmental conditions experienced by each respective strain. To identify the Cpx regulon members that contribute to zinc resistance in EPEC, combined proteomic and transcriptomic analysis of wild-type, *cpxA*,* and *cpxR* mutant cells exposed to excess zinc or grown in metal-limited conditions could aid the identification of proteins that increase or decrease in abundance in a Cpx-dependent manner under different metal conditions. Quantitative real-time polymerase chain reaction (qRT-PCR) will identify relative changes in transcript levels in each condition and strain while proteomic analysis will also identify post-transcriptional modifications to proteins that regulate activity. Each of these analyses will augment our knowledge of how the Cpx response is linked to metal homeostasis, a finding of this study based on the analysis of Cpx- and zinc-regulated reporter genes, together with phenotypes associated with various *cpx* alleles in the presence of zinc.

Recently, the Goulian lab identified a strain of commensal *E. coli* that stably colonizes the mouse intestine and identified two-component systems that are required for colonization. Only three response regulators were required: *arcB, rcsB,* and *cpxR* (13). Seemingly in contradiction with these observations, activation of the Cpx regulon in EPEC results in the down-regulation of cell surface structures that aid in the attachment of EPEC to gut epithelial cells (15, 16, 31). However, though CpxR represses the expression of adhesive pili required to initiate infection in animal hosts in EPEC, the basal transcription of Cpx-regulated chaperones and proteases such as CpxP and DegP is also required for the proper elaboration of these pili (31). Therefore, commensal strains of *E. coli* might have a greater need for the Cpx response during colonization while pathogenic strains must balance the expression of Cpx to ensure that

virulence factors are expressed when they are needed. This work suggests that the CpxR-mediated expression of *cpxP* is critical for determining the survival of laboratory strain *E. coli* in the presence of toxic levels of zinc (Chapters 2 and 3). Perhaps another critical role of the Cpx system during colonization by commensal strains of *E. coli* is the maintenance of metal homeostasis to ensure that *E. coli* is metabolically prepared for colonization and a stable association with the host.

5.5. CpxP is an auxiliary regulator that integrates multiple signals

CpxP has two known functions: CpxP acts as an inhibitor of the Cpx response and as a periplasmic chaperone (11, 24). Misfolded proteins are thought to allosterically inactivate the ability of CpxP to interact with CpxA, converting CpxP from an inhibitor into a chaperone that is capable of re-folding proteins and/or ushering them to the periplasmic protease DegP (5, 11, 30) (Figure 5-1). The mutational analyses in this work could support this model because mutations in the conserved N-terminal LTXXQ motif of CpxP enhance chaperone function without affecting inhibitor function (Figures 2-2 and 2-3A), suggesting that the residues that affect the chaperone and inhibitor activities of CpxP are distinct. Additionally, CpxPR_{60}Q exhibits weak inhibitor activity but enhanced chaperone activity relative to the wild-type protein. These findings establish that enhanced chaperone activity of CpxP does not ablate CpxP-mediated inhibition of the Cpx response. If strong chaperone activity was sufficient to remove CpxP from CpxA, then the CpxPAA and CpxPR_{60}Q mutants that are stronger chaperones than the wild-type protein *in vitro* (Figure 3-3) would be expected to have the weaker inhibitor activity than the wild-type protein *in vivo.* However, the over-expression of CpxPAA was able to mediate inhibiton of the Cpx response while CpxPR_{60}Q could not (Figure 3-4A) (Table 5-1), though the proteins exhibit similar chaperone activities *in vitro* (Figure 3-3). Therefore, the enhanced chaperone activity of

these mutants does not render CpxP incapable of inhibiting the Cpx pathway as might be expected if chaperone activity were sufficient to ablate inhibitor function. CpxP appears to retain the ability to interact with the sensor kinase, even when it is mutationally activated to bind and refold substrates better or more efficiently. However, we are observing the ability of these proteins to inhibit upon over-expression where the copies of CpxP are very high. pTrc99A is present in approximately 20 copies per cell (Pharmacia). Therefore, it would be useful to examine the activity of the *cpxP'-'lacZ* reporter with these mutations under the control of the native promoter, in single copy on the chromosome. In this manner, we could better examine the physiological effects of these mutations when they are expressed at levels that are comparable to the endogenous protein. Furthermore, introducing these mutations on to the chromosome would also allow us to over-express the NlpE protein to examine the ability of these mutant proteins to inhibit the Cpx response in the presence of characterized inducing cues and potential substrates for CpxP *in vivo*.

5.6. CpxP is a metal-binding signal transduction protein like its paralogues

This work also sought to determine whether CpxP binds metals and to determine the physiological significance of metal-binding, if any. CpxP-containing samples were enriched for zinc as compared to samples that contained only buffer (Table 3-3), suggesting that CpxP may bind zinc. We attempted to identify zinc-binding residues by examining the crystal structure (29) and identified E_{79} and H_{136} as putative zinc-binding residues. Mutation of the putative zinc-binding residue E_{79} only modestly decreased the inhibitor activity of CpxP and had no effect on chaperone activity *in vitro* (Figures 3-4A and 3-3) (Table 5-1). We confirmed that introducing alanines into the putative zinc-binding residues does not affect the stability of the expressed proteins in the cytoplasm (Figure 3-2). Therefore, mutation of the putative zinc-binding residue

E_{79} does not ablate either of the known activities of CpxP. We propose that this residue might participate in maintaining the interaction between CpxP and CpxA through subtle rearrangements in the α2 and α4 helices and the connecting unstructured loop. We were unable to assess the inhibitory activity of pCpxPH_{136}A *in vivo* but a similar mutation, CpxPH_{136}Q, was also not as effective at inhibiting the Cpx pathway as wild-type CpxP (32). Thus, mutations in the putative zinc-binding residues or zinc limitation may render the protein less able to bind CpxA. If correct, one prediction of this model is that a mutation in the positively-charged residues thought to mediate an interaction between CpxP and CpxA should be epistatic to a mutation in E_{79} or H_{136}. For example, over-expression of *cpxP*R_{67}Q exhibits 75% inhibitor activity of the wild-type protein, similar to the activity of a strain over-expressing the CpxPE_{79}A mutant protein. A *cpxP*R_{67}Q/E_{79}A mutant should have the same activity as the R_{67}Q single mutant. If the putative zinc-binding residues contribute to inhibitor activity through some other mechanism, then the double mutant should exhibit less inhibitor activity than either of the single mutants. For instance, if these residues contribute to the oligomer status of CpxP, while the basic amino acids that mediate inhibition do not, then the signaling phenotypes of these mutants would be additive. Regardless of the mechanism, this work establishes that CpxP likely binds zinc, thereby supporting a role for CpxP in the trafficking of intracellular zinc.

Conversely, mutations to the variable XX residues in the conserved N-terminal LTXXQ motifs of CpxP and Spy impact the chaperone activities of the proteins. CpxPAA is a stronger chaperone *in vitro* than CpxP and exhibits similar inhibitor activity as the wild-type CpxP protein (Figure 2-2 and 2-3A). In contrast, SpyEH is a weaker chaperone than wild-type Spy and, unlike wild-type Spy, cannot mediate a decrease in Cpx activity upon over-expression in the absence of *cpxP*. Thus, the variable XX residues of the N-terminal LTXXQ motif affects only the chaperone

activity of CpxP but affects both the chaperone and signaling activities of Spy. Unlike CpxP, the signaling activity of Spy might be mediated at least in part by the chaperone activity of the protein. The decrease in Cpx activity upon *spy* over-expression depends on the identity of the variable XX residues of Spy (Figure 3-4) and these residues also affect the chaperone activity of Spy (Figure 3-3). Furthermore, the Cpx response up-regulates the expression of a number of protein folding and degrading factors but only CpxP and Spy can mediate decreases in Cpx activity (Figure 3-4B). One possible explanation for these observations is that CpxP and Spy refold a specific periplasmic substrate and the misfolding of that substrate is involved in the activation of the Cpx pathway. No natural substrates for these proteins are known. One approach to identifying CpxP substrates would be to look for periplasmic proteins that are no longer stable in the absence of *cpxP* and *spy.* Periplasmic extracts collected from wild-type, *cpxP*, *spy*, and *cpxP spy* mutants could be subjected to high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) to identify all of the proteins present in the presence or absence of *cpxP* and/or *spy.* Proteins that are not present in the mutant backgrounds may represent natural substrates for those proteins. Identifying the substrates of CpxP and Spy will provide the means to assess chaperone activity of the proteins *in vivo* and compare the inhibitor and chaperone activities under physiologically relevant conditions. Alternatively, Spy may be able to interact with the periplasmic sensing domain of CpxA only in the absence of the specific inhibitor CpxP and mutation of the XX residues renders the protein incapable of doing this. This model is less likely because there are no gross structural rearrangements mediated by mutations in these residues (Figure 3-2) and the mutation of these residues in CpxP does not eliminate inhibitor activity (Figure 3-4A). Thus, this work establishes a previously unknown signaling role for Spy

in the absence of *cpxP* and confirms the role of the variable XX residues in the conserved LTXXQ motifs of CpxP and Spy in the chaperone activities of the proteins.

5.6. CpxP connects the regulation of the Cpx response to zinc homeostasis

Previous studies in *E. coli* and *Salmonella* have shown that the Cpx pathway is induced by excess zinc but these studies have not determined how the Cpx regulon contributes to survival in excess zinc (7, 14, 23) . This work reveals a novel function for CpxP in zinc homeostasis. Deletion of *cpxP* increases the expression of the zinc efflux protein *zntA* and confers zinc resistance on solid medium (Figure 2-5). Over-expression of wild-type CpxP or mutants capable of inhibiting the Cpx pathway restores zinc sensitivity to the *cpxP* mutant, suggesting that zinc resistance is mediated at least in part by elevated Cpx regulon expression. Furthermore, zinc efflux and resistance is abolished in a *cpxR* mutant (Figure 2-6) despite the low levels of *cpxP* transcription in the absence of CpxR (Figure 2-3). The increase in zinc efflux in a *cpxP* mutant background, therefore, depends on other CpxR-regulated factors. One model that is consistent with our data posits the excess periplasmic zinc accumulated in the absence of CpxP would be bound by ZnuA, delivered to ZnuBC at the membrane, and imported into the cytoplasm. Excess zinc would bind Zur to repress the expression of zinc importers and to ZntR to activate the expression of *zntA*. Cpx-regulated periplasmic proteins could assist in zinc efflux by enhancing the activity of inner membrane transporters or by ushering zinc from inner membrane efflux proteins to porins or efflux components at the outer membrane. Alternatively, efflux components regulated by the Cpx pathway, such as the inner membrane fusion protein EmrA, may assist ZntA in effluxing zinc from the cell. Indeed, EmrA is homologous to proteins that efflux cobalt, nickel, and zinc in *Rhizobium meliloti* (27) and associates with the outer membrane efflux protein TolC. TolC is known to participate in the efflux of the siderophore enterobactin across

the outer membrane of *E. coli* (2), suggesting that an EmrAB-TolC complex could potentially assist in the maintenance of periplasmic metal homeostasis.

An important future direction for this project is to identify the Cpx-regulated factors required to increase zinc efflux in a *cpxP* mutant background. Deletion of *spy* or *zraP* does not abolish zinc resistance singly or in combination with a *cpxP* mutation, suggesting that periplasmic chaperones that are related to CpxP are not responsible for inducing zinc efflux (Figure 2-3D). RNA-seq analysis of wild-type, *cpxP*, and *cpxR* mutants grown in the presence or absence of zinc may identify the Cpx-regulated factors that contribute to zinc resistance. A targeted screen where individual Cpx-regulated genes are overexpressed in a *cpxR* background and examined for zinc resistance may also be useful in identifying the Cpx-regulated genes that contribute to this phenotype. These analyses would complement the analysis of envelope proteins in *cpxP* and *spy* mutants proposed in section 5.6.

Much of the recent research into metal homeostasis in pathogens has focused on either the clinical relevance of bacterial metal acquisition systems during animal infection or the survival of environmental isolates from metal-contaminated environments. However, an association between heavy metal resistance and antibiotic resistance in bacteria isolated from agricultural environments has been documented since the 1960s. Environmental isolates of penicillin-resistant *Staphylococcus aureus* carried genes for zinc, cadmium, bismuth, mercury, and arsenate-resistance on the same plasmid that encoded β-lactam resistance (22). Similarliy, 74% of methicillin resistant *S. aureus* (MRSA) isolated from farmed pigs were also resistant to zinc and cadmium (4). In *S. aureus,* a metal-specific efflux pump is predicted to be encoded on the same mobile genetic element that carries the genes for antibiotic resistance (28). Further, the BaeSR-regulated efflux components *acrD, tolC,* and *mdtD* are involved in both heavy metal and

antibiotic resistance in *Salmonella enterica*, suggesting that metals can be effluxed by the same structures that rid the cell of antibiotics (21). Multi-drug efflux pumps help bacteria evade antibiotics and host-derived antimicrobial compounds during infection but the same structures may be co-opted for heavy metal resistance in the environment where local concentrations of metals fluctuate widely (18). Therefore, there is selective pressure for the pairing of metal and antibiotic resistance in pathogenic bacterial species.

Activation of the Cpx pathway confers resistance to specific antibiotics (17) (Bernal, M., unpublished observations). Mutations in *cpxP* and the paralogues *spy* and *zraP* result in sensitivity to the cationic antimicrobial peptide polymixin B in *S.* Typhimurium (1) but the mutants were not examined for resistance to other antibiotics. This work could be extended to include an analysis of antibiotic resistance in the *cpxP* mutant with special attention to β-lactams, which have a documented association with zinc resistance in *S. aureus* (22) and with Cpx pathway activation (Bernal, M., unpublished observations). Confoundingly, deletion of *cpxP* confers zinc resistance only in the lab strain of *E. coli* and not in EPEC. Therefore, these experiments will inform our understanding of how commensal *E. coli* copes with the possible antibiotic and metal challenges that occur in the human gut during infection or dietary changes, respectively.

5.7. NuoF and EfeB are novel *cpxP*-dependent signal inputs for the Cpx system

CpxP inhibits the Cpx system through a direct interaction with the periplasmic sensing domain of CpxA but removal of CpxP is not sufficient to maximally induce Cpx activity (24). Part of this work sought to understand how inhibition of the Cpx pathway is achieved by screening for novel Cpx-regulated envelope-localized inhbitors of the Cpx pathway. The cytoplasmic NADH-binding subunit of the NADH dehydrogenase I complex, NuoF, inhibits the

Cpx pathway in a manner that does not require other components of the Nuo complex (Figure 4-5B). Similarly, the heme peroxidase EfeB inhibits the Cpx pathway in a manner that does not require the heme permease encoded upstream of *efeB* (Chapter 4). Unexpectedly, both NuoF and EfeB require *cpxP* to inhibit the Cpx pathway, despite different cellular locations.

A mechanism for how cytoplasmic NuoF signals to periplasmic CpxP is lacking. One way the mechanism of NuoF-mediated inhibition could be investigated is by performing pull-down assays in *nuoA-M* mutant cells over-expressing pCA-*nuoF*, which encodes an N-terminal fusion of the His_6 affinity tag to *nuoF* (12). Identifying the potential interacting partners of NuoF may help us understand the function of *nuoF* in the absence of a functional Nuo complex. Similarly, introducing the $E_{95}Q$ mutation into NuoF lowers the affinity for NADH and prevents the release of NAD^+, resulting in inefficient NADH oxidase activity (6). Even if NuoF associates with another complex, such as the NADH dehydrogenase II protein Ndh, the NuoF$E_{95}Q$ mutant would be less efficient at catalyzing the oxidation of NADH. Examination of the *cpxP'-'lacZ* activity of cells over-expressing wild-type or *nuoF*$E_{95}Q$ will determine whether the NADH-binding activity of NuoF is essential for inhibition of the Cpx pathway.

Our assays did not detect a phenotype for *efeB* mutants, even when measuring Cpx activity under conditions where EfeB is highly expressed. The heme permease EfeU is cryptic in *E. coli* K-12 due to a frameshift mutation within the open reading frame but both the accessory proteins EfeO and EfeB are thought to be expressed in K-12 (3). Perhaps specific conditions must be present for EfeB to exert an effect, such as low iron conditions or the presence of other redox active metals such as copper. EPEC is expected to express the heme permease and the accessory proteins (3). Therefore, it would be interesting to examine the ability of EfeB to inhibit the Cpx pathway in an EPEC background and the contribution of heme transport to the

signaling phenotype. Certainly, the same analyses suggested for NuoF could be applied to EfeB. To examine the interacting partners of EfeB, *efeB* must be expressed from a vector that encodes a C-terminal affinity tag such as FLAG or His_6 and then pull-down assays and LC-MS/MS to identify the proteins that co-elute with EfeB. Additionally, the two catalytic activities of EfeB are separable by mutations in two adjacent amino acids. The *efeB*D_{235}N mutation abolishes only the ability to extract iron from heme while the G_{236}A mutation abolishes both the deferrochelatase and guaiacol peroxidase activities of EfeB. Over-expression of both of these mutations will help us determine which of the known activities of EfeB, if any, are required for inhibition of the Cpx pathway. Furthermore, EfeB may only be required under conditions when iron is limiting. Perhaps growth in the iron-specific chelator bipyridyl would allow us to observe a signaling or growth phenotype in *efeB* mutants or growth on minimal medium with a non-fermentable carbon source such as succinate.

This work sought to identify novel inhibitors of the Cpx pathway and unexpectedly identified the first *cpxP*-dependent signals. That NuoF largely depends on CpxP for signaling to the Cpx pathway is surprising and difficult to understand with only data from these studies. We favour the model that NuoF is altering NADH/NAD^+ levels in the cell, resulting in a change in the levels of an unidentified metabolite that induces the Cpx pathway in the periplasm. Mutation of *tolC* activates the Cpx pathway in a CpxA-dependent manner due to the accumulation of an endogenous metabolite that causes envelope stress in the absence of a functional *tolC* (26). Metabolomic studies to examine the effect of *nuoF* over-expression may help us identify potential metabolites that affect Cpx signaling.

The novel inhibitors identified in this work potentially link signal sensing by the Cpx system to aerobic respiration and iron acquisition, expanding the possible cellular roles for the

Cpx system and extending the signal-sensing abilities of CpxP from only protein-folding signals to global cellular processes.

5.8. Concluding remarks

The purpose of this thesis was to characterize the cellular role of the periplasmic protein CpxP in *E. coli*. We have shown that CpxP is a zinc-binding protein that affects cellular zinc trafficking in addition to assisting in periplasmic protein folding as a chaperone. CpxP also senses uncharacterized signals generated by high-level expression of NuoF and EfeB, suggesting that CpxP can sense specific metabolic signals. This work suggests that the cellular role of CpxP extends beyond protein-folding and that CpxP can integrate metal, respiratory, and protein-folding signals to the CpxA sensor kinase to modulate activation of the Cpx response (Figure 5-1).

Balancing the activity of the Cpx two-component system is important for the expression of virulence traits in a number of pathogens and CpxP is a specific inhibitor of the Cpx response in the Enterobacteriaceae. A better understanding of how CpxP senses stress and communicates with the CpxA sensor kinase has implications for the development of small molecule inhibitors of the Cpx response and for our understanding of the role of auxiliary regulators in bacterial two-component signal transduction.

5.9. Tables

Table 5-1: Summary of CpxP mutants and associated phenotypes

Allele	% Chaperone activity	% Inhibitor activity	% Expression	Reference
Wild-type	100	100	100	Chapter 2
$E_{79}A$	100	72	100	Chapter 2
$H_{136}A$	140	ND	ND	Chapter 2
$E_{53}A/H_{54}A$ (AA)	269	107	100	Chapter 2
$R_{60}Q$	237	21	100	Chapter 2
Spy	365	105	ND	Chapter 2
SpyEH	209	-24	ND	Chapter 2
$H_{136}Q$	ND	66.3	59	(32)
$E_{79}R$	ND	36.5	39	(32)
$R_{67}Q$	ND	75	98	(32)
$R_{56}Q$	ND	-13	96	(32)

CpxP mutant alleles and phenotypes obtained or discussed in this study. % chaperone, inhibitor, and expression were calculated from the values in Figures 2-2, 2-3A, and Western Blot analysis of cells over-expressing pCpxP, pSpy, or their derivatives. (not shown) or as indicated. Each of the values represents the % activity as compared to the wild-type protein within each assay given.

5.10. Figures

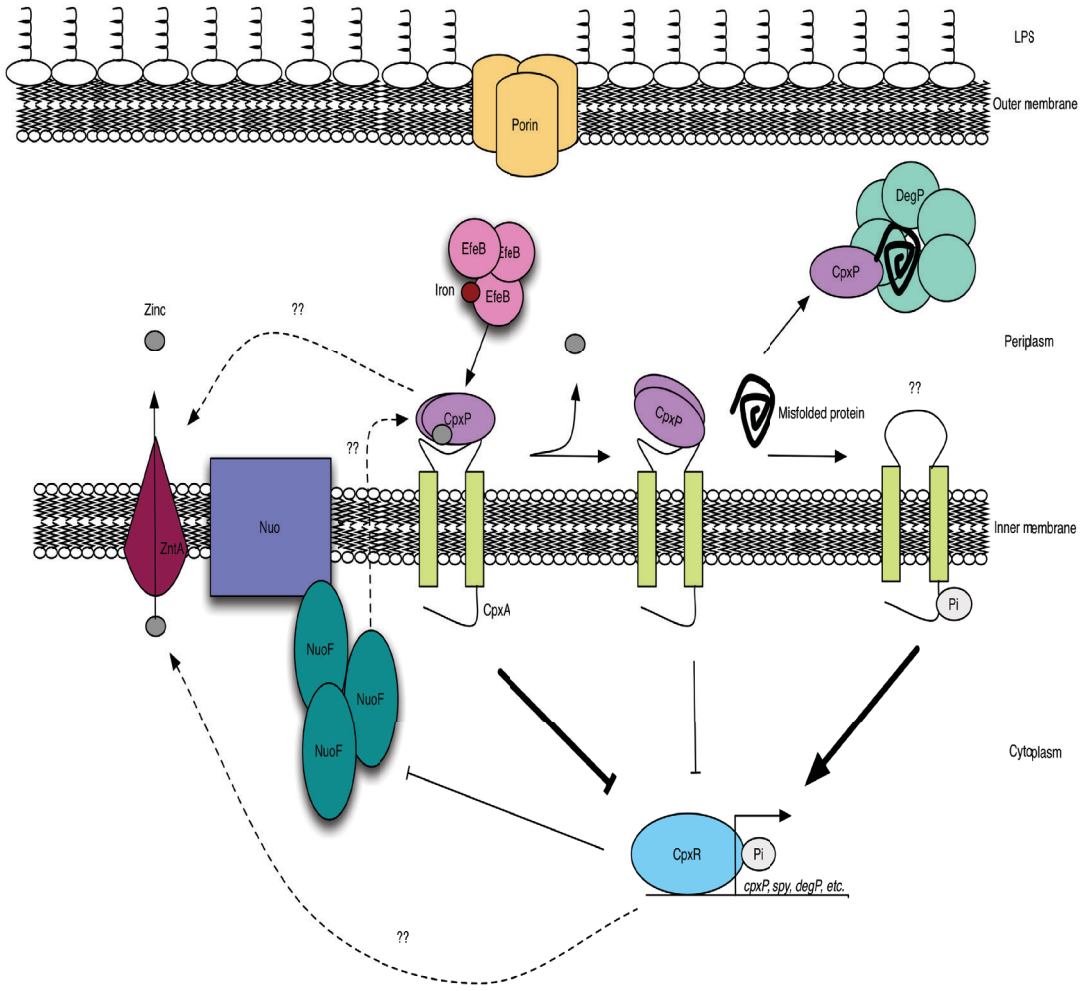


Figure 5-1: Summary model for the cellular role of CpxP.

CpxP inhibits the Cpx pathway, likely through a direct interaction that is stabilized in the presence of zinc. EfeB and NuoF also contribute to Cpx inhibition through CpxP, though the signaling mechanisms are not known. Over-expression of CpxP can also negatively regulate zinc efflux through an uncharacterized mechanism. Mutations at the putative zinc-binding site of CpxP prime CpxP to leave CpxA and the presence of misfolded proteins may provide a second signal that titrates CpxP from the sensing domain of CpxA. CpxA then senses signals and activates the Cpx response through autophosphorylation and phosphorylation of CpxR.

Phosphorylated CpxR up-regulates the expression of *cpxP* and other envelope protein-folding factors and down-regulates the expression of *nuo* and *efeB*. As stress subides, CpxP re-associates with CpxA to inhibit the Cpx pathway and attenuate the response.

5.11. References

1. **Appia-Ayme C., A. Hall, E. Patrick, S. Rajadurai, T. A. Clarke, and G. Rowley.** 2012. ZraP is a periplasmic molecular chaperone and a repressor of the zinc-responsive two-component regulator ZraSR. Biochem J **442**:85-93.

2. **Bleuel C., C. Grosse, N. Taudte, J. Scherer, D. Wesenberg, G. J. Krauss, D. H. Nies, and G. Grass.** 2005. TolC is involved in enterobactin efflux across the outer membrane of *Escherichia coli*. J Bacteriol **187**:6701-6707.

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

4. **Cavaco L. M., H. Hasman, M. Stegger, P. S. Andersen, R. Skov, A. C. Fluit, T. Ito, and F. M. Aarestrup.** 2010. Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob Agents Chemother **54**:3605-3608.

5. **Danese P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*. J Bacteriol **180**:5875-5884.

6. **Euro L., G. Belevich, D. A. Bloch, M. I. Verkhovsky, M. Wikström, and M. Verkhovskaya.** 2009. The role of the invariant glutamate 95 in the catalytic site of Complex I from *Escherichia coli*. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1787**:68-73.

7. **Graham A. I., G. Sanguinetti, N. Bramall, C. W. McLeod, and R. K. Poole.** 2012. Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of the bacterial

response to zinc reveals transient behaviour of the Fur and SoxS regulators. Microbiology **158**:284-292.

8. **Graham A. I., S. Hunt, S. L. Stokes, N. Bramall, J. Bunch, A. G. Cox, C. W. McLeod, and R. K. Poole.** 2009. Severe zinc depletion of Escherichia coli: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins. J Biol Chem **284**:18377-18389.

9. **Hayes K. F., and S. J. Traina.** 1998. Metal Ion Speciation and its Significance in Ecosystem Health. Soil Chemistry and Ecosystem Health :45-84.

10. **Hood M. I., and E. P. Skaar.** 2012. Nutritional immunity: transition metals at the pathogen-host interface. Nat Rev Microbiol **10**:525-537.

11. **Isaac D. D., J. S. Pinkner, S. J. Hultgren, and T. J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc Natl Acad Sci U S A **102**:17775-17779.

12. **Kitagawa M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori.** 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**:291-299.

13. **Lasaro M., Z. Liu, R. Bishar, K. Kelly, S. Chattopadhyay, S. Paul, E. Sokurenko, J. Zhu, and M. Goulian.** 2014. *Escherichia coli* isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts. J Bacteriol **196**:1723-1732.

14. **Lee L. J., J. A. Barrett, and R. K. Poole.** 2005. Genome-wide transcriptional response of chemostat-cultured Escherichia coli to zinc. J Bacteriol **187**:1124-1134.

15. **MacRitchie D. M., J. D. Ward, A. Z. Nevesinjac, and T. L. Raivio.** 2008. Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic *Escherichia coli*. Infect Immun **76**:1465-1475.

16. **MacRitchie D. M., N. Acosta, and T. L. Raivio.** 2012. DegP is involved in Cpx-mediated posttranscriptional regulation of the type III secretion apparatus in enteropathogenic *Escherichia coli*. Infect Immun **80**:1766-1772.

17. **Mahoney T. F., and T. J. Silhavy.** 2013. The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. J Bacteriol **195**:1869-1874.

18. **Martinez J. L., M. B. Sánchez, L. Martínez-Solano, A. Hernandez, L. Garmendia, A. Fajardo, and C. Alvarez-Ortega.** 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev **33**:430-449.

19. **Mellies J. L., K. Thomas, M. Turvey, N. R. Evans, J. Crane, E. Boedeker, and G. C. Benison.** 2012. Zinc-induced envelope stress diminishes type III secretion in enteropathogenic *Escherichia coli*. BMC Microbiol **12**:123.

20. **Morel F. M., R. J. Hudson, and N. M. Price.** 1991. Limitation of productivity by trace metals in the sea. Limnology and Oceanography **36**:1742-1755.

21. **Nishino K., E. Nikaido, and A. Yamaguchi.** 2007. Regulation of multidrug efflux systems involved in multidrug and metal resistance of *Salmonella enterica* serovar Typhimurium. J Bacteriol **189**:9066-9075.

22. **Novick R. P., and C. Roth.** 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J Bacteriol **95**:1335-1342.

23. **Pontel L. B., N. L. Scampoli, S. Porwollik, S. K. Checa, M. McClelland, and F. C. Soncini.** 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**:1659-1669.

24. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

25. **Raivio T. L., S. K. Leblanc, and N. L. Price.** 2013. The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. J Bacteriol **195**:2755-2767.

26. **Rosner J. L., and R. G. Martin.** 2013. Reduction of cellular stress by TolC-dependent efflux pumps in *Escherichia coli* indicated by BaeSR and CpxARP activation of *spy* in efflux mutants. J Bacteriol **195**:1042-1050.

27. **Saier M. H., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol **11**:841-847.

28. **Seiler C., and T. U. Berendonk.** 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol **3**:399.

29. **Thede G. L., D. C. Arthur, R. A. Edwards, D. R. Buelow, J. L. Wong, T. L. Raivio, and J. N. Glover.** 2011. Structure of the periplasmic stress response protein CpxP. J Bacteriol **193**:2149-2157.

30. **Tschauner K., P. Hörnschemeyer, V. S. Müller, and S. Hunke.** 2014. Dynamic Interaction between the CpxA Sensor Kinase and the Periplasmic Accessory Protein CpxP Mediates Signal Recognition in *E. coli*. PLoS One **9**:e107383.

31. **Vogt S. L., A. Z. Nevesinjac, R. M. Humphries, M. S. Donnenberg, G. D. Armstrong, and T. L. Raivio.** 2010. The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. Mol Microbiol **76**:1095-1110.

32. **Zhou X., R. Keller, R. Volkmer, N. Krauss, P. Scheerer, and S. Hunke.** 2011. Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. J Biol Chem **286**:9805-9814.

Appendix A: A genetic screen for proteases that affect the stability of CpxP in *Escherichia coli*

A1. Introduction

The envelope is the first structure to encounter adverse environmental conditions in Gram-negative bacteria. A number of cell surface structures aid cells in adhering to surfaces or other cells, secreting effectors to aid pathogenesis or survival, and protecting the cell from antimicrobial molecules (23). A network of chaperones, proteases, and other protein-folding factors monitors the dense protein traffic in the envelope, including those controlled by the Cpx two-component system. The Cpx system senses protein-folding perturbations and increases the expression of ATP-independent chaperones such as CpxP and Spy, proteases such as DegP, and protein-folding factors such as the disulfide bond oxidase DsbA and the peptidyl-prolyl isomerase PpiA (17). DegP degrades or refolds misfolded outer membrane porins and off-pathway pilins (2, 5) , as well as the periplasmic inhibitor-chaperone CpxP (3, 12) . CpxP is thought to inhibit the Cpx pathway through a direct interaction with the periplasmic sensing domain of CpxA (20, 26, 29) but activation of the Cpx pathway leads to DegP-mediated degradation that contributes to Cpx activation (3). However, neither *cpxP* nor *degP* is required for the activation of the Cpx pathway (6).

DegP is a prototypical oligomeric protein that defines a class of ATP-independent proteases (13, 15). At 30°C, DegP is found mainly as trimers and hexamers that are proteolytically inactive and exhibit chaperone activity *in vitro* (24). At 37°C, DegP oligomerizes in to 12- and 24-mers that are proteolytically active and do not exhibit chaperone activity (13, 15) . Intriguingly, the degradation of CpxP by DegP has never been reconstituted *in vitro*. Another protease, the tail-specific protease Tsp interacts with the C-terminus of CpxP in pull-down assays (27). Tsp degrades substrates with hydrophobic C-termini *in vitro* (22) and processes the C-terminus of penicillin-binding protein 3 in *E. coli*, which cooperates with the

major transpeptidase PBP1a in *E. coli* to cross-link murein peptides during cell division (10). The functional significance of the interaction between Tsp and CpxP remains unknown. We wondered whether there are other periplasmic proteases that affect Cpx activity. In this work, we conducted a genetic screen to look for proteases that affect the activity of the Cpx response and attempted to characterize the effect of protease over-expression on the stability of the inhibitor of the response, CpxP.

A2. Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table A1. Bacterial strains were constructed using standard genetic techniques.

Media and growth conditions. Wild-type and *cpxP* mutant strains over-expressing periplasmic proteases were grown in Luria-Bertani broth (LB) at 37°C with aeration by fixing a test tube rack to an orbital shaker set to 225 rpm. Strains bearing *degP, ptrA, sohB,* or *tsp* mutations were grown at 30°C with aeration. Antibiotics were used at the following concentrations: 25 μg/mL chloramphenicol, 50 μg/mL kanamycin, and 100 μg/mL ampicillin (all Sigma). LB was buffered to pH 8.0 or 5.8 by adding 100 mM dibasic sodium phosphate (final concentration, all from Sigma).

Luminescence assays. Single isolated colonies were inoculated into 2 mL of LB plus chloramphenicol in 5 mL plating tubes in triplicate and grown at 37°C overnight with aeration by fixing a test tube rack to the platform of an orbital shaker set to 225 rpm. The next morning, cultures were diluted 1:50 into 2 mL of fresh LB with chloramphenicol in 5 mL glass plating tubes and grown for 2 h in the same fashion. 0.1 mM IPTG was added to induce protease expression and cultures were returned to the incubator for an additional 2 h of growth at 37°C with aeration. 200 μL aliquots of each culture were transferred to a 96-well microtitre plate and

luminescence and growth (A_{600}) were measured on a Wallac^2 1420 platereader (Perkin Elmer) after a total of four hours of growth (A_{600}~0.7-0.8). Results are given as average luminescence normalized to growth plus the standard deviation of triplicate samples.

Western blots. Single isolated colonies were inoculated into 5 mL LB in 10 mL glass plating tubes broth plus antibiotics and grown at 30°C with aeration, as described above. The next morning, cultures were diluted 1:50 into fresh 5 mL LB broth plus antibiotics and 0.1 mM IPTG and grown to OD_{600} ~0.6-0.7. Whole cell lysates were harvested by centrifugation at 16 100 *g* for 10 minutes in a benchtop centrifuge and resuspended in 50 µL 2X sample buffer. Samples were incubated for 10 minutes in a boiling water bath and loaded into 12% denaturing polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose at 10 V overnight and blocked in 5% skim milk for 3 h. Blots were washed overnight in 1X Tris-buffered saline (1 M Tris, 0.9 M NaCl) plus 0.2% (v/v) Tween 20 (1X WS) (all Sigma), shaking at room temperature (~200 rpm). Primary anti-CpxP-maltose-binding protein (MBP) antisera or anti-bacterial alkaline phosphatase (BAP) antibody was diluted 1:50 000 in 5% skim milk and applied to blots for one hour while shaking at room temperature. After three 20-minute washes at room temperature in 1X WS, anti-rabbit Ig antibody conjugated to alkaline phosphatase was diluted 1:50 000 in 5% skim milk was applied to blots for one hour while shaking at room temperature. The Immunstar AP Substrate Pack (Bio-Rad) was used to develop blots after three more 20-minute washes in 1X WS at room temperature. Blots were imaged on a Chemi-Doc Imager (Bio-Rad).

In vitro degradation assays. Whole membrane preparations were prepared as described by Lobos and Mora (1991) (16). Briefly, single isolated colonies were inoculated into 5 mL LB broth plus antibiotics in 10 mL glass plating tubes and grown overnight at 37°C with aeration, as

described above. The next morning, cultures were diluted 1:50 into fresh LB plus antibiotics and 0.1 mM IPTG and grown to an OD_{600}~1.0 in the same fashion. Cells were pelleted at 16 100 *g* at room temperature in a benchtop centrifuge and resuspended in 1 mL 50 mM Tris-HCl pH 8.0. Samples were kept on ice and lysed by sonication at low setting (setting 1-1.5), five times one minute or until clear. Cell debris was pelleted by centrifugation at 10 000 *g* for 5 minutes at 4°C. 900 µL of supernatant was transferred to a fresh 1.5 mL eppendorf tube and centrifuged at 13 000 *g* for 45 minutes at 4°C. The pellet was either resuspended in 100 µL 100 mM Tris-HCl, pH 7.5 or 100 µL of periplasmic extracts prepared as described below.

Periplasmic extracts were prepared from cells grown as described above but cell pellets were resuspended in 100 µL lysis buffer (20% sucrose, 10mg lysozyme, 30 µM Tris HCl pH 8.5, and 2 µM EDTA). Cells were incubated on ice for 1-2 hours then pelleted to remove cellular debris and the supernatant was used as the periplasmic fraction in degradation reactions. Degradation reactions were performed in a final volume of 100 µL with a 20-40 µg of CpxP (as indicated) diluted into each reaction and incubated at 37°C overnight. Reactions were precipitated in 10% trichloroacetic acid on ice for 30 minutes followed by centrifugation at 13 200 rpm at room temperature and removal of the supernatant, then air-dried at room temperature. Reaction pellets were resuspended in 10-15 µL 2X sample buffer and run on 12% denaturing acrylamide gels. Gels were stained with Coomassie Brilliant Blue (Bio-Rad) and de-stained. Gels were imaged on the Chemi-Doc Imager (Bio-Rad).

A3. Results and Discussion

A3.1. Over-expression of many envelope proteases affect Cpx pathway activity

We were interested in identifying proteases that modify Cpx activity in the envelope where CpxP is localized. To identify envelope-localized proteases, we examined the Ecogene

database (http://www.ecogene.org) for predicted proteases and peptidases with known or predicted localizations to the inner membrane, outer membrane, or periplasm. For proteases localized to the inner membrane, we did not differentiate between proteases with catalytic sites on the periplasmic or the cytoplasmic side of the membrane. Furthermore, lytic transglycosylases and transpeptidases were not included in this analysis, precluding the identification of cell wall enzymes that might impact Cpx activity.

Nevertheless, 32 candidate plasmids from the ASKA over-expression library (Table A2) were transformed into a strain of *E. coli* bearing *cpxP-lux* reporter where the promoter of *cpxP* has been fused to the *luxCDABE* genes to drive light production when *cpxP* is expressed (17). It has been demonstrated that the *cpxP* promoter is a very sensitive read-out of the activity of the CpxRA two-component system (6). In the presence of IPTG to induce over-expression of envelope proteases, we observed that 23 out of 32 plasmids produced statistically significant changes in Cpx activity as compared to the vector control strain, including those that contained *degP* and *tsp* (Figure A1). Notably, the over-expression of some predicted or confirmed proteases resulted in strong decreases in *cpxP-lux* activity, such as the over-expression of *sohB* (Figure A1). DegP is both a protease and a chaperone (24) and therefore it is possible that some of the predicted proteases may assist in stabilizing CpxP through chaperone activity rather than proteolyzing CpxP and degrading it. Alternatively, over-expression of certain proteases may remove misfolded substrates from the envelope that CpxP recognizes, thereby indirectly stabilizing the interaction between CpxP and CpxA. Nevertheless, the over-expression of many proteases affected *cpxP-lux* activity in our assay.

A3.2. CpxP is highly stable when over-expressed from the *trc* promoter

CpxP is an inhibitor of the Cpx response that is degraded by the protease DegP in the presence of stress (3, 12). We hypothesized that some of the proteases might be affecting the activation of the Cpx response through alterations in the stability of the inhibitor CpxP. Therefore, we narrowed our focus to the two proteases that are known to interact with CpxP *in vivo,* DegP and Tsp (3, 12, 28), as well as two other proteases that activated the response (Spr, PtrA) and one protease that inhibited the response (SohB) (Figure A2). Pitrylisin A (PtrA) is a peptidase that is conserved in animals and plants and degrades insulin in humans (7). Suppressor of HtrA (SohB) is a multi-copy suppressor of the thermosensitivity of a high-temperature requirement A (HtrA or DegP) mutant (1) and participates in the elaboration of peritrichous pili on the surface of the food-borne pathogen *Campylobacter jejuni* (8). Since proteolysis is known to regulate the Cpx response via alterations in the stability of the inhibitor CpxP, we first sought to examine the effect of protease mutations on the stability of CpxP. Accordingly, we introduced mutations into a strain of *E. coli* that also carries the high-copy IPTG-inducible pCpxP plasmid (19). CpxP protein is detected by the CpxP-maltose-binding protein (MBP) antisera only when the protein is over-expressed (Figure A2A, compare lanes 1 and 2). We collected whole cell lysates from wild-type and protease-deficient *E. coli* over-expressing CpxP and assessed CpxP stability by Western blot using antisera directed against CpxP-MBP. We did not observe any differences in CpxP stability in Western blots (Figure A2A), perhaps due to the high levels of CpxP protein present in the cell upon over-expression. Additionally, deletion of *degP* resulted in decreased levels of CpxP protein (Figure A2B). Previous experiments that examined the stability of a CpxP-β-lactamase fusion protein by Western blot against β-lactamase and observed increased levels of CpxP in the absence of DegP (3). Other groups also observed DegP-

dependent alterations to the levels of endogenous CpxP expressed from the chromosome in the presence of the constitutively active *cpxA24* allele and the over-expression of pilin subunits in the absence of their cognate chaperones (12). In this work, however, we examined the stability of untagged CpxP using CpxP-MBP antisera and consistently observed a decrease in CpxP levels in a *degP* mutant, even in the presence of the Cpx activating signal alkaline pH (Figure A2B). Possibly, *degP* is required to stabilize periplasmic pools of CpxP when over-expressed from the high-copy vector pTrc99A by its chaperone activity, but we have not tested the dependence of this phenotype on the protease activity of DegP.

A3.3. The *in vitro* degradation of CpxP by DegP is enhanced by membranes

Therefore, we sought to develop an alternative *in vitro* assay to assess degradation of purified CpxP protein. As stated in the introduction, previous attempts to reconstitute the degradation of purified CpxP by purified DegP protein were unsuccessful. We purified DegP-His_6 from the periplasm of *E. coli* and added 10 μg of DegP to 10 μg of CpxP protein and incubated the reaction at 37°C overnight. We hypothesized that purified DegP required allosteric activation by misfolded substrates to degrade CpxP. DegP degrades the major pilin subunit of the bundle-forming pilus in enteropathogenic *E. coli in vivo* and *in vitro*, but requires the addition of dithithreitol (DTT) to degrade bundlin *in vitro* (11). Therefore, we combined purified bundlin, CpxP, and DegP *in vitro* with and without DTT and incubated the reaction overnight at 37°C, but observed no degradation of CpxP (data not shown).

DegP might be associated with membranes in its catalytically-active form (21). We hypothesized that membranes might assist in the degradation of purified CpxP. Therefore, we added periplasmic and/or membrane extracts from cells over-expressing *degP* or carrying the vector control with 40 μg of purified CpxP protein at 37°C (Figure A3A), precipitated the

reactions in TCA, then ran the reactions on denaturing polyacrylamide gels and stained the gels with Coomassie Brillant Blue. In the absence of any cell extracts, CpxP was present in two forms: a full-length 17kDa form ($CpxP_{20-166}$) and a lower molecular weight form (Figure A3A, lane 5). We hypothesize that the lower molecular weight band is the stable $CpxP_{40-151}$ fragment that was purified and used to solve the crystal structure of CpxP (25) but we have not confirmed this hypothesis by tandem mass spectrometry. Treatment of purified CpxP with periplasmic extracts from the vector control strain reduced the stability of the lower molecular weight species but not full-length CpxP (Figure A3A, lane 6). Treatment of 40 μg of purified CpxP with periplasmic extracts from a strain over-expressing DegP or with membranes from the vector control strain had no impact on CpxP stability (Figure A3A, lanes 7 and 8). However, treatment with membranes from the strain over-expressing *degP* decreased CpxP stability when incubated with periplasmic extracts, regardless of their origin (Figure A3A, lanes 10 and 11). However, incubation of purified CpxP with membranes from the vector control strain did not impact CpxP stability (Figure A3A, lanes 8 and 12). Therefore, the presence of membranes might enhance the degradation of purified CpxP protein when *degP* is over-expressed. We noted the presence of a band in periplasmic and membrane extracts that corresponds to the size of CpxP or Spy and complicated our interpretation of the stability of the lower molecular weight band observed in the purified protein preparation (see lanes 1 and 3 of Figure A4A). We hypothesized that those bands might correspond to increased expression of endogenous CpxP or Spy. Therefore, we transformed all plasmids into a *cpxP spy* double mutant for the following analysis.

The catalytic S_{210} residue of DegP is required for degradation of substrates. The $DegPS_{210}A$ protein is proteolytically inactive but maintains chaperone activity *in vivo* and *in vitro* (24). To test whether the degradation of CpxP is dependent on the protease activity of DegP, we collected

membranes from strains over-expressing *degP* from the high-copy IPTG inducible vectors pCA24N or pCS19 (24) and incubated membranes with 20μg CpxP protein. The pCS20 and pCS21 vectors encode periplasmically-expressed wild-type and *degP*S210A expressed from the IPTG-inducible p*Trc* promoter, respectively and were a gift from Michael Ehrmann (Universitat Duisburg). These plasmids differ in replication origin, antibiotic resistance, and copy number (14, 24) such that pCS19 is present in lower copy number than pCA24N, but both plasmids produce IPTG-inducible expression of *degP*. Incubation of purified CpxP with membranes from a strain over-expressing wild-type DegP from pCA-*degP* resulted in a decrease in CpxP stability as compared to reactions containing membranes from the vector control strain (Figure A3). Incubation of CpxP with membranes from a strain over-expressing wild-type DegP from pCS19 resulted in a decrease in CpxP stability as compared to reactions containing membranes from a strain over-expressing *degP*S210A (Figure A3). We do not possess the vector control pCS19 but the addition of membranes from a strain containing the vector controls for pCS20 and 21 would be a helpful addition to these experiments. This experiment was performed only once and provides preliminary evidence that the protease activity of DegP mediates the decrease in CpxP levels observed upon incubation with membranes.

A3.4. Future Directions

An extension of this analysis should include membranes from strains over-expressing the *ptrA, sohB,* and *tsp* proteases to examine effect of these proteins on CpxP stability. However, all assays should be repeated with an appropriate loading control, such as bovine serum albumin (BSA), assuming that BSA does not affect catalytic efficiency nor compete with CpxP for DegP. Additionally, the membrane preparations in this assay are crude and may include membrane proteases from the cytosol that could impact CpxP stability *in vitro*. Therefore, analysis with

clean membrane preparations obtained after multiple ultracentrifugation steps will strengthen the conclusions that might be made from these experiments. Finally, inhibition of DegP activity by chemical means may also strengthen these analyses, such as incorporating a protease inhibitor to chemically antagonize the protease activity of wild-type DegP instead of eliminating catalytic activity genetically.

Our studies were further complicated by the fact that *degP* mutants are temperature-sensitive (1) and must be grown at 30°C. Therefore, we grew all protease-deficient strains at 30°C to ensure that all strains were viable under the conditions tested. High temperature activates the proteolytic activity of DegP (24) and likely other envelope proteases and it is possible that some of the proteases that affect the stability of CpxP are not active under the conditions we tested here. Thus, it would be useful to assess CpxP stability only under conditions where the proteases are over-expressed to circumvent the temperature-sensitivity of protease mutants or to perform this screen in a strain of *E. coli* where *degP* is not required for growth at 37°C, such as in enteropathogenic *E. coli* (A. Nevesinjac, unpublished observations).

A recent study used site-specific cross-linking of amino acids near the catalytic site of DegP to examine the substrate profile of the enzyme *in vivo* and found that OmpC, A, F, X, and W all cross-linked with DegP at high temperatures, suggesting that DegP mainly functions to degrade misfolded OMPs in the periplasm (9). A similar approach might be used to identify interacting partners of CpxP by using a photoactivated site-specific cross-linker incorporated as a synthetic amino acid into CpxP by genetic manipulation. This approach is less biased than our approach, which was limited to the power of annotations in Ecogene.

This work attempted to elucidate the connections between CpxP and the proteolytic network that maintains protein-folding homeostasis in the Gram-negative envelope. Proteolysis

of envelope structures is important for bacterial virulence and survival, therefore, understanding how the auxiliary regulator CpxP interacts with envelope proteases will help us characterize the molecular interactions that occur to aid bacterial adaptation to stress.

A4. Tables

Table A1: Bacterial strains and plasmids used in this study.

Strain	Genotype or Description	Reference
DB12	TR50 pTrc99A	(3)
DB252	TR50 *tsp:kn*	This study
DB254	TR50 *ptrA:kn*	This study
DB260	TR50 *degP:kn*	This study
JLW7	TR50 pCA24N	This study
JLW200	DB252 pCpxP	This study
JLW201	DB254 pCpxP	This study
JLW202	DB258 pCpxP	This study
JLW203	DB260 pCpxP	This study
JLW215	TR50 *sohB:kn*	This study
JLW219	JLW215 pCpxP	This study
JLW261	TR50 pJW25	This study
JLW262	TR50 *spr:kn*	This study
JLW269	TR50 pCA-*degP*	This study
JLW274	JLW261 pCA-*sohB*	This study
JLW275	JLW261 pCA-*ptrB*	This study
JLW276	JLW261 pCA-*htpX*	This study
JLW277	JLW261 pCA-*ftsH*	This study
JLW278	JLW261 pCA-*gspO*	This study
JLW279	JLW261 pCA-*hflC*	This study
JLW280	JLW261 pCA-*ompT*	This study
JLW281	JLW261 pCA-*lepB*	This study
JLW282	JLW261 pCA-*lspA*	This study
JLW283	JLW261 pCA-*nlpC*	This study
JLW284	JLW261 pCA24N	This study
JLW286	JLW262 pCpxP	This study
JLW299	JLW261 pCA-*qmcA*	This study
JLW300	JLW261 pCA-*imp*	This study
JLW301	JLW261 pCA-*bamA*	This study
JLW302	JLW261 pCA-*yggG*	This study
JLW303	JLW261 pCA-*ytfB*	This study
JLW304	JLW261 pCA-*yfhR*	This study
JLW305	JLW261 pCA-*degS*	This study
JLW306	JLW261 pCA-*yafL*	This study
JLW307	JLW261 pCA-*yfbL*	This study
JLW308	JLW261 pCA-*rseP*	This study
JLW309	JLW261 pCA-*pppA*	This study
JLW310	JLW261 pCA-*pepN*	This study
JLW311	JLW261 pCA-*sppA*	This study
JLW313	JLW261 pCA-*yacL*	This study
JLW333	JLW261 pCA-*degP*	This study

JLW334	JLW261 pCA-*spr*	This study
JLW335	JLW261 pCA-*yebA*	This study
JLW336	JLW261 pCA-*degQ*	This study
JLW337	JLW261 pCA-*ptrA*	This study
JLW338	JLW261 pCA-*ygeR*	This study
JLW339	JLW261 pCA-*envC*	This study
JLW340	JLW261 pCA-*tsp*	This study
JLW627a	TR50 Δ*cpxP* Δ*spy*	This study
JLW694	JLW627a pCA24N	This study
JLW695	JLW627a pCA-*degP*	This study
JLW696	JLW627a pCS20	This study
JLW697	JLW627a pCS21	This study
MC4100	F^- *araD139* Δ(*argF-lac*)U169 *rpsL150*(Str^r) *relA1 flbB5301 decC1 ptsF25 rbsR*	(4)
TR50	MC4100 *cpxP'-'lacZ* at λRS88	(18)
Plasmids	**Description**	**Source or Reference**
pCA24N	High-copy IPTG-inducible plasmid for expression of envelope proteases, Cam^R	(14)
pJW25	Vector encoding *cpxP* promoter cloned upstream of *luxCDABE* genes to drive light production	(17)
pCS20	High-copy IPTG-inducible plasmid for expression of DegP-His_6 in the periplasm	(24)
pCS21	High-copy IPTG-inducible plasmid for expression of $DegPS_{210}A$-His_6 in the periplasm	(24)

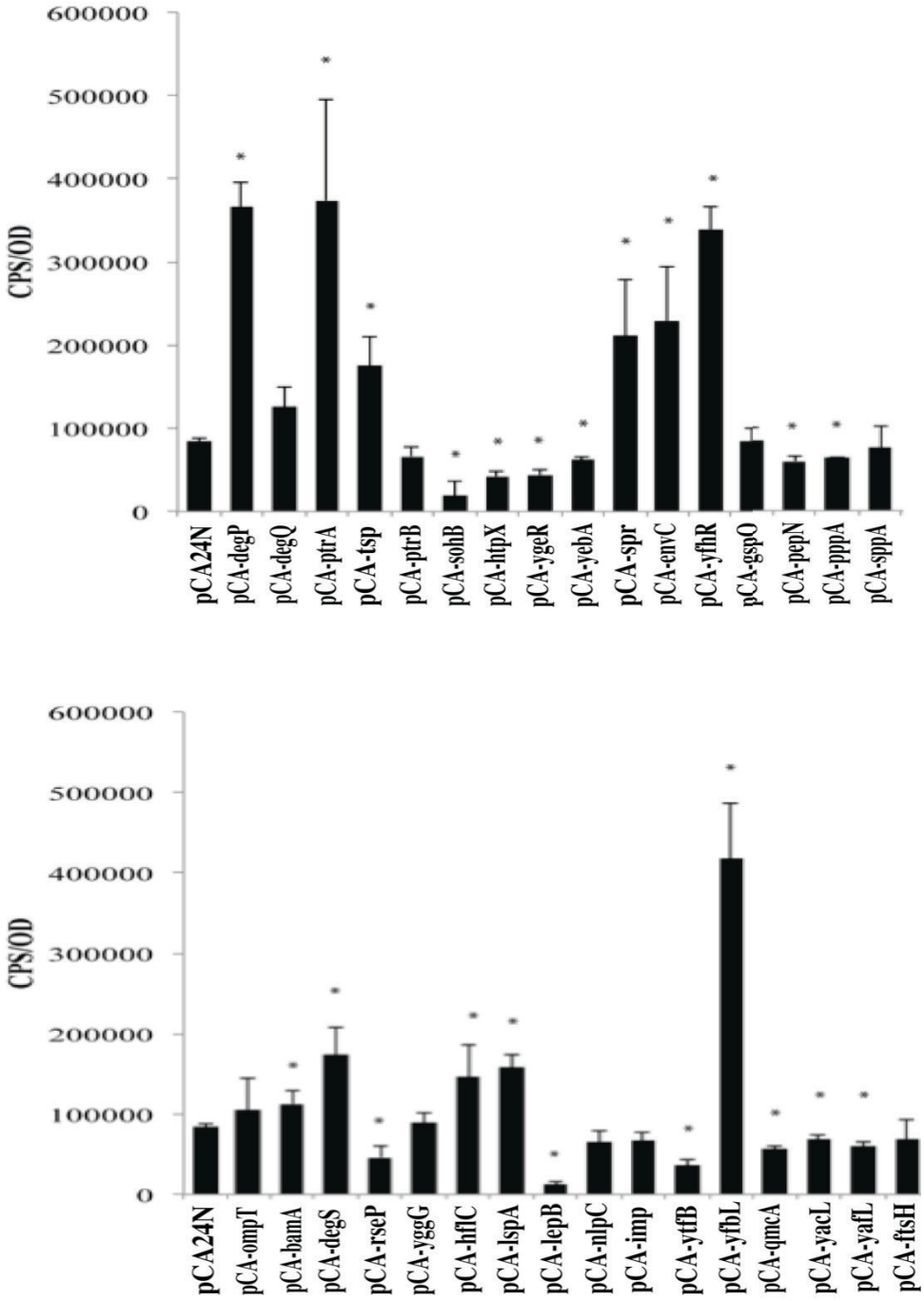
Table A2: Summary of envelope proteases investigated in this study

Candidate protease	Cellular Location	Location of catalytic site	Description
BamA	Outer membrane	Periplasm, outer membrane	An essential outer membrane protein that assembles and inserts nascent OMPs into the outer membrane
DegP	Periplasm, inner membrane	Periplasm	Oligomeric protease that degrades OMPs and pilins
DegQ	Inner membrane, periplasm	Periplasm	Multi-copy suppressor of *tsp* mutants, increases in activity during anaerobic growth at pH 8.5, degrades various misfolded substrates as a dodecamer, like DegP
DegS	Inner membrane	Periplasm	Protease that senses misfolded OMPs as part of a regulated intramembrane proteolytic cascade
EnvC	Inner membrane, periplasm	Periplasm	Protease that processes the murein amidases AmiA and AmiB to activate their activity
FtsH	Inner membrane	Cytosol	Zinc metalloprotease driven by a combination of ATP hydrolysis and proton-motive force; thought to contribute to inner membrane protein quality control
GspO	Inner membrane	Cytosol	Cryptic pre-pilin peptidase for type IV pilus that is homologous to the Pul pilus in *Klebsiella oxytoca*
HflC	Inner membrane	Unknown	Protein that regulates the ATPase activity of the inner membrane protease activity of FtsH
HtpX	Inner membrane	Cytosol	Cpx-regulated zinc metalloprotease that degrades misfolded proteins
Imp	Outer membrane	Periplasm, outer membrane	LptD, an essential outer membrane lipoprotein that cooperates with LptE to assemble lipopolysaccharide
LepB	Inner membrane	Cytosol	Signal peptidase
LspA	Inner membrane	Cytosol	Pro-lipoprotein signal peptidase
NlpC	Inner membrane, periplasm	Periplasm	Lipoprotein with homology to cell wall peptidases in *Bacillus subtilis*
OmpT	Outer membrane	Periplasm	Outer membrane protease that cleaves after paired basic amino acids; cleaves colicins (ColE2) and is regulated by the sRNAs *omrA* and *omrB*
PepN	Inner membrane	Unknown	Aminopeptidase N, inducible expression during growth on non-

			fermentable carbon substrates and phosphate starvation
PppA	Inner membrane	Periplasm	Degrades heterologously expressed pilins
PtrA	Periplasm, cytosol	Periplasm	Zinc metalloprotease that degrades small peptides such as MalE and β-lactamase
PtrB	Cytosol, inner membrane	Cytosol	Oligopeptidase B cleaves after basic residues, especially RR motifs
QmcA	Inner membrane	Unknown	Quality control-associated membrane complex protein A, multi-copy suppressor of *ftsH htpX* double mutants
RseP	Inner membrane	Inner membrane	Zinc metalloprotease that cleaves the anti-sigma factor RseA in the inner membrane as part of a regulated intramembrane proteolytic cascade
SohB	Inner membrane	Periplasm	Multi-copy suppressor of the thermosensitivity of a *degP* mutant
SppA	Inner membrane	Cytosol	Degrades lipoprotein signal peptides cleaved from pro-lipoproteins at the inner membrane
Spr	Outer membrane	Periplasm	D,D-endopeptidase (lipoprotein) that cleaves D-Ala-*m*-DAP crosslinks of peptidoglycan
Tsp	Periplasm, inner membrane	Periplasm	Protease that processes proteins after non-polar residues, including penicillin-binding protein 3 (PBP3)
YacL	Cytosol	Cytosol	Unknown
YafL	Inner membrane	Unknown	Unknown
YebA	Outer membrane	Periplasm	D,D-endopeptidase that cleaves between D-Ala-*m*-DAP crosslinks in peptidoglycan
YfbL	Membranes	Unknown	Unknown
YfhR	Inner membrane	Unknown	Unknown
YgeR	Inner membrane	Periplasm	May assist in the same cell-wall associated functions as *envC* and *nlpD*
YggG	Outer membrane	Periplasm	LoiP (low osmolarity- induced protease) Outer membrane metalloprotease that cleaves between Phe-Phe residues and may be regulated by the Rcs system
YtfB	Extracellular space, membranes	Unknown	Over-expression of *ytfB* causes filamentation

Summary of the functions and cellular locations of the envelope proteases investigated in this study. Locations and descriptions were modified from information available on Ecocyc (http://ecocyc.org).

A5. Figures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pCA-ptrB
pCA-sohB
pCA-htpX
pCA-ygeR
pCA-yebA
pCA-spr
pCA-envC
pCA-yfhR
pCA-gspO
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pCA-bamA
pCA-degS
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pCA-nlpC
pCA-imp
pCA-ytfB
pCA-yfbL
pCA-qmcA
pCA-yacL
pCA-yafL
pCA-ftsH

Figure A1: Envelope protease over-expression affects Cpx activity.

Luminescence of TR50 over-expressing envelope proteases normalized to growth. The average luminescence and standard deviation normalized to growth of triplicate samples is plotted above. Asterisks mark activities that are differ in a statistically significant manner from the isogenic vector control strain as determined by a Student's t-test (p-values <0.05). A representative result of two independent experiments is given.

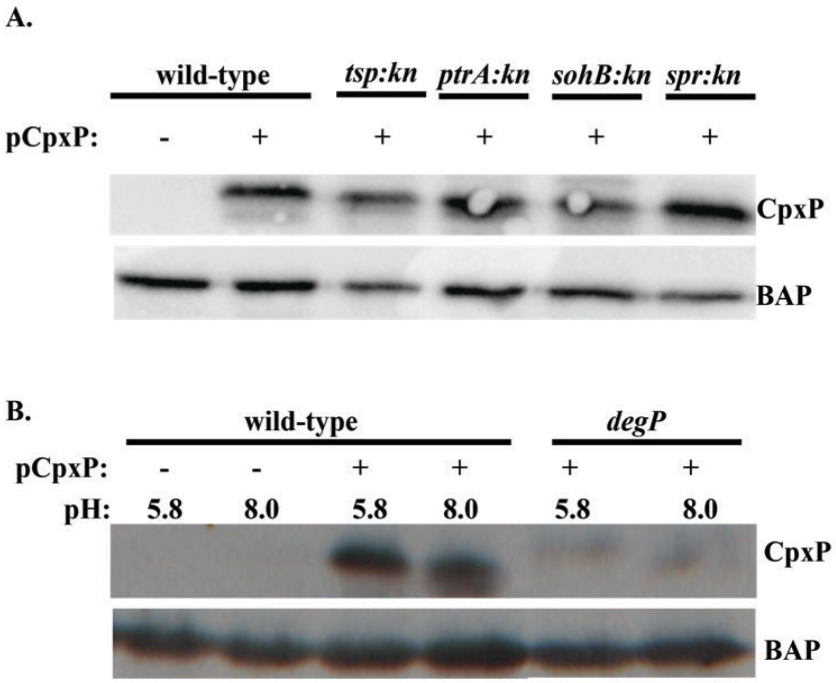


Figure A2: CpxP has altered stability only in a *degP* mutant background when over-expressed from the *trc* promoter.

A. CpxP is highly stable in protease-deficient backgrounds. Single isolated colonies of wild-type (JLW8), *tsp:kn* (JLW200), *ptrA:kn* (JLW201), *sohB:kn* (JLW219), and *spr:kn* (JLW286) cells over-expressing *cpxP* or the vector control (DB12) were grown to late exponential phase and whole cell lysates were collected and run on denaturing polyacrylamide gels. Proteins were transferred to nitrocellulose and Western blots were performed using CpxP-MBP antisera or anti-bacterial alkaline phosphatase (BAP, loading control). CpxP runs at approximately 17kDa and bacterial alkaline phosphatase (BAP) runs at approximately 49kDa. **B.** CpxP is unstable in the absence of *degP* when over-expressed from pCpxP. Wild-type (JLW8) or *degP* mutant strains (JLW203) 1mL equivalents were harvested, run, and blotted as described in A. A representative result of 3 independent experiments is shown.

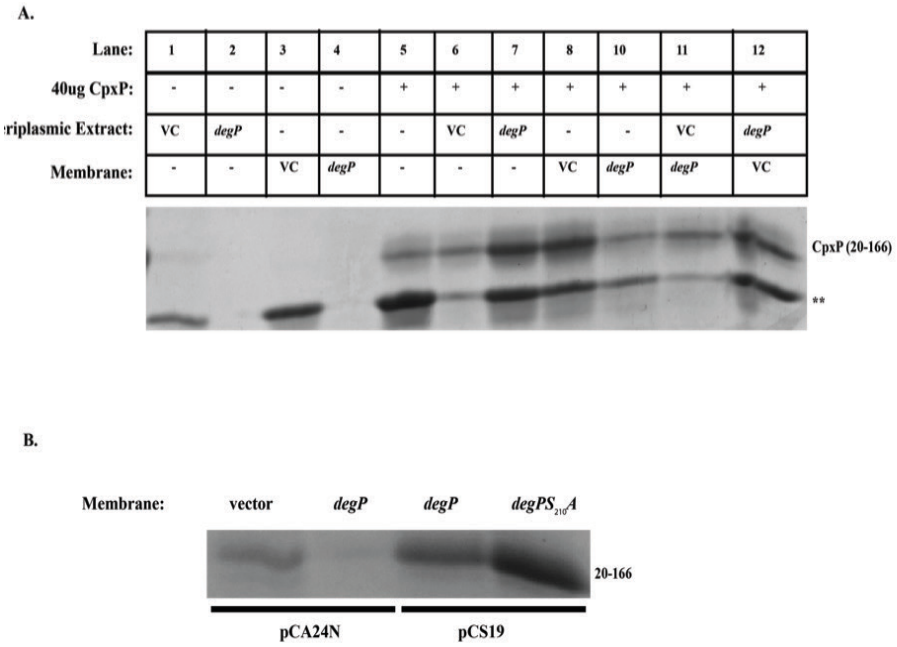


Figure A3: Membranes facilitate the degradation of CpxP by DegP *in vitro*.

A. Membranes from strains over-expressing DegP decrease CpxP stability *in vitro.* Periplasmic extracts, membranes, or combined periplasmic extract and membranes from strains over-expressing *degP* (JLW269) or the vector control (JLW7) were added to 40 μg of purified CpxP in 100 μL reactions (final volume) and incubated overnight at 37°C to reconstitute the degradation of CpxP by DegP *in vitro*. The origin of the cellular fraction(s) in each reaction is given above the gel, the full-length $CpxP_{20\text{-}166}$ or an unidentified low molecular weight species (**) is indicated on the right of the gel. VC stands for vector control and *degP* stands for strains over-expressing pCA-*degP.* A representative result of two independent experiments is given here. **B.** The protease activity of DegP contributes to degradation of CpxP *in vitro*. Membranes from cells over-expressing *degP* from pCA24N (JLW694 vector control strain; JLW695 for over-expression of *degP*) or pCS19 (JLW696 for wild-type *degP*; JLW697 for the *degP*S_{210}A) were collected and incubated with purified CpxP as described in A. Protease-deficient

degPS_{210}A was over-expressed from pCS19. Preliminary results from a single experiment are shown.

A6. References

1. **Baird L., B. Lipinska, S. Raina, and C. Georgopoulos.** 1991. Identification of the *Escherichia coli sohB* gene, a multicopy suppressor of the *htrA* (*degP*) null phenotype. J Bacteriol **173**:5763-5770.

2. **Bakker D., C. E. Vader, B. Roosendaal, F. R. Mooi, B. Oudega, and F. K. de Graaf.** 1991. Structure and function of periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. Mol Microbiol **5**:875-886.

3. **Buelow D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J Bacteriol **187**:6622-6630.

4. **Casadaban M. J., and S. N. Cohen.** 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 **76**:4530-4533.

5. **CastilloKeller M., and R. Misra.** 2003. Protease-deficient DegP suppresses lethal effects of a mutant OmpC protein by its capture. J Bacteriol **185**:148-154.

6. **DiGiuseppe P. A., and T. J. Silhavy.** 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol **185**:2432-2440.

7. **Ding L., A. B. Becker, A. Suzuki, and R. A. Roth.** 1992. Comparison of the enzymatic and biochemical properties of human insulin-degrading enzyme and *Escherichia coli* protease III. J Biol Chem **267**:2414-2420.

8. **Doig P., R. Yao, D. H. Burr, P. Guerry, and T. J. Trust.** 1996. An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. Mol Microbiol **20**:885-894.

9. **Ge X., R. Wang, J. Ma, Y. Liu, A. N. Ezemaduka, P. R. Chen, X. Fu, and Z. Chang.** 2013. DegP primarily functions as a protease for the biogenesis of β-barrel outer membrane proteins in the Gram-negative bacterium *Escherichia coli*. FEBS J **281**: 1226-40.

10. **Hara H., Y. Nishimura, J. Kato, H. Suzuki, H. Nagasawa, A. Suzuki, and Y. Hirota.** 1989. Genetic analyses of processing involving C-terminal cleavage in penicillin-binding protein 3 of *Escherichia coli*. J Bacteriol **171**:5882-5889.

11. **Humphries R. M., T. P. Griener, S. L. Vogt, G. L. Mulvey, T. Raivio, M. S. Donnenberg, P. I. Kitov, M. Surette, and G. D. Armstrong.** 2010. N-acetyllactosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic *Escherichia coli*. Mol Microbiol **76**:1111-1126.

12. **Isaac D. D., J. S. Pinkner, S. J. Hultgren, and T. J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc Natl Acad Sci U S A **102**:17775-17779.

13. **Jiang J., X. Zhang, Y. Chen, Y. Wu, Z. H. Zhou, Z. Chang, and S. F. Sui.** 2008. Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. Proc Natl Acad Sci U S A **105**:11939-11944.

14. **Kitagawa M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori.** 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**:291-299.

15. **Krojer T., J. Sawa, E. Schäfer, H. R. Saibil, M. Ehrmann, and T. Clausen.** 2008. Structural basis for the regulated protease and chaperone function of DegP. Nature **453**:885-890.

16. **Lobos S. R., and G. C. Mora.** 1991. Alteration in the electrophoretic mobility of OmpC due to variations in the ammonium persulfate concentration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis **12**:448-450.

17. **Price N. L., and T. L. Raivio.** 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol **191**:1798-1815.

18. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

19. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

20. **Raivio T. L., M. W. Laird, J. C. Joly, and T. J. Silhavy.** 2000. Tethering of CpxP to the inner membrane prevents spheroplast induction of the Cpx envelope stress response. Mol Microbiol **37**:1186-1197.

21. **Shen Q. T., X. C. Bai, L. F. Chang, Y. Wu, H. W. Wang, and S. F. Sui.** 2009. Bowl-shaped oligomeric structures on membranes as DegP's new functional forms in protein quality control. Proc Natl Acad Sci U S A **106**:4858-4863.

22. **Silber K. R., K. C. Keiler, and R. T. Sauer.** 1992. Tsp: a tail-specific protease that selectively degrades proteins with nonpolar C termini. Proc Natl Acad Sci U S A **89**:295-299.

23. **Silhavy T. J., D. Kahne, and S. Walker.** 2010. The Bacterial Cell Envelope. Cold Spring Harbor Perspectives in Biology **2**:a000414.

24. **Spiess C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97**:339-347.

25. **Thede G. L., D. C. Arthur, R. A. Edwards, D. R. Buelow, J. L. Wong, T. L. Raivio, and J. N. Glover.** 2011. Structure of the periplasmic stress response protein CpxP. J Bacteriol **193**:2149-2157.

26. **Tschauner K., P. Hörnschemeyer, V. S. Müller, and S. Hunke.** 2014. Dynamic Interaction between the CpxA Sensor Kinase and the Periplasmic Accessory Protein CpxP Mediates Signal Recognition in *E. coli.* PLoS One **9**:e107383.

27. **Weski J., and M. Ehrmann.** 2012. Genetic analysis of 15 protein folding factors and proteases of the *Escherichia coli* cell envelope. J Bacteriol **194**:3225-3233.

28. **Weski J., and M. Ehrmann.** 2012. Genetic analysis of 15 protein folding factors and proteases of the *Escherichia coli* cell envelope. J Bacteriol **194**:3225-3233.

29. **Zhou X., R. Keller, R. Volkmer, N. Krauss, P. Scheerer, and S. Hunke.** 2011. Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. J Biol Chem **286**:9805-9814.

Appendix B: Mutational analysis of CpxP and related proteins in *Escherichia coli*

B1. Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a Gram-negative human pathogen that infects the small intestine and causes diarrheal illness that is responsible for up to 11% of deaths in infants under the age of five annually (7). EPEC possesses a number of virulence factors, including the plasmid-encoded bundle-forming pilus (BFP) (12). The BFP mediate cell-cell interactions between EPEC cells as well as the initial attachment of EPEC to intestinal epithelial cells (11). Following 'loose attachment' mediated by the BFP, 'intimate adherence' is facilitated by the chromosomally-encoded type III secretion system, which secretes effectors into the host cell (4), including the EPEC receptor intimin (19). Intimate adherence is associated with the effacement of the gut villi that is thought to alter the absorption of fluids in the gut and lead to diarrheal illness (6).

The Cpx two-component system both positively and negatively affects BFP expression (22). The Cpx two-component system senses envelope stress through the sensor kinase CpxA and transduces the signal to CpxR through a conserved phosphotransfer (14). Phosphorylated CpxR activates the expression of chaperones and proteases, including *cpxP*. Upon activation of the Cpx system, CpxR represses the transcription of the genes encoding the BFP either directly or indirectly. In the absence of *cpxR*, decreased expression of Cpx-regulated chaperones and proteases prevents the elaboration of BFP at a post-transcriptional level (22). Accordingly, it is thought that Cpx-regulated protein-folding factors and/or proteases are involved in the biogenesis of the BFP. One such folding factor is likely CpxP, since in the absence of *cpxP*, levels of BFP expression are decreased (22).

CpxP is a periplasmic protein that contains two conserved LTXXQ motifs of unknown function (Figure B1). Mutations close to the N-terminal LTXXQ motifs negatively affect the

ability of CpxP to inhibit the Cpx signal transduction through interactions with the periplasmic sensing domain of the CpxA sensor kinase while a mutation in the glutamine of the C-terminal LTXXQ motif alters the stability of the protein (1). *E. coli* CpxP (EcCpxP) and its closely related paralogue Spy (EcSpy) share two LTXXQ motifs, the C-terminal of which is absolutely conserved (16) (Figure B1). Similarly, the *Vibrio cholerae* CpxP (VcCpxP) homologue contains two LTXXQ motifs, both of which possess amino acids at the XX positions different from those of EcCpxP (Figure B1). We speculated that the EcCpxP and VcCpxP homologues may have similar chaperone functions.

In this work, we sought to determine if we could measure CpxP chaperone activity *in vivo* by analyzing BFP production. Using this assay, we examined the effect of mutations in the conserved LTXXQ motifs of the *E. coli* and *V. cholerae* CpxP homologues as well as *E. coli* Spy. This preliminary work suggests that the BFP are native substrates for the chaperone activity of CpxP in EPEC and that the XX residues found in the conserved LTXXQ motifs of CpxP homologues play an important role in this function.

B2. Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table B1. Mutant plasmids were constructed using the Quikchange Lightning Mutagenesis Kit (Agilent) as per the manufacturer's instructions.

Growth conditions and media. Unless otherwise specified, cells were grown in Luria-Bertani broth (LB broth) at 37°C with aeration by fixing a test tube rack to the platform of an orbital shaker set to 225 rpm. Antibiotics at the following concentrations were used, where appropriate: 50 μg/mL kanamycin and 100 μg/mL ampicillin. Stocks of 0.1 M IPTG and 1 M L-

arabinose (both Sigma) were diluted 1:1000 into cultures to induce expression of proteins from pTrc99A and pBAD33, respectively.

Western blots. Single isolated colonies were inoculated into 5 mL Luria-Bertani (LB) broth plus antibiotics in 10 mL glass tubes and grown overnight at 37°C with aeration, as described above. The next morning, cultures were diluted 1:50 into 10 mL fresh Tris-buffered Dulbecco's Modified Eagle Medium (DMEM) F12, pH 7.5 (Gibco) plus antibiotics and 0.1 mM IPTG or 1 mM L-arabinose (Sigma) in a 50 mL Erlenmeyer flask and grown to an OD ~0.6-0.7 with aeration. 1 mL equivalents of whole cell lysates were harvested and resuspended in 50 µL 2X sample buffer. 5 µL samples were run on a 12% denaturing polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose overnight. The next morning, the membranes were blocked in 5% skim milk for 3 h, shaking at room temperature, and washed overnight in 1X Tris-buffered saline (1 M Tris, 0.9 M NaCl) plus 0.2% Tween 20 (Sigma) (1X WS), shaking at room temperature. The next morning, anti-BfpB antibody diluted 1:10 000 and anti-CpxP-maltose-binding protein (MBP) antisera diluted 1:50 000 in 5% skim milk was applied to blots and incubated at room temperature for an hour with shaking. After three 15-minute washes at room temperature in 1X WS, anti-rabbit Ig secondary antibody conjugated to alkaline phosphatase diluted 1:25 000 was applied to anti-BfpB blots and secondary antibody diluted 1:50 000 was applied to anti-CpxP blots. After three more 15-minute washes at room temperature in 1X TS, blots were developed using the Immunstar AP Substrate Pack (Bio-Rad). Blots were imaged using the Chemi-Doc Imager (Bio-Rad).

Luminescence assays. Luminescence assays were performed as described previously (21). Briefly, single isolated colonies were inoculated into 2 mL LB plus antibiotics in 5 mL glass plating tubes in triplicate and grown at 37°C overnight with aeration, as described above.

The next morning, cultures were diluted 1:100 into fresh LB broth plus antibiotics in 5 mL plating tubes and grown for 1 h at 37°C with aeration. 0.1 mM IPTG was added to strains over-expressing EcCpxP or EcSpy and 1 mM L-arabinose was added to cultures over-expressing VcCpxP to induce over-expression of the gene cloned. 200 μL aliquots of each sample were transferred to a black 96-well microtitre plate (Corning). Luminescence and growth (A_{600}) was measured every 2 h for 6 h on a Wallac^2 1420 platereader (Perkin Elmer) and cultures were grown at 37°C with aeration (225 rpm). The average luminescence normalized to growth and standard deviations were plotted against time and only the 4 h time point is shown, for simplicity.

B3. Results and Discussion

We wondered if we could use BFP production as an *in vivo* measure of chaperone activity. The BFP is a type IV pilus and BfpB is the outer membrane secretin from which the BFP filaments are extruded (18). Levels of BfpB generally correlate with the overall stability of the entire pilus (17) and may therefore serve as a measure of the efficiency of BFP expression. To test this idea, we examined levels of BFP production using Western blots with antibody directed against the outer membrane secretin BfpB in wild-type EPEC and its isogenic *cpxP*:*kn* mutant derivative over-expressing EcCpxP, EcSpy, or VcCpxP. As a negative control, we also examined the levels of BfpB in EPEC that lacks the genes for BFP synthesis and assembly, JPN15.

Deletion of *cpxP* had variable effects on BFP levels between experiments (Figure B2, compare top and bottom panels). This might reflect the the conflicting positive and negative roles of CpxP in BFP biogenesis as both a regulator of Cpx signaling and a chaperone that impacts BFP assembly or stability (22). Thus, we would expect that deletion of *cpxP* would

activate the Cpx response, thereby simultaneously inhibiting the transcription of the *bfp* genes and providing elevated levels of Cpx-regulated chaperones that facilitate BFP assembly (22). We consistently observed that over-expression of EcCpxP or VcCpxP had little to no effect on BFP biogenesis, as measured by the levels of BfpB expression (Figure B2, compare lanes 3 and 4, top and bottom, and lanes 9 and 10, bottom). Conversely, the over-expression of EcSpy consistently led to a dramatic decrease in BfpB levels, indicative of a disruption in BfpB assembly (Figure B2, compare lanes 3 and 9, top panel). These results may suggest that the more efficient chaperone activity of Spy (13) somehow interferes with normal BFP assembly.

To investigate the effect of mutations in the conserved LTXXQ motifs of CpxP, we mutagenized the N- and C-terminal XX residues of EcCpxP, EcSpy, and VcCpxP. The XX residues of EcCpxP were replaced by alanine residues, while the XX residues of EcSpy and VcCpxP were replaced by the cognate residues of EcCpxP (Figure B1). These mutations were introduced into the high-copy IPTG-inducible plasmid pTrc99A bearing *cpxP* or *spy* for the *E. coli* homologues. Mutations in VcCpxP were introduced into pBAD33 bearing *cpxP* from *V. cholerae* (20). We then compared the effects of over-expressing the mutant versions of EcCpxP, EcSpy, and VcCpxP on BFP production to those of the wild-type proteins. We transformed the mutagenized plasmids into the EPEC *cpxP:kn* mutant and examined levels of BfpB, the outer membrane secretin for the BFP, by Western Blot. Over-expression of EcCpxP bearing an $E_{53}A$ mutation in the N-terminal LTXXQ motif had no effect on BfpB levels as compared to wild-type EcCpxP (Figure B2, compare lanes 4 and 5). In some experiments, strains over-expressing a CpxP variant carrying an $H_{54}A$ mutation showed diminished BfpB production (Figure B2, compare lanes 4 and 6, top and bottom panels). Interestingly, over-expression of the double CpxP$E_{53}A$, $H_{54}A$ mutant (CpxPAA) always resulted in diminished BfpB production (Figure B2,

compare lanes 4 and 7, top and bottom panels). This phenotype is similar to the levels of BfpB that result when wild-type Spy is over-expressed and suggests that the elevated chaperone activity of this mutant protein (Figure 3-3) may have a deleterious impact on BFP assembly. Mutation of the C-terminal P_{126} residue of CpxP had little or no impact on BfpB levels (Figure B2, compare lanes 4 and 8).

Over-expression of wild-type *spy* resulted in undetectable levels of BfpB protein, (Figure B2, compare lanes 3 and 9, top panel). Similarly, over-expression of proteins bearing single mutations in the N-terminal LTXXQ motif that introduced the cognate residues from CpxP at these positions ($D_{59}E$ or $A_{60}H$) resulted in dramatically reduced BfpB levels (Figure B2, compare lane 3 to lanes 10 and 11, top panel). This suggests that, individually, these amino acids have little impact on the *in vivo* chaperone activity of Spy. In contrast, over-expression of a Spy variant carrying both the $D_{59}E$ and $A_{60}H$ mutations (SpyEH) failed to completely inhibit the production of BfpB as compared to the strain over-expressing wild-type Spy (Figure B2, compare lanes 9 and 12). Although, over-expression of SpyEH still led to decreased BfpB levels as compared to the isogenic vector control strain (Figure 3B, compare lanes 3 and 12, top panel). These results, coupled with the observation that the SpyEH mutant protein exhibits diminished chaperone activity *in vitro* (Figure 3-3), suggest that it is the chaperone activity of Spy that interferes with BfpB production. Further, when the N-terminal LTXXQ motif of Spy is converted to that of CpxP, the protein behaves more like CpxP by exhibiting diminished chaperone activity *in vitro* (Figure 3-3), and failing to impact BFP biogenesis upon over-expression.

Over-expression of *V. cholerae cpxP* does not change BFP levels as compared to the isogenic vector control strain (Figure B2, compare lanes 9 and 10, lower panel). Similarly, when

the N-terminal X residue of the LTXXQ motif was mutated ($A_{55}E$) or both of the XX residues ($A_{55}E$, $E_{56}H$) to the identity of the cognate residues in EcCpxP, BfpB levels were unaffected, relative to the vector control strain (Figure B2, compare lane 10 to lanes 11 and 13, lower panel). This result was also seen when the $A_{135}P$ mutation was introduced into the C-terminal LTXXQ motif of VcCpxP (Figure B2, compare lanes 10 and 14, lower panel). In contrast, the single mutation $E_{56}H$ in VcCpxP led to a reduction in BfpB levels as compared to the vector control and wild-type VcCpxP over-expression strain (Figure B2, compare lane 9 to lanes 10 and 12, lower panel). Intriguingly, the VcCpxPEH protein was recognized by the EcCpxP polyclonal antisera (Figure B2, lane 13, lower panel), suggesting that this variant adopts a conformation with epitopes that are shared between the two proteins.

Altogether, these experiments suggest that the chaperone activity of Spy interferes with BFP elaboration. When mutations are introduced into Spy that mimic the amino acids found in the N-terminal LTXXQ motif of CpxP (SpyEH), the protein exhibits diminished chaperone activity *in vitro* (Figure 3-3) and *spy* over-expression no longer impacts BfpB levels, similar to what is seen with the wild-type CpxP. Further, when mutations are introduced into the N-terminal LTXXQ motif of EcCpxP that lead to an increase in chaperone activity (Figure 3-3), over-expression of this mutant variant behaves more like Spy, causing a reduction in the amount of BfpB and presumably a defect in BFP elaboration. These data constitute strong evidence for a role of the variable XX residues of the N-terminal LTXXQ motif in these homologues in the chaperone activity of the proteins. This conclusion is further supported by the observation that mutation of the same N-terminal LTXXQ motif in VcCpxP can convert this protein into one that resembles Spy in that it also leads to BfpB expression defects.

The Cpx pathway represses the transcription of the BFP, though it is not known whether CpxR binds upstream of the *bfp* operon to repress transcription directly (22). One explanation for the above results could be that some of the EcCpxP, EcSpy, and VcCpxP variants cause reductions in BFP levels by impacting transcription of the *bfpB* gene. To determine if this was the case, we transformed the wild-type and mutant plasmids into EPEC bearing a *bfp-lux* reporter in which the promoter for the *bfp* operon has been cloned upstream of the *luxCDABE* genes to produce light (9, 22). Over-expression of wild-type and mutant *cpxP* resulted in similar small decreases in *bfp* reporter expression that did not correlate with the levels of BfpB seen by Western Blot (compare Figures B2 and B3A). For example, over-expression of CpxPAA had similar effects on *bfp* transcription as the wild-type protein (Figure B3A) but resulted in diminished BfpB levels as compared to the over-expression of the wild-type protein in Western Blots for BfpB expression (Figure B2). The over-expression of EcSpy, VcCpxP, and their mutant derivatives had no or slightly stimulatory effects on *bfp-lux* transcription (Figure B3A). Thus, the over-expression of EcCpxP, EcSpy, and VcCpxP or their mutant derivatives had minimal impacts on the transcription of the *bfpA-L* operon that did not correlate with the changes in BfpB protein observed by Western blot (compare figures B2 and B3A). We conclude that the changes in BfpB expression observed upon over-expression of these mutant proteins, therefore, occurs post-transcriptionally and likely at the level of BFP assembly.

To determine whether the over-expression of mutant *cpxP* and *spy* plasmids was exerting an effect on BFP transcription through altered Cpx signaling, we also examined *cpxP-lux* activity. CpxP is one of the most inducible members of the Cpx regulon and is commonly used as a measure of the activity of the Cpx response (2). We transformed wild-type EPEC bearing a *cpxP-lux* reporter with the wild-type and mutant EcCpxP, EcSpy, and VcCpxP plasmids and

monitored luminescence and growth. In the presence of wild-type *cpxP* or any mutant derivative, a strong decrease in luminescence as compared to the vector control was observed (Figure B3B), suggesting that mutation of any of the residues in the LTXXQ motifs does not impact the ability of EcCpxP to inhibit the Cpx pathway in EPEC (Figure B3B). In contrast, though over-expression of wild-type *spy* could inhibit Cpx activity, over-expression of any of the *spy* mutants resulted in increased Cpx activity relative to strains over-expressing wild-type *spy* (Figure B3B). Surprisingly, over-expression of *V. cholerae cpxP* strongly decreased Cpx expression in EPEC, suggesting that VcCpxP can inhibit the Cpx pathway in EPEC. We have previously observed a 2-fold increase in Cpx pathway activity in the lab strain of *E. coli* when *V. cholerae cpxP* is over-expressed (Malpica, R., unpublished observatoins). Therefore, VcCpxP might have strain-specific impacts on Cpx signaling. However, it is important to note that the vector control for VcCpxP expression was not included in this analysis.

As noted for the transcription of the *bfp* operon (Figure B3A), the *cpxP* and *spy* mutations do not have the same effects on signaling as they do on BFP protein levels. For instance, though all of the *E. coli cpxP* mutants can inhibit the Cpx pathway to a similar extent as wild-type *cpxP*, only over-expression of *cpxPAA* resulted in decreased BfpB levels as compared to the over-expression of wild-type EcCpxP. Similarly, though wild-type Spy had similar effects as Spy bearing mutations in the N-terminal XX residues on BfpB stability, only wild-type *spy* can inhibit the Cpx pathway in EPEC.

Therefore, we believe that mutations of the XX residues are not impacting BFP expression solely by modulating Cpx activity or transcription of the *bfpA-L* operon, but may be participating in the post-transcriptional regulation of BFP expression. In light of the mutational analysis in Chapter 2 of this work, it is likely that mutations in the N-terminal XX residues are

impacting chaperone activity. Interestingly, Spy is strong chaperone *in vitro* (Figure 3-3) but over-expression of *spy* decreases BFP stability (Figure B2, compare lanes 3 and 9, top panel). Similarly, CpxPAA is a stronger chaperone than wild-type CpxP (Figure 3-3) but results in decreased BfpB levels as compared to wild-type EPEC (Figure B2, compare lanes 4 and 7), suggesting that mutations that positively impact *in vitro* chaperone activity might negatively impact the activity of CpxP that positively affects BFP expression. We speculate the CpxPAA might have higher affinity for misfolded substrates which allows for enhanced chaperone activity *in vitro*. However, perhaps this increased affinity prevents the efficient release of substrates *in vivo*, leading to decreased assembly of the BFP. It seems possible that both CpxPAA and wild-type Spy proteins could behave similarly in this regard, given their similar effects on BfpB and that wild-type Spy expression antagonizes BFP expression (Figure B3, compare lane 3 to lanes 7 and 9). Further study is required to confirm the effect of CpxP and Spy on BFP expression in EPEC and how these mutations impact CpxP function on native substrates in *E. coli*. A better understanding of how BFP are elaborated will help us understand the initial steps in EPEC pathogenesis and, potentially, the development of antimicrobial molecules that can antagonize the expression of virulence factors such as the BFP.

B4. Acknowledgements.

We are grateful to Matthew Waldor (Harvard University) for the gift of the pBAD33 and pVcCpxP plasmids.

B5. Tables

Table B1. Bacterial strains and plasmids used in this study

Strain	Genotype or Description	Reference or source
ALN194	E2348/69 *cpxP:kn*	(10, 22)
E2348/69	Prototypical enteropathogenic *E. coli* strain	(8)
JLW101	SV37 pTrc99A	This study
JLW102	SV37 pCpxP	This study
JLW108	NLP94 pTrc99A	This study
JLW413	ALN194 pCpxPE_{53}A	This study
JLW414	ALN194 pCpxPH_{54}A	This study
JLW415	ALN194 pCpxPAA	This study
JLW416	ALN194 pCpxPP_{126}A	This study
JLW417	ALN194 pSpy	This study
JLW418	ALN194 pSpyD_{59}E	This study
JLW419	ALN194 pSpyA_{60}H	This study
JLW420	ALN194 pSpyEH	This study
JLW421	ALN194 pBAD33	This study
JLW422	ALN194 pVcCpxP	This study
JLW423	ALN194 pVcCpxPA_{55}E	This study
JLW424	ALN194 pVcCpxPE_{56}H	This study
JLW425	ALN194 pVcCpxPEH	This study
JLW426	ALN194 pVcCpxPA_{135}P	This study
JLW439	SV37 pCpxPE_{53}A	This study
JLW440	SV37 pCpxPH_{54}A	This study
JLW441	SV37 pCpxPAA	This study
JLW442	SV37 pCpxPP_{126}A	This study
JLW443	SV37 pSpy	This study
JLW444	SV37 pSpyD_{59}E	This study
JLW445	SV37 pSpyA_{60}H	This study
JLW446	SV37 pSpyEH	This study
JLW447	SV37 pBAD33	This study
JLW449	SV37 pVcCpxP	This study
JLW450	SV37 pVcCpxPA_{55}E	This study
JLW451	SV37 pVcCpxPE_{56}H	This study
JLW452	SV37 pVcCpxPEH	This study
JLW453	SV37 pVcCpxPA_{135}P	This study
JLW453*	NLP94 pCpxP	This study
JLW454	NLP94 pCpxPE_{53}A	This study
JLW455	NLP94 pCpxPH_{54}A	This study
JLW456	NLP94 pCpxPAA	This study
JLW457	NLP94 pCpxPP_{126}A	This study
JLW459	NLP94 pSpy	This study
JLW460	NLP94 pSpyD_{59}E	This study

JLW461	NLP94 pSpyA$_{60}$H	This study
JLW462	NLP94 pSpyEH	This study
JLW463	NLP94 pBAD33	This study
JLW464	NLP94 pVcCpxP	This study
JLW465	NLP94 pVcCpxPA$_{55}$E	This study
JLW466	NLP94 pVcCpxPE$_{56}$H	This study
JLW467	NLP94 pVcCpxPEH	This study
JLW468	NLP94 pVcCpxPA$_{135}$P	This study
JPN15	E2348/69 cured of the EPEC adherence factor plasmid carrying the genes for *bfp*	(5)
NLP94	E2348/69 pJW25	This study
SV28	ALN194 pTrc99A	This study
SV29	ALN194 pCpxP	This study
SV37	E2348/69 pJW23	This study
Plasmids	**Description**	**Source or Reference**
pTrc99A	High-copy IPTG-inducible vector for expression, Amp^R	Pharmacia
pCpxP	Vector for over-expressing *cpxP*	(15)
pSpy	Vector for over-expressing *spy*	(1)
pBAD33	High-copy arabinose inducible vector for expression, Amp^R	(3)
pVcCpxP	Vector for over-expressing Vc*cpxP*	(20)

Table B2: Primers used in this study

Primer name	Sequence (5' to 3')	Description or reference
pBAD24-for	CTGTTTCTCCATACCCGTT	Forward primer for sequencing into the multiple-cloning site of pBAD33
pBAD24-rv	GGCTGAAAATCTTCTCT	Reverse primer for sequencing into the multiple-cloning site of pBAD33
pTrc99Aseq-for	TGCAGGTCGTAAATCACTGC	Forward primer for sequencing into the multiple cloning site of pTrc99A
pTrc99Aseq-rv	CTGGCAGTTCCCTACTCTCG	Reverse primer for sequencing into the multiple cloning site of pTrc99A
VcCpxPA55E-for	AAACAGCTTGATTTAACCGAGCACCAGCAAGCCCAACTCAAAGAGAT	Forward mutagenic primer for pVcCpxPA55E
VcCpxPA55E-rev	TTTGAGTTGGGCTTGCTGGTGCTCGGTTAAATCAAGCTGTTTCCAG	Reverse mutagenic primer for pVcCpxPA55E
VcCpxPE56H-for	CAGCTTGATTTAACCGCTCACCAGCAAGCCCAACTCAA	Forward mutagenic primer for pVcCpxPE56H
VcCpxPE56H-rv	CCACCAAATGATGAGCATTTTGACTCCCGAACAGAAGC	Reverse mutagenic primer for pVcCpxPE56H
VcCpxPA135P-for	CCACCAAATGATGAGCATTTTGACTCCCGAACAGAAGC	Forward mutagenic primer for pVcCpxPA135P
VcCpxPA135P-rev	GCTTTCTGTTCGGGAGTCAAAATGCTCATTTGGTGG	Reverse mutagenic primer for pVcCpxPA135P
cpxPE53A-for	CGCATAAGTTTAACCGCACATCAGCGTCAGCAGA	Forward mutagenic primer for pCpxPE53A
cpxPH53A-rev	TCTGCTGACGCTGATGTGCGGTTAAACTTATGCCG	Reverse mutagenic primer for pCpxPE53A
cpxPH54A-for	TCGACGGCATAAGTTTAACCGAAGCTCAGCGTCAGCAGAT	Forward mutagenic primer for pCpxPH54A
cpxPH54A-rev	ATCTGCTGACGCTGAGCTTCGGTTAAACTTATGCCGTCG	Reverse mutagenic primer for pCpxPH54A
cpxPE53AH54A-rev	GACGGCATAAGTTTAACCGCAGCTCAGCGTCAGCAGATGCGA	Forward mutagenic primer for pCpxPAA
cpxPE53AH54A-rev	TCGATCTGCTGACGCTGAGCTGCGGTTAAACTTATGCCGTC	Reverse mutagenic primer for pCpxPAA

cpxPP126A-for	TCGCCTGTTAACGGCGGAGCAGCAAGC	Forward mutagenic primer for pCpxPP126A
cpxPP126A-rev	GCTTGCTGCTCCGCCGTTAACAGGCGA	Forward mutagenic primer for pCpxPP126A
spyD59E-for2	AAGACCTGAACCTGACCGAGGCGCAGAAAC	Forward mutagenic primer for pSpyD59E
spyD59E-rev2	GTTTCTGCGCCTCGGTCAGGTTCAGGTCTT	Reverse mutagenic primer for pSpyD59E
SDMspyA60H-for	ACCTGAACCTGACCGACCACCAGAAACAGCAGATCCG	
SDMspyA60H-rev	CGGATCTGCTGTTTCTGGTGGTCGGTCAGGTTCAGGT	
EcSpyD59E/A60H-for	AGACCTGAACCTGACCGAGCACCAGAAACAGCAGATCCGC	Forward mutagenic primer for pSpyEH
EcSpyD59E/A60H-rev	GCGGATCTGCTGTTTCTGGTGCTCGGTCAGGTTCAGGTCT	Reverse mutagenic primer for pSpyEH

B6. Figures

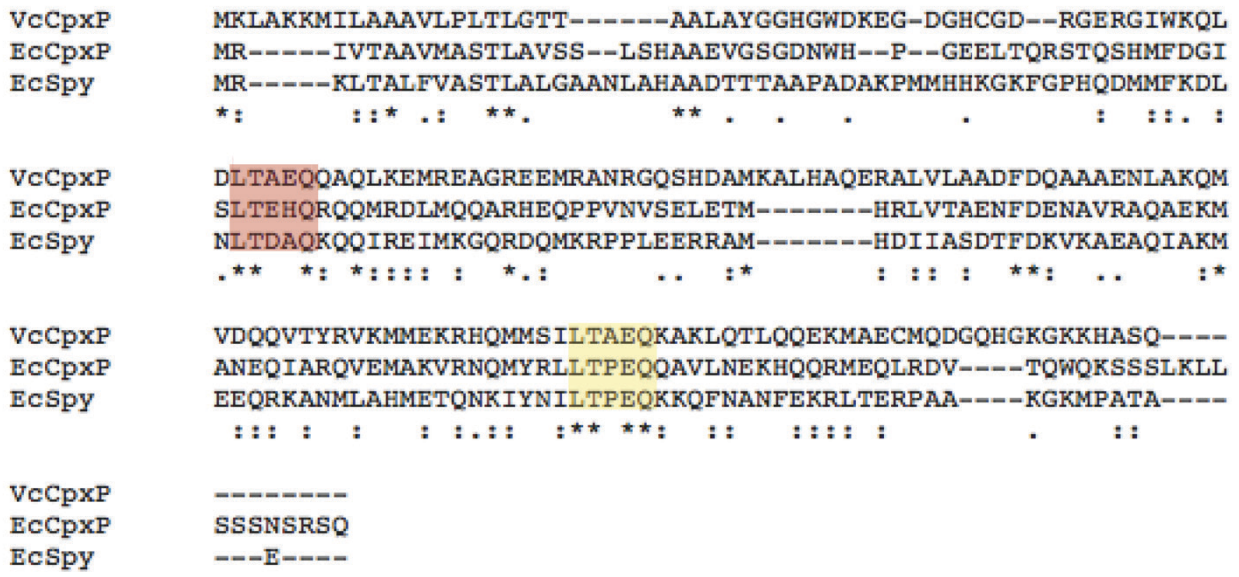


Figure B1. The conserved LTXXQ motifs define CpxP homologues.

Protein sequence alignment of CpxP from *E. coli* (EcCpxP), Spy from *E. coli* (EcSpy) and CpxP from *V. cholerae* (VcCpxP). The N-terminal LTXXQ motif is highlighted in red and the C-terminal LTXXQ motif is highlighted in yellow. The alignment was generated in Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Asterisks marked absolutely conserved residues in all three sequences, colons and periods mark high and low sequence conservation, respectively. Gaps in sequences are marked with hyphens.

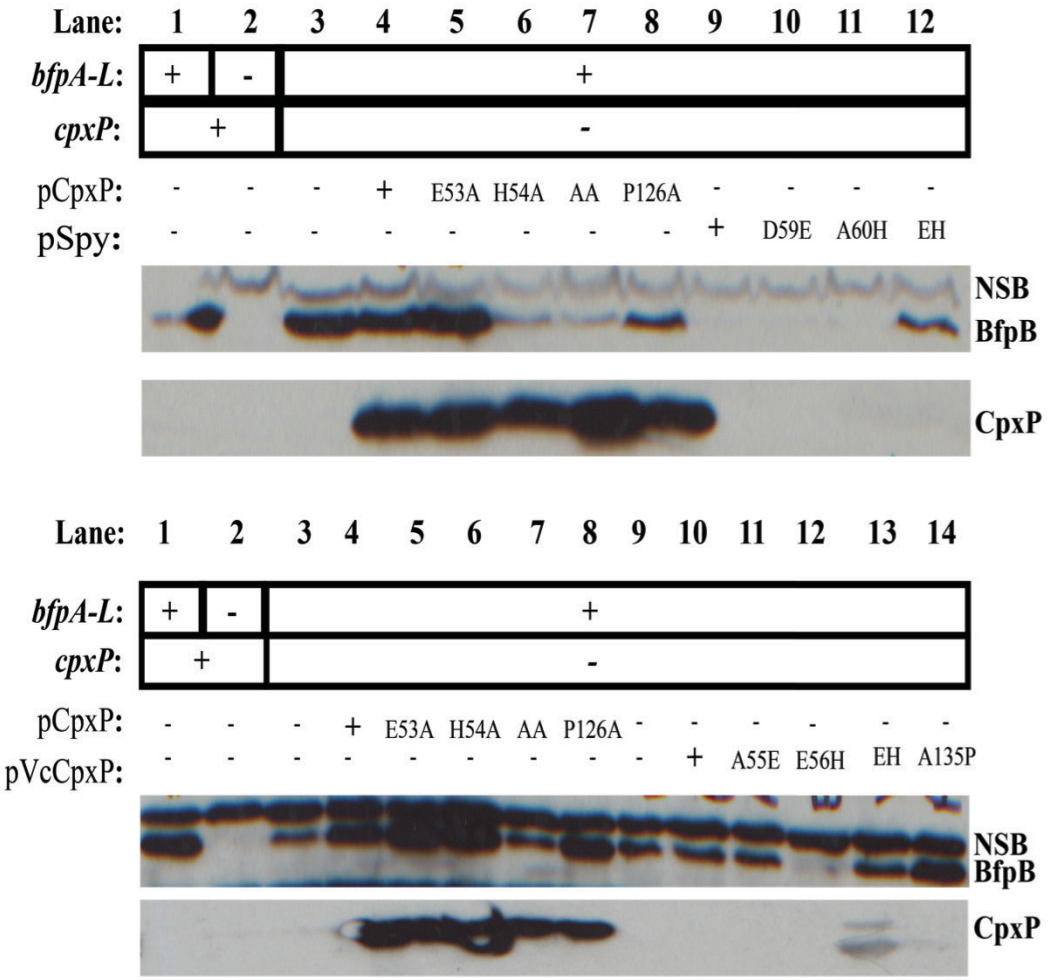
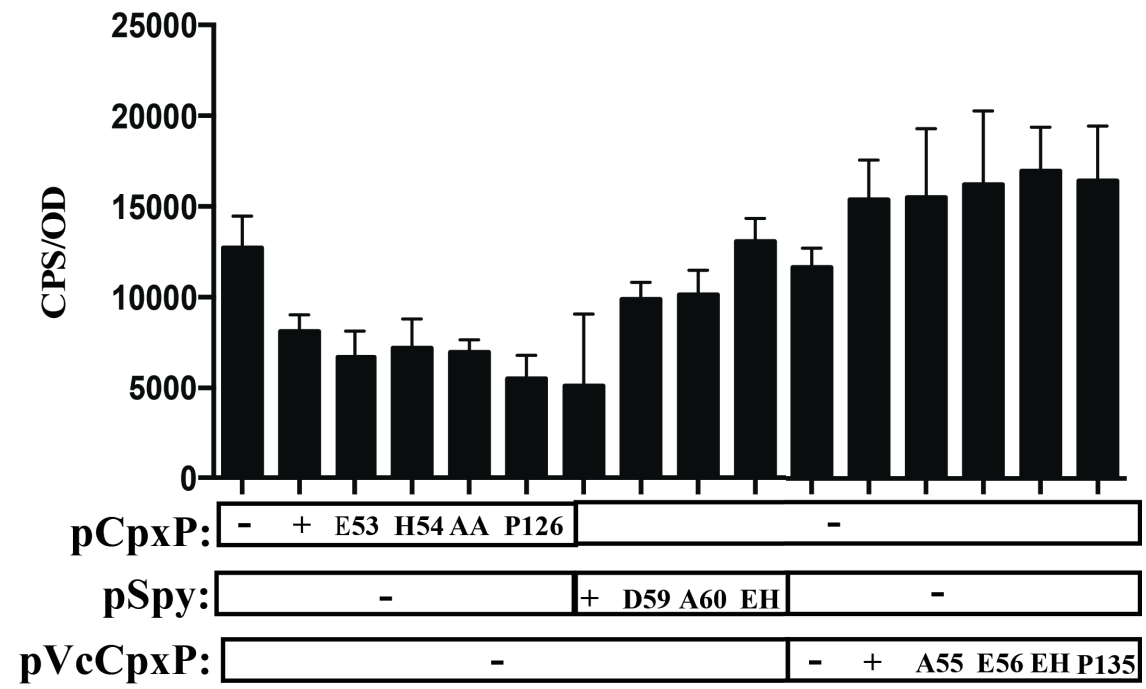


Figure B2. The stability of BfpB is altered by the over-expression of *cpxP* and *spy* mutant derivatives.

The expression of the secretin BfpB in wild-type EPEC (E2348/69), EPEC lacking the genes for *bfp* expression (JPN15), *cpxP:kn* mutant EPEC over-expressing pTrc99A (SV28), pCpxP (SV29), or mutant derivatives of EcCpxP, EcSpy, or VcCpxP (JLW413 through JLW426) was assessed by Western Blot as described in Materials and Methods. The bands corresponding to BfpB and CpxP are marked on the right of the figure. NSB stands for a non-specific band that serves as a loading control for this experiment. A set of representative results of five independent experiments is given here.

A.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20000
15000
10000
5000
0
CPS/OD
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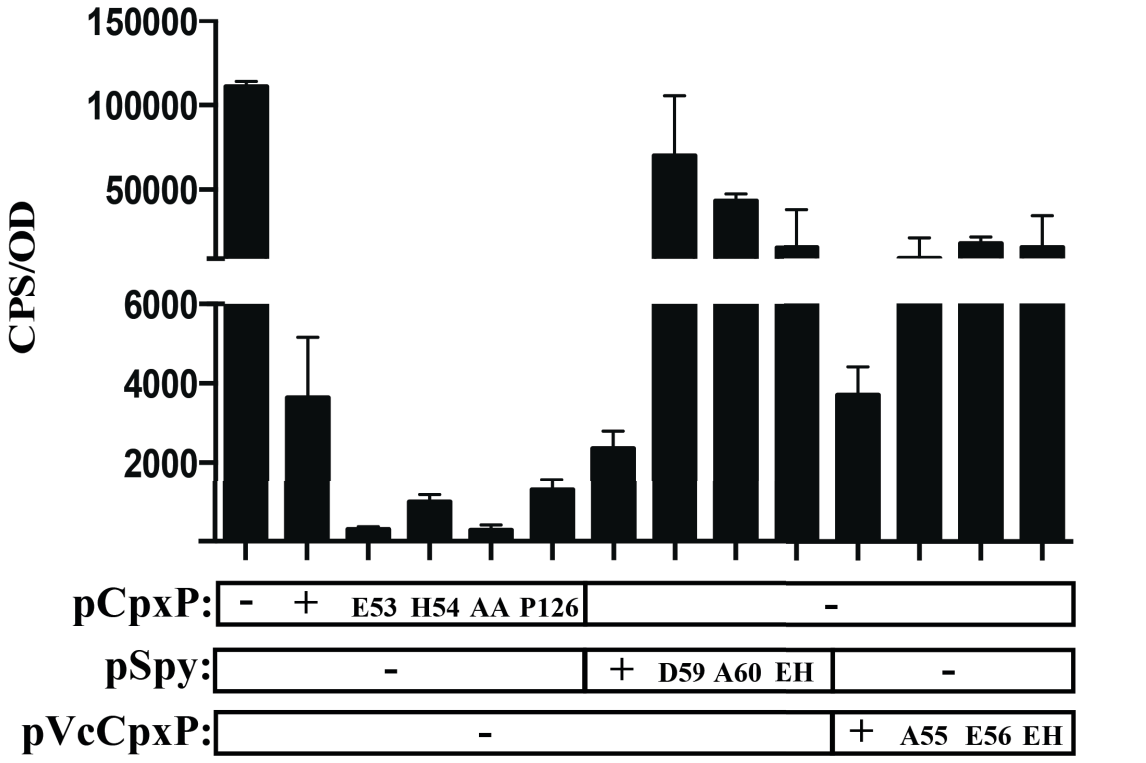
- + E53 H54 AA P126
-
pSpy:
-
+ D59 A60 EH
-
pVcCpxP:
-
- + A55 E56 EH P135

B.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150000
100000
50000
6000
4000
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CPS/OD
pCpxP:
- + E53 H54 AA P126
-
pSpy:
-
+ D59 A60 EH
-
pVcCpxP:
-
+ A55 E56 EH

Figure B3. Over-expression of *cpxP* and *spy* mutations alters Cpx activity and *bfp* transcription.

A. Over-expression of *cpxP* and *spy* from *E. coli* and *V. cholerae* affects *bfp-lux* and **B.** *cpxP-lux* activity. The residues mutated are indicated, except for the double mutants, which are marked as AA for CpxPAA, EH for SpyEH and EH for VcCpxPEH. The luminescence at 4 hours normalized to the A_{600} plus the standard deviation of triplicate cultures is given above. A representative result of two-independent experiments for each of A and B is shown here.

B7. References

1. **Buelow D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J Bacteriol **187**:6622-6630.

2. **DiGiuseppe P. A., and T. J. Silhavy.** 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol **185**:2432-2440.

3. **Guzman L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177**:4121-4130.

4. **Jarvis K. G., J. A. Girón, A. E. Jerse, T. K. McDaniel, M. S. Donnenberg, and J. B. Kaper.** 1995. Enteropathogenic *Escherichia col*i contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc Natl Acad Sci U S A **92**:7996-8000.

5. **Jerse A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci U S A **87**:7839-7843.

6. **Kenny B., and B. B. Finlay.** 1997. Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C-gamma1. Infect Immun **65**:2528-2536.

7. **Lanata C. F., C. L. Fischer-Walker, A. C. Olascoaga, C. X. Torres, M. J. Aryee, R. E. Black, and Child Health Epidemiology Reference Group of the World Health Organization and UNICEF.** 2013. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. PLoS One **8**:e72788.

8. **Levine M., D. Nalin, R. Hornick, E. Bergquist, D. Waterman, C. Young, S. Sotman, and B. Rowe.** 1978. Escherichia coli strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. The Lancet **311**:1119-1122.

9. **MacRitchie D. M., J. D. Ward, A. Z. Nevesinjac, and T. L. Raivio.** 2008. Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic *Escherichia coli*. Infect Immun **76**:1465-1475.

10. **MacRitchie D. M., N. Acosta, and T. L. Raivio.** 2012. DegP is involved in Cpx-mediated posttranscriptional regulation of the type III secretion apparatus in enteropathogenic *Escherichia coli*. Infect Immun **80**:1766-1772.

11. **Nataro J. P., I. C. Scaletsky, J. B. Kaper, M. M. Levine, and L. R. Trabulsi.** 1985. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. Infect Immun **48**:378-383.

12. **Williams, P. H., Evans, N., Turner, P., George, R. H., and A. S. McNeish.** 1977. Plasmid mediating mucosal adherence in human enteropathogenic *Escherichia coli*. Lancet **8022**: 1151.

13. **Quan S., P. Koldewey, T. Tapley, N. Kirsch, K. M. Ruane, J. Pfizenmaier, R. Shi, S. Hofmann, L. Foit, G. Ren, U. Jakob, Z. Xu, M. Cygler, and J. C. Bardwell.** 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat Struct Mol Biol **18**:262-269.

14. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

15. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

16. **Raivio T. L., M. W. Laird, J. C. Joly, and T. J. Silhavy.** 2000. Tethering of CpxP to the inner membrane prevents spheroplast induction of the Cpx envelope stress response. Mol Microbiol **37**:1186-1197.

17. **Ramer S. W., D. Bieber, and G. K. Schoolnik.** 1996. BfpB, an outer membrane lipoprotein required for the biogenesis of bundle-forming pili in enteropathogenic *Escherichia coli*. J Bacteriol **178**:6555-6563.

18. **Schmidt S. A., D. Bieber, S. W. Ramer, J. Hwang, C. Y. Wu, and G. Schoolnik.** 2001. Structure-function analysis of BfpB, a secretin-like protein encoded by the bundle-forming-pilus operon of enteropathogenic *Escherichia coli*. J Bacteriol **183**:4848-4859.

19. **Schoolnik G. K.** 1993. Intimin and the intimate attachment of bacteria to human cells. The Journal of Clinical Investigation **92**:1117-1118.

20. **Slamti L., and M. K. Waldor.** 2009. Genetic analysis of activation of the *Vibrio cholerae* Cpx pathway. J Bacteriol **191**:5044-5056.

21. **Wong, J. L., Vogt, S. L., and T. L. Raivio.** 2012. Using reporter genes and the *Escherichia coli* ASKA over-expression library in screens for regulators of the Gram-negative envelope stress response. Methods. Mol. Biol. **966:** 337-57.

22. **Vogt S. L., A. Z. Nevesinjac, R. M. Humphries, M. S. Donnenberg, G. D. Armstrong, and T. L. Raivio.** 2010. The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. Mol Microbiol **76**:1095-1110.

References

1. **Abramson J., S. Riistama, G. Larsson, A. Jasaitis, M. Svensson-Ek, L. Laakkonen, A. Puustinen, S. Iwata, and M. Wikström.** 2000. The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site. Nature Structural Molecular Biology **7**:910.

2. **Achard M. E. S., J. J. Tree, J. A. Holden, K. R. Simpfendorfer, O. L. C. Wijburg, R. A. Strugnell, M. A. Schembri, M. J. Sweet, M. P. Jennings, and A. G. McEwan.** 2010. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. Infect Immun **78**:2312-2319.

3. **Acosta N., S. Pukatzki, and T. L. Raivio.** 2014. The Vibrio cholerae Cpx envelope stress response senses and mediates adaptation to low iron. J Bacteriol. **197(2)**: 226-76.

4. **Ahmer B. M., M. G. Thomas, R. A. Larsen, and K. Postle.** 1995. Characterization of the *exbBD* operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and stability. J Bacteriol **177**:4742-4747.

5. **Anjem A., S. Varghese, and J. A. Imlay.** 2009. Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli. Mol Microbiol **72**:844-858.

6. **Anraku Y., and R. B. Gennis.** 1987. The aerobic respiratory chain of *Escherichia coli*. Trends in Biochemical Sciences **12**:262-266.

7. **Appia-Ayme C., A. Hall, E. Patrick, S. Rajadurai, T. A. Clarke, and G. Rowley.** 2012. ZraP is a periplasmic molecular chaperone and a repressor of the zinc-responsive two-component regulator ZraSR. Biochem J **442**:85-93.

8. **Archibald F.** 1986. Manganese: its acquisition by and function in the lactic acid bacteria. Crit Rev Microbiol **13**:63-109.

9. **Auriol C., G. Bestel-Corre, J. -B. Claude, P. Soucaille, and I. Meynial-Salles.** 2011. Stress-induced evolution of *Escherichia coli* points to original concepts in respiratory cofactor selectivity. Proceedings of the National Academy of Sciences **108**:1278-1283.
[NO STYLE for: Wong 2013].

11. **Baird L., B. Lipinska, S. Raina, and C. Georgopoulos.** 1991. Identification of the *Escherichia coli sohB* gene, a multicopy suppressor of the HtrA (DegP) null phenotype. J Bacteriol **173**:5763-5770.

12. **Bakker D., C. E. Vader, B. Roosendaal, F. R. Mooi, B. Oudega, and F. K. de Graaf.** 1991. Structure and function of periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. Mol Microbiol **5**:875-886.

13. **Baranova N., and H. Nikaido.** 2002. The *baeSR* two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate. J Bacteriol **184**:4168-4176.

14. **Bassford P. J., C. Bradbeer, R. J. Kadner, and C. A. Schnaitman.** 1976. Transport of vitamin B12 in *tonB* mutants of *Escherichia coli*. J Bacteriol **128**:242-247.

15. **Bauer B. W., T. Shemesh, Y. Chen, and T. A. Rapoport.** 2014. A "push and slide" mechanism allows sequence-insensitive translocation of secretory proteins by the SecA ATPase. Cell **157**:1416-1429.

16. **Beard S. J., M. N. Hughes, and R. K. Poole.** 1995. Inhibition of the cytochrome bd-terminated NADH oxidase system in *Escherichia coli* K-12 by divalent metal cations. FEMS Microbiol Lett **131**:205-210.

17. **Beard S. J., R. Hashim, J. Membrillo-Hernández, M. N. Hughes, and R. K. Poole.** 1997. Zinc(II) tolerance in *Escherichia coli* K-12: evidence that the *zntA* gene (o732) encodes a cation transport ATPase. Mol Microbiol **25**:883-891.

18. **Becker K. W., and E. P. Skaar.** 2014. Metal limitation and toxicity at the interface between host and pathogen. FEMS Microbiol Rev .

19. **Becker L. A., I. -S. Bang, M. -L. Crouch, and F. C. Fang.** 2005. Compensatory role of PspA, a member of the phage shock protein operon, in *rpoE* mutant *Salmonella enterica* serovar Typhimurium. Mol Microbiol **56**:1004-1016.

20. **Beckman D. L., D. R. Trawick, and R. G. Kranz.** 1992. Bacterial cytochromes c biogenesis. Genes Dev **6**:268-283.

21. **Bina X. R., D. Provenzano, N. Nguyen, and J. E. Bina.** 2008. *Vibrio cholerae* RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. Infect Immun **76**:3595-3605.

22. **Bleuel C., C. Grosse, N. Taudte, J. Scherer, D. Wesenberg, G. J. Krauss, D. H. Nies, and G. Grass.** 2005. TolC is involved in enterobactin efflux across the outer membrane of *Escherichia coli*. J Bacteriol **187**:6701-6707.

23. **Boer J. L., S. B. Mulrooney, and R. P. Hausinger.** 2014. Nickel-dependent metalloenzymes. Arch Biochem Biophys **544**:142-152.

24. **Botella H., P. Peyron, F. Levillain, R. Poincloux, Y. Poquet, I. Brandli, C. Wang, L. Tailleux, S. Tilleul, G. M. Charrière, S. J. Waddell, M. Foti, G. Lugo-Villarino, Q. Gao, I.**

Maridonneau-Parini, P. D. Butcher, P. R. Castagnoli, B. Gicquel, C. de Chastellier, and O. Neyrolles. 2011. Mycobacterial p(1)-type ATPases mediate resistance to zinc poisoning in human macrophages. Cell Host Microbe **10**:248-259.

25. **Bouchart F., G. Boussemart, A. F. Prouvost, V. Cogez, E. Madec, O. Vidal, B. Delrue, J. P. Bohin, and J. -M. Lacroix.** 2010. The virulence of a *Dickeya dadantii* 3937 mutant devoid of osmoregulated periplasmic glucans is restored by inactivation of the RcsCD-RcsB phosphorelay. J Bacteriol **192**:3484-3490.

26. **Braun M., S. Bungert, and T. Friedrich.** 1998. Characterization of the Overproduced NADH Dehydrogenase Fragment of the NADH:Ubiquinone Oxidoreductase (Complex I) from *Escherichia coli*. Biochemistry **37**:1861-1867.

27. **Breyton C., W. Haase, T. A. Rapoport, W. Kühlbrandt, and I. Collinson.** 2002. Three-dimensional structure of the bacterial protein-translocation complex SecYEG. Nature **418**:662-665.

28. **Brissette J. L., L. Weiner, T. L. Ripmaster, and P. Model.** 1991. Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. J Mol Biol **220**:35-48.

29. **Brocklehurst K. R., J. L. Hobman, B. Lawley, L. Blank, S. J. Marshall, N. L. Brown, and A. P. Morby.** 1999. ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. Mol Microbiol **31**:893-902.

30. **Buelow D. R., and T. L. Raivio.** 2005. Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. J Bacteriol **187**:6622-6630.

31. **Butcher J., S. Sarvan, J. S. Brunzelle, J. -F. Couture, and A. Stintzi.** 2012. Structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation. Proc Natl Acad Sci U S A **109**:10047-10052.

32. **Büttner F. M., S. Zoll, M. Nega, F. Götz, and T. Stehle.** 2014. Structure-Function Analysis of *Staphylococcus aureus* Amidase Reveals the Determinants of Peptidoglycan Recognition and Cleavage. J Biol Chem **289**:11083-11094.

33. **Calhoun M. W., and R. B. Gennis.** 1993. Demonstration of separate genetic loci encoding distinct membrane-bound respiratory NADH dehydrogenases in *Escherichia coli*. J Bacteriol **175**:3013-3019.

34. **Caly D. L., P. W. O'Toole, and S. A. Moore.** 2010. The 2.2-Å structure of the HP0958 protein from *Helicobacter pylori* reveals a kinked anti-parallel coiled-coil hairpin domain and a highly conserved Zn-ribbon domain. J Mol Biol **403**:405-419.

35. **Campoy S., M. Jara, N. Busquets, A. M. Pérez De Rozas, I. Badiola, and J. Barbé.** 2002. Role of the high-affinity zinc uptake *znuABC* system in *Salmonella enterica* serovar Typhimurium virulence. Infect Immun **70**:4721-4725.

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

37. **Carpenter B. M., J. J. Gilbreath, O. Q. Pich, A. M. McKelvey, E. L. Maynard, Z. -Z. Li, and D. S. Merrell.** 2013. Identification and characterization of novel *Helicobacter pylori* apo-Fur-regulated target genes. J Bacteriol **195**:5526-5539.

38. **Casadaban M. J., and S. N. Cohen.** 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 **76**:4530-4533.

39. **CastilloKeller M., and R. Misra.** 2003. Protease-deficient DegP suppresses lethal effects of a mutant OmpC protein by its capture. J Bacteriol **185**:148-154.

40. **Cavaco L. M., H. Hasman, M. Stegger, P. S. Andersen, R. Skov, A. C. Fluit, T. Ito, and F. M. Aarestrup.** 2010. Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. Antimicrob Agents Chemother **54**:3605-3608.

41. **Cerasi M., J. Z. Liu, S. Ammendola, A. J. Poe, P. Petrarca, M. Pesciaroli, P. Pasquali, M. Raffatellu, and A. Battistoni.** 2014. The ZupT transporter plays an important role in zinc homeostasis and contributes to *Salmonella enterica* virulence. Metallomics **6**:845-853.

42. **Cerasi M., S. Ammendola, and A. Battistoni.** 2013. Competition for zinc binding in the host-pathogen interaction. Front Cell Infect Microbiol **3**:108.

43. **Chaba R., B. M. Alba, M. S. Guo, J. Sohn, N. Ahuja, R. T. Sauer, and C. A. Gross.** 2011. Signal integration by DegS and RseB governs the σ^E-mediated envelope stress response in *Escherichia coli*. Proc Natl Acad Sci U S A **108**:2106-2111.

44. **Chandra B. R., M. Yogavel, and A. Sharma.** 2007. Structural analysis of ABC-family periplasmic zinc binding protein provides new insights into mechanism of ligand uptake and release. J Mol Biol **367**:970-982.

45. **Changela A., K. Chen, Y. Xue, J. Holschen, C. E. Outten, T. V. O'Halloran, and A. Mondragón.** 2003. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science **301**:1383-1387.

46. **Chao Y., and D. Fu.** 2004. Thermodynamic studies of the mechanism of metal binding to the *Escherichia coli* zinc transporter YiiP. J Biol Chem **279**:17173-17180.

47. **Chen N. G., K. Gregory, Y. Sun, and V. Golovlev.** 2011. Transient model of thermal deactivation of enzymes. Biochim Biophys Acta **1814**:1318-1324.

48. **Cheung J., C. A. Bingman, M. Reyngold, W. A. Hendrickson, and C. D. Waldburger.** 2008. Crystal structure of a functional dimer of the PhoQ sensor domain. J Biol Chem **283**:13762-13770.

49. **Chung K. C. C., and D. B. Zamble.** 2011. The *Escherichia coli* metal-binding chaperone SlyD interacts with the large subunit of [NiFe]-hydrogenase 3. FEBS Lett **585**:291-294.

50. **Connolly L., A. De Las Penas, B. M. Alba, and C. A. Gross.** 1997. The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. Genes Dev **11**:2012-2021.

51. **Coppola D., D. Giordano, M. Tinajero-Trejo, G. di Prisco, P. Ascenzi, R. K. Poole, and C. Verde.** 2013. Antarctic bacterial haemoglobin and its role in the protection against nitrogen reactive species. Biochim Biophys Acta **1834**:1923-1931.

52. **Corbett D., J. Wang, S. Schuler, G. Lopez-Castejon, S. Glenn, D. Brough, P. W. Andrew, J. S. Cavet, and I. S. Roberts.** 2012. Two zinc uptake systems contribute to the full virulence of *Listeria monocytogenes* during growth in vitro and in vivo. Infect Immun **80**:14-21.

53. **Cosma C. L., P. N. Danese, J. H. Carlson, T. J. Silhavy, and W. B. Snyder.** 1995. Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope-associated stresses. Mol Microbiol **18**:491-505.

54. **Costerton J. W., J. M. Ingram, and K. J. Cheng.** 1974. Structure and function of the cell envelope of Gram-negative bacteria. Bacteriol Rev **38**:87-110.

55. **Daly M. J., E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai, A. Venkateswaran, M. Hess, M. V. Omelchenko, H. M. Kostandarithes, K. S. Makarova, L. P.**

Wackett, J. K. Fredrickson, and D. Ghosal. 2004. Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science **306**:1025-1028.

56. **Danese P. N., G. R. Oliver, K. Barr, G. D. Bowman, P. D. Rick, and T. J. Silhavy.** 1998. Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*. J Bacteriol **180**:5875-5884.

57. **Danese P. N., W. B. Snyder, C. L. Cosma, L. J. Davis, and T. J. Silhavy.** 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. Genes Dev **9**:387-398.

58. **Darwin A. J., and V. L. Miller.** 2001. The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth in vitro when the Ysc type III secretion system is produced. Mol Microbiol **39**:429-444.

59. **David G., K. Blondeau, M. Schiltz, S. Penel, and A. Lewit-Bentley.** 2003. YodA from Escherichia coli is a metal-binding, lipocalin-like protein. J Biol Chem **278**:43728-43735.

60. **Davidson V. L., and L. H. Jones.** 1996. Electron transfer from copper to heme within the methylamine dehydrogenase--amicyanin--cytochrome c-551i complex. Biochemistry **35**:8120-8125.

61. **Davies J. K., and P. Reeves.** 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group B. J Bacteriol **123**:96-101.

62. **Debnath I., J. P. Norton, A. E. Barber, E. M. Ott, B. K. Dhakal, R. R. Kulesus, and M. A. Mulvey.** 2013. The Cpx stress response system potentiates the fitness and virulence of uropathogenic *Escherichia coli*. Infect Immun **81**:1450-1459.

63. **DebRoy S., M. Gebbie, A. Ramesh, J. R. Goodson, M. R. Cruz, A. van Hoof, W. C. Winkler, and D. A. Garsin.** 2014. Riboswitches. A riboswitch-containing sRNA controls gene expression by sequestration of a response regulator. Science **345**:937-940.

64. **DeChavigny A., P. N. Heacock, and W. Dowhan.** 1991. Sequence and inactivation of the pss gene of *Escherichia coli*. Phosphatidylethanolamine may not be essential for cell viability. J Biol Chem **266**:5323-5332.

65. **Desnoyers G., A. Morissette, K. Prévost, and E. Massé.** 2009. Small RNA-induced differential degradation of the polycistronic mRNA *iscRSUA*. EMBO J **28**:1551-1561.

66. **DiGiuseppe P. A., and T. J. Silhavy.** 2003. Signal detection and target gene induction by the CpxRA two-component system. J Bacteriol **185**:2432-2440.

67. **Ding L., A. B. Becker, A. Suzuki, and R. A. Roth.** 1992. Comparison of the enzymatic and biochemical properties of human insulin-degrading enzyme and *Escherichia coli* protease III. J Biol Chem **267**:2414-2420.

68. **Doig P., R. Yao, D. H. Burr, P. Guerry, and T. J. Trust.** 1996. An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. Mol Microbiol **20**:885-894.

69. **Donnenberg M. S., J. A. Girón, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol Microbiol **6**:3427-3437.

70. **Douchin V., C. Bohn, and P. Bouloc.** 2006. Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*. J Biol Chem **281**:12253-12259.

71. **Dowd G. C., P. G. Casey, M. Begley, C. Hill, and C. G. M. Gahan.** 2012. Investigation of the role of ZurR in the physiology and pathogenesis of *Listeria monocytogenes*. FEMS Microbiol Lett **327**:118-125.

72. **Ebel W., and J. E. Trempy.** 1999. *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions to activate its own expression. J Bacteriol **181**:577-584.

73. **Euro L., G. Belevich, D. A. Bloch, M. I. Verkhovsky, M. Wikström, and M. Verkhovskaya.** 2009. The role of the invariant glutamate 95 in the catalytic site of Complex I from *Escherichia coli.* Biochimica et Biophysica Acta (BBA) - Bioenergetics **1787**:68-73.

74. **Euro L., G. Belevich, D. A. Bloch, M. I. Verkhovsky, M. Wikström, and M. Verkhovskaya.** 2009. The role of the invariant glutamate 95 in the catalytic site of Complex I from *Escherichia coli*. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1787**:68-73.

75. **Fan B., and B. P. Rosen.** 2002. Biochemical characterization of CopA, the *Escherichia coli* Cu(I)-translocating P-type ATPase. J Biol Chem **277**:46987-46992.

76. **Ferrières L., A. Thompson, and D. J. Clarke.** 2009. Elevated levels of sigma S inhibit biofilm formation in *Escherichia coli*: a role for the Rcs phosphorelay. Microbiology **155**:3544-3553.

77. **Flynn J. M., I. Levchenko, R. T. Sauer, and T. A. Baker.** 2004. Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. Genes Dev **18**:2292-2301.

78. **Francis C. L., A. E. Jerse, J. B. Kaper, and S. Falkow.** 1991. Characterization of interactions of enteropathogenic *Escherichia coli* O127:H6 with mammalian cells in vitro. J Infect Dis **164**:693-703.

79. **Franke S., G. Grass, C. Rensing, and D. H. Nies.** 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol **185**:3804-3812.

80. **Fredericks C. E., S. Shibata, S. -I. Aizawa, S. A. Reimann, and A. J. Wolfe.** 2006. Acetyl phosphate-sensitive regulation of flagellar biogenesis and capsular biosynthesis depends on the Rcs phosphorelay. Mol Microbiol **61**:734-747.

81. **Fridén H., and L. Hederstedt.** 1990. Role of His residues in *Bacillus subtilis* cytochrome b558 for haem binding and assembly of succinate: quinone oxidoreductase (Complex II). Mol Microbiol **4**:1045-1056.

82. **Friedrich T., and D. Scheide.** 2000. The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. FEBS Lett **479**:1-5.

83. **Gabbianelli R., R. Scotti, S. Ammendola, P. Petrarca, L. Nicolini, and A. Battistoni.** 2011. Role of ZnuABC and ZinT in *Escherichia coli* O157:H7 zinc acquisition and interaction with epithelial cells. BMC Microbiol **11**:36.

84. **García-Calderón C. B., M. García-Quintanilla, J. Casadesús, and F. Ramos-Morales.** 2005. Virulence attenuation in *Salmonella enterica* rcsC mutants with constitutive activation of the Rcs system. Microbiology **151**:579-588.

85. **Gerken H., E. S. Charlson, E. M. Cicirelli, L. J. Kenney, and R. Misra.** 2009. MzrA: a novel modulator of the EnvZ/OmpR two-component regulon. Mol Microbiol **72**:1408-1422.

86. **Gervais F. G., and G. R. Drapeau.** 1992. Identification, cloning, and characterization of rcsF, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation *ftsZ84* in *Escherichia coli* K-12. J Bacteriol **174**:8016-8022.

87. **Ge X., R. Wang, J. Ma, Y. Liu, A. N. Ezemaduka, P. R. Chen, X. Fu, and Z. Chang.** 2013. DegP primarily functions as a protease for the biogenesis of β-barrel outer membrane proteins in the Gram-negative bacterium *Escherichia coli*. FEBS J **281**: 1226-40.

88. **Gold V. A. M., A. Robson, H. Bao, T. Romantsov, F. Duong, and I. Collinson.** 2010. The action of cardiolipin on the bacterial translocon. Proc Natl Acad Sci U S A **107**:10044-10049.
89. **Gotoh Y., Y. Eguchi, T. Watanabe, S. Okamoto, A. Doi, and R. Utsumi.** 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. Curr Opin Microbiol **13**:232-239.
90. **Gottesman S., and V. Stout.** 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. Mol Microbiol **5**:1599-1606.
91. **Gottesman S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J Bacteriol **162**:1111-1119.
92. **Gómez-Duarte O. G., and J. B. Kaper.** 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. Infect Immun **63**:1767-1776.
93. **Graham A. I., G. Sanguinetti, N. Bramall, C. W. McLeod, and R. K. Poole.** 2012. Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of the bacterial response to zinc reveals transient behaviour of the Fur and SoxS regulators. Microbiology **158**:284-292.
94. **Graham A. I., S. Hunt, S. L. Stokes, N. Bramall, J. Bunch, A. G. Cox, C. W. McLeod, and R. K. Poole.** 2009. Severe zinc depletion of *Escherichia coli*: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins. J Biol Chem **284**:18377-18389.
95. **Grass G., B. Fan, B. P. Rosen, S. Franke, D. H. Nies, and C. Rensing.** 2001. ZitB (YbgR), a member of the cation diffusion facilitator family, is an additional zinc transporter in *Escherichia coli*. J Bacteriol **183**:4664-4667.
96. **Grass G., C. Grosse, and D. H. Nies.** 2000. Regulation of the *cnr* cobalt and nickel resistance determinant from *Ralstonia* sp. strain CH34. J Bacteriol **182**:1390-1398.
97. **Grass G., K. Thakali, P. E. Klebba, D. Thieme, A. Müller, G. F. Wildner, and C. Rensing.** 2004. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. J Bacteriol **186**:5826-5833.
98. **Grass G., M. D. Wong, B. P. Rosen, R. L. Smith, and C. Rensing.** 2002. ZupT is a Zn(II) uptake system in *Escherichia coli*. J Bacteriol **184**:864-866.

99. **Grass G., S. Franke, N. Taudte, D. H. Nies, L. M. Kucharski, M. E. Maguire, and C. Rensing.** 2005. The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. J Bacteriol **187**:1604-1611.

100. **Green J., and J. R. Guest.** 1994. Regulation of transcription at the ndh promoter of *Escherichia coli* by FNR and novel factors. Mol Microbiol **12**:433-444.

101. **Green R. C., and A. J. Darwin.** 2004. PspG, a new member of the *Yersinia enterocolitica* phage shock protein regulon. J Bacteriol **186**:4910-4920.

102. **Grishin N. V.** 2000. C-terminal domains of *Escherichia coli* topoisomerase I belong to the zinc-ribbon superfamily. J Mol Biol **299**:1165-1177.

103. **Groisman E. A., C. Parra-Lopez, M. Salcedo, C. J. Lipps, and F. Heffron.** 1992. Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. Proc Natl Acad Sci U S A **89**:11939-11943.

104. **Guo M. S., T. B. Updegrove, E. B. Gogol, S. A. Shabalina, C. A. Gross, and G. Storz.** 2014. MicL, a new σE-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein. Genes Dev **28**:1620-1634.

105. **Gupta S., J. Chai, J. Cheng, R. D'Mello, M. R. Chance, and D. Fu.** 2014. Visualizing the kinetic power stroke that drives proton-coupled zinc(II) transport. Nature .

106. **Guzman L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177**:4121-4130.

107. **Haag L. -M., A. Fischer, B. Otto, R. Plickert, A. A. Kühl, U. B. Göbel, S. Bereswill, and M. M. Heimesaat.** 2012. Intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogate colonization resistance against *Campylobacter jejuni* in mice. PLoS One **7**:e35988.

108. **Halliwell B., and J. M. Gutteridge.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J **219**:1-14.

109. **Hantke K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol Gen Genet **182**:288-292.

110. **Hara H., Y. Nishimura, J. Kato, H. Suzuki, H. Nagasawa, A. Suzuki, and Y. Hirota.** 1989. Genetic analyses of processing involving C-terminal cleavage in penicillin-binding protein 3 of *Escherichia coli.* J Bacteriol **171**:5882-5889.

111. **Haurat M. F., J. Aduse-Opoku, M. Rangarajan, L. Dorobantu, M. R. Gray, M. A. Curtis, and M. F. Feldman.** 2011. Selective sorting of cargo proteins into bacterial membrane vesicles. J Biol Chem **286**:1269-1276.

112. **Hayes K. F., and S. J. Traina.** 1998. Metal Ion Speciation and its Significance in Ecosystem Health. Soil Chemistry and Ecosystem Health :45-84.

113. **Härd T., A. Rak, P. Allard, L. Kloo, and M. Garber.** 2000. The solution structure of ribosomal protein L36 from *Thermus thermophilus* reveals a zinc-ribbon-like fold. J Mol Biol **296**:169-180.

114. **Heddle J., D. J. Scott, S. Unzai, S. -Y. Park, and J. R. H. Tame.** 2003. Crystal structures of the liganded and unliganded nickel-binding protein NikA from *Escherichia coli*. J Biol Chem **278**:50322-50329.

115. **Helander I. M., Y. Kato, I. Kilpeläinen, R. Kostiainen, B. Lindner, K. Nummila, T. Sugiyama, and T. Yokochi.** 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. Eur J Biochem **237**:272-278.

116. **Higgins C. F., I. D. Hiles, K. Whalley, and D. J. Jamieson.** 1985. Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent transport systems. EMBO J **4**:1033-1039.

117. **Hirano Y., M. M. Hossain, K. Takeda, H. Tokuda, and K. Miki.** 2007. Structural studies of the Cpx pathway activator NlpE on the outer membrane of *Escherichia coli*. Structure **15**:963-976.

118. **Hoch E., W. Lin, J. Chai, M. Hershfinkel, D. Fu, and I. Sekler.** 2012. Histidine pairing at the metal transport site of mammalian ZnT transporters controls Zn^{2+} over Cd^{2+} selectivity. Proc Natl Acad Sci U S A **109**:7202-7207.

119. **Hood M. I., and E. P. Skaar.** 2012. Nutritional immunity: transition metals at the pathogen-host interface. Nat Rev Microbiol **10**:525-537.

120. **Hoyland C. N., C. Aldridge, R. M. Cleverley, M. -C. Duchêne, G. Minasov, O. Onopriyenko, K. Sidiq, P. J. Stogios, W. F. Anderson, R. A. Daniel, A. Savchenko, W. Vollmer, and R. J. Lewis.** 2014. Structure of the LdcB LD-carboxypeptidase reveals the molecular basis of peptidoglycan recognition. Structure **22**:949-960.

121. **Huang J. M., Y. F. Wei, Y. H. Kim, L. Osterberg, and H. R. Matthews.** 1991. Purification of a protein histidine kinase from the yeast *Saccharomyces cerevisiae*. The first member of this class of protein kinases. J Biol Chem **266**:9023-9031.

122. **Huja S., Y. Oren, D. Biran, S. Meyer, U. Dobrindt, J. Bernhard, D. Becher, M. Hecker, R. Sorek, and E. Z. Ron.** 2014. Fur is the master regulator of the extraintestinal pathogenic *Escherichia coli* response to serum. MBio **5**.

123. **Humphreys S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts.** 1999. The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella typhimurium*. Infect Immun **67**:1560-1568.

124. **Humphries R. M., T. P. Griener, S. L. Vogt, G. L. Mulvey, T. Raivio, M. S. Donnenberg, P. I. Kitov, M. Surette, and G. D. Armstrong.** 2010. N-acetyllactosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic *Escherichia coli*. Mol Microbiol **76**:1111-1126.

125. **Ilari A., F. Alaleona, G. Tria, P. Petrarca, A. Battistoni, C. Zamparelli, D. Verzili, M. Falconi, and E. Chiancone.** 2014. The *Salmonella enterica* ZinT structure, zinc affinity and interaction with the high-affinity uptake protein ZnuA provide insight into the management of periplasmic zinc. Biochim Biophys Acta **1840**:535-544.

126. **Imlay J. A.** 2006. Iron-sulphur clusters and the problem with oxygen. Mol Microbiol **59**:1073-1082.

127. **Isaac D. D., J. S. Pinkner, S. J. Hultgren, and T. J. Silhavy.** 2005. The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. Proc Natl Acad Sci U S A **102**:17775-17779.

128. **Iwig J. S., J. L. Rowe, and P. T. Chivers.** 2006. Nickel homeostasis in *Escherichia coli* - the *rcnR-rcnA* efflux pathway and its linkage to NikR function. Mol Microbiol **62**:252-262.

129. **Jacques J. -F., S. Jang, K. Prévost, G. Desnoyers, M. Desmarais, J. Imlay, and E. Massé.** 2006. RyhB small RNA modulates the free intracellular iron pool and is essential for normal growth during iron limitation in *Escherichia coli*. Mol Microbiol **62**:1181-1190.

130. **Janssen P. J., R. Van Houdt, H. Moors, P. Monsieurs, N. Morin, A. Michaux, M. A. Benotmane, N. Leys, T. Vallaeys, A. Lapidus, S. Monchy, C. Médigue, S. Taghavi, S. McCorkle, J. Dunn, D. van der Lelie, and M. Mergeay.** 2010. The complete genome sequence

of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. PLoS One **5**:e10433.

131. **Jarvis K. G., J. A. Girón, A. E. Jerse, T. K. McDaniel, M. S. Donnenberg, and J. B. Kaper.** 1995. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc Natl Acad Sci U S A **92**:7996-8000.

132. **Jerse A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci U S A **87**:7839-7843.

133. **Jiang J., X. Zhang, Y. Chen, Y. Wu, Z. H. Zhou, Z. Chang, and S. -F. Sui.** 2008. Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. Proc Natl Acad Sci U S A **105**:11939-11944.

134. **Jin T., and M. Inouye.** 1993. Ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the signaling domain of the hybrid *Escherichia coli* transmembrane receptor, Taz1. J Mol Biol **232**:484-492.

135. **Jones C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren.** 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J **16**:6394-6406.

136. **Jones S. E., L. J. Lloyd, K. K. Tan, and M. Buck.** 2003. Secretion defects that activate the phage shock response of *Escherichia coli*. J Bacteriol **185**:6707-6711.

137. **Jordan I., D. A. Natale, and M. Y. Galperin.** 2000. Copper chaperones in bacteria: association with copper-transporting ATPases. Trends in Biochemical Sciences **25**:480-481.

138. **Jovanovic G., L. Weiner, and P. Model.** 1996. Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress-induced psp operon. J Bacteriol **178**:1936-1945.

139. **Jovanovic G., P. Mehta, L. Ying, and M. Buck.** 2014. Anionic lipids and the cytoskeletal proteins MreB and RodZ define the spatio-temporal distribution and function of membrane stress controller PspA in *Escherichia coli*. Microbiology **160**:2374-2386.

140. **Jubelin G., A. Vianney, C. Beloin, J. -M. Ghigo, J. -C. Lazzaroni, P. Lejeune, and C. Dorel.** 2005. CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. J Bacteriol **187**:2038-2049.

141. **Kadurugamuwa J. L., and T. J. Beveridge.** 1996. Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. J Bacteriol **178**:2767-2774.

142. **Kammann M., J. Laufs, J. Schell, and B. Gronenborn.** 1989. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). Nucleic Acids Res **17**:5404.

143. **Karlinsey J. E., M. E. Maguire, L. A. Becker, M. -L. V. Crouch, and F. C. Fang.** 2010. The phage shock protein PspA facilitates divalent metal transport and is required for virulence of *Salmonella enterica* sv. Typhimurium. Mol Microbiol **78**:669-685.

144. **Karlsson M., K. Hannavy, and C. F. Higgins.** 1993. ExbB acts as a chaperone-like protein to stabilize TonB in the cytoplasm. Mol Microbiol **8**:389-396.

145. **Karsisiotis A. I., C. F. Damblon, and G. C. K. Roberts.** 2014. A variety of roles for versatile zinc in metallo-β-lactamases. Metallomics **6**:1181-1197.

146. **Kawahara K., H. Tsukano, H. Watanabe, B. Lindner, and M. Matsuura.** 2002. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. Infect Immun **70**:4092-4098.

147. **Keele B. B., J. M. McCord, and I. Fridovich.** 1970. Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. J Biol Chem **245**:6176-6181.

148. **Kenny B., and B. B. Finlay.** 1997. Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C-gamma1. Infect Immun **65**:2528-2536.

149. **Kershaw C. J., N. L. Brown, and J. L. Hobman.** 2007. Zinc dependence of *zinT* (*yodA*) mutants and binding of zinc, cadmium and mercury by ZinT. Biochem Biophys Res Commun **364**:66-71.

150. **Kershaw C. J., N. L. Brown, C. Constantinidou, M. D. Patel, and J. L. Hobman.** 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. Microbiology **151**:1187-1198.

151. **Kesty N. C., and M. J. Kuehn.** 2004. Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles. J Biol Chem **279**:2069-2076.

152. **Kim C., W. W. Lorenz, J. T. Hoopes, and J. F. Dean.** 2001. Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J Bacteriol **183**:4866-4875.

153. **Kim S., K. Watanabe, T. Shirahata, and M. Watarai.** 2004. Zinc uptake system (znuA locus) of *Brucella abortus* is essential for intracellular survival and virulence in mice. J Vet Med Sci **66**:1059-1063.

154. **Kirsten A., M. Herzberg, A. Voigt, J. Seravalli, G. Grass, J. Scherer, and D. H. Nies.** 2011. Contributions of five secondary metal uptake systems to metal homeostasis of *Cupriavidus metallidurans* CH34. J Bacteriol **193**:4652-4663.

155. **Kitagawa M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, and H. Mori.** 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**:291-299.

156. **Klein G., B. Lindner, W. Brabetz, H. Brade, and S. Raina.** 2009. *Escherichia coli* K-12 Suppressor-free Mutants Lacking Early Glycosyltransferases and Late Acyltransferases: minimal lipopolysaccharide structure and induction of envelope stress response. J Biol Chem **284**:15369-15389.

157. **Komeda H., M. Kobayashi, and S. Shimizu.** 1997. A novel transporter involved in cobalt uptake. Proc Natl Acad Sci U S A **94**:36-41.

158. **Koretke K. K., A. N. Lupas, P. V. Warren, M. Rosenberg, and J. R. Brown.** 2000. Evolution of two-component signal transduction. Mol Biol Evol **17**:1956-1970.

159. **Krojer T., J. Sawa, E. Schäfer, H. R. Saibil, M. Ehrmann, and T. Clausen.** 2008. Structural basis for the regulated protease and chaperone function of DegP. Nature **453**:885-890.

160. **Kumar A., S. Kumar, D. Kumar, A. Mishra, R. P. Dewangan, P. Shrivastava, S. Ramachandran, and B. Taneja.** 2013. The structure of Rv3717 reveals a novel amidase from *Mycobacterium tuberculosis*. Acta Crystallogr D Biol Crystallogr **69**:2543-2554.

161. **Kumazaki K., S. Chiba, M. Takemoto, A. Furukawa, K. I. Nishiyama, Y. Sugano, T. Mori, N. Dohmae, K. Hirata, Y. Nakada-Nakura, A. D. Maturana, Y. Tanaka, H. Mori, Y. Sugita, F. Arisaka, K. Ito, R. Ishitani, T. Tsukazaki, and O. Nureki.** 2014. Structural basis of Sec-independent membrane protein insertion by YidC. Nature **509**:516-520.

162. **Lanata C. F., C. L. Fischer-Walker, A. C. Olascoaga, C. X. Torres, M. J. Aryee, R. E. Black, and Child Health Epidemiology Reference Group of the World Health Organization and UNICEF.** 2013. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. PLoS One **8**:e72788.

163. **Williams, P. H., Evans, N., Turner, P., George, R. H., and A. S. McNeish**. 1977. Plasmid mediating mucosal adherence in human enteropathogenic *Escherichia coli.* Lancet **1**: 1151.

164. **Larsen R. A., M. G. Thomas, and K. Postle.** 1999. Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. Mol Microbiol **31**:1809-1824.

165. **Lasaro M., Z. Liu, R. Bishar, K. Kelly, S. Chattopadhyay, S. Paul, E. Sokurenko, J. Zhu, and M. Goulian.** 2014. *Escherichia coli* isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts. J Bacteriol **196**:1723-1732.

166. **De Las Peñas A., L. Connolly, and C. A. Gross.** 1997. The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. Mol Microbiol **24**:373-385.

167. **Laubacher M. E., and S. E. Ades.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J Bacteriol **190**:2065-2074.

168. **Leblanc S. K. D., C. W. Oates, and T. L. Raivio.** 2011. Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. J Bacteriol **193**:3367-3375.

169. **Lee J. -H., W. -S. Yeo, and J. -H. Roe.** 2004. Induction of the *sufA* operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: involvement of OxyR, IHF and an unidentified oxidant-responsive factor. Mol Microbiol **51**:1745-1755.

170. **Lee L. J., J. A. Barrett, and R. K. Poole.** 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J Bacteriol **187**:1124-1134.

171. **Lehninger A., Nelson, D. L., and M. M. Cox.** 2008. Lehninger Principles Of Biochemistry. W. H. Freeman and Sons. New York, New York.

172. **Leimbach A., J. Hacker, and U. Dobrindt.** 2013. *E. coli* as an all-rounder: the thin line between commensalism and pathogenicity. Curr Top Microbiol Immunol **358**:3-32.

173. **Leonhartsberger S., A. Huber, F. Lottspeich, and A. Böck.** 2001. The *hydH/G* Genes from *Escherichia coli* code for a zinc and lead responsive two-component regulatory system. J Mol Biol **307**:93-105.

174. **Lessard I. A., and C. T. Walsh.** 1999. Mutational analysis of active-site residues of the enterococcal D-ala-D-Ala dipeptidase VanX and comparison with *Escherichia coli* D-ala-D-Ala ligase and D-ala-D-Ala carboxypeptidase VanY. Chem Biol **6**:177-187.

175. **Levine M., D. Nalin, R. Hornick, E. Bergquist, D. Waterman, C. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. The Lancet **311**:1119-1122.

176. **Levine M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J Infect Dis **152**:550-559.

177. **Lewis C., H. Skovierova, G. Rowley, B. Rezuchova, D. Homerova, A. Stevenson, A. Sherry, J. Kormanec, and M. Roberts.** 2008. Small outer-membrane lipoprotein, SmpA, is regulated by sigmaE and has a role in cell envelope integrity and virulence of *Salmonella enterica* serovar Typhimurium. Microbiology **154**:979-988.

178. **Lewis D. A., J. Klesney-Tait, S. R. Lumbley, C. K. Ward, J. L. Latimer, C. A. Ison, and E. J. Hansen.** 1999. Identification of the *znuA*-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. Infect Immun **67**:5060-5068.

179. **Lima S., M. S. Guo, R. Chaba, C. A. Gross, and R. T. Sauer.** 2013. Dual molecular signals mediate the bacterial response to outer-membrane stress. Science **340**:837-841.

180. **Lin H., M. A. Fischbach, D. R. Liu, and C. T. Walsh.** 2005. In vitro characterization of salmochelin and enterobactin trilactone hydrolases IroD, IroE, and Fes. J Am Chem Soc **127**:11075-11084.

181. **Liu X., Q. Du, Z. Wang, D. Zhu, Y. Huang, N. Li, T. Wei, S. Xu, and L. Gu.** 2011. Crystal structure and biochemical features of EfeB/YcdB from *Escherichia coli* O157: ASP235 plays divergent roles in different enzyme-catalyzed processes. J Biol Chem **286**:14922-14931.

182. **Li W., Y. Liu, X. Sheng, P. Yin, F. Hu, Y. Liu, C. Chen, Q. Li, C. Yan, and J. Wang.** 2014. Structure and mechanism of a type III secretion protease, NleC. Acta Crystallogr D Biol Crystallogr **70**:40-47.

183. **Lloyd L. J., S. E. Jones, G. Jovanovic, P. Gyaneshwar, M. D. Rolfe, A. Thompson, J. C. Hinton, and M. Buck.** 2004. Identification of a new member of the phage shock protein response in Escherichia coli, the phage shock protein G (PspG). J Biol Chem **279**:55707-55714.

184. **Lobos S. R., and G. C. Mora.** 1991. Alteration in the electrophoretic mobility of OmpC due to variations in the ammonium persulfate concentration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis **12**:448-450.

185. **Lucarelli D., S. Russo, E. Garman, A. Milano, W. Meyer-Klaucke, and E. Pohl.** 2007. Crystal structure and function of the zinc uptake regulator FurB from *Mycobacterium tuberculosis*. J Biol Chem **282**:9914-9922.

186. **Lukat G. S., B. H. Lee, J. M. Mottonen, A. M. Stock, and J. B. Stock.** 1991. Roles of the highly conserved aspartate and lysine residues in the response regulator of bacterial chemotaxis. J Biol Chem **266**:8348-8354.

187. **Lu M., and D. Fu.** 2007. Structure of the zinc transporter YiiP. Science **317**:1746-1748.

188. **Lu M., J. Chai, and D. Fu.** 2009. Structural basis for autoregulation of the zinc transporter YiiP. Nat Struct Mol Biol **16**:1063-1067.

189. **Maciąg A., E. Dainese, G. M. Rodriguez, A. Milano, R. Provvedi, M. R. Pasca, I. Smith, G. Palù, G. Riccardi, and R. Manganelli.** 2007. Global Analysis of the *Mycobacterium tuberculosis* Zur (FurB) Regulon. J Bacteriol **189**:730-740.

190. **MacRitchie D. M., J. D. Ward, A. Z. Nevesinjac, and T. L. Raivio.** 2008. Activation of the Cpx Envelope Stress Response Down-Regulates Expression of Several Locus of Enterocyte Effacement-Encoded Genes in Enteropathogenic *Escherichia coli*. Infect Immun **76**:1465-1475.

191. **MacRitchie D. M., N. Acosta, and T. L. Raivio.** 2012. DegP is involved in Cpx-mediated posttranscriptional regulation of the type III secretion apparatus in enteropathogenic *Escherichia coli*. Infect Immun **80**:1766-1772.

192. **Mahoney T. F., and T. J. Silhavy.** 2013. The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. J Bacteriol **195**:1869-1874.

193. **Majdalani N., and S. Gottesman.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu Rev Microbiol **59**:379-405.

194. **Makui H., E. Roig, S. T. Cole, J. D. Helmann, P. Gros, and M. F. Cellier.** 2000. Identification of the *Escherichia coli* K-12 Nramp orthologue (MntH) as a selective divalent metal ion transporter. Mol Microbiol **35**:1065-1078.

195. **Malpica R., B. Franco, C. Rodriguez, O. Kwon, and D. Georgellis.** 2004. Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc Natl Acad Sci U S A **101**:13318-13323.

196. **Manganelli R., M. I. Voskuil, G. K. Schoolnik, and I. Smith.** 2001. The *Mycobacterium tuberculosis* ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol Microbiol **41**:423-437.

197. **Maret W.** 2004. Zinc and sulfur: a critical biological partnership. Biochemistry **43**:3301-3309.

198. **Martinez J. L., M. B. Sánchez, L. Martínez-Solano, A. Hernandez, L. Garmendia, A. Fajardo, and C. Alvarez-Ortega.** 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev **33**:430-449.

199. **Martino L., Y. He, K. L. D. Hands-Taylor, E. R. Valentine, G. Kelly, C. Giancola, and M. R. Conte.** 2009. The interaction of the *Escherichia coli* protein SlyD with nickel ions illuminates the mechanism of regulation of its peptidyl-prolyl isomerase activity. FEBS J **276**:4529-4544.

200. **Massé E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A **99**:4620-4625.

201. **Massé E., C. K. Vanderpool, and S. Gottesman.** 2005. Effect of RyhB Small RNA on Global Iron Use in *Escherichia coli*. J Bacteriol **187**:6962-6971.

202. **Massé E., F. E. Escorcia, and S. Gottesman.** 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes Dev **17**:2374-2383.

203. **Matsushita K., T. Ohnishi, and H. R. Kaback.** 1987. NADH-ubiquinone oxidoreductases of the *Escherichia coli* aerobic respiratory chain. Biochemistry **26**:7732-7737.

204. **Matsuyama S., T. Tajima, and H. Tokuda.** 1995. A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. EMBO J **14**:3365-3372.

205. **Maurer L. M., E. Yohannes, S. S. Bondurant, M. Radmacher, and J. L. Slonczewski.** 2005. pH Regulates Genes for Flagellar Motility, Catabolism, and Oxidative Stress in *Escherichia coli* K-12. J Bacteriol **187**:304-319.

206. **Maxson M. E., and A. J. Darwin.** 2004. Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the RpoE and Cpx extracytoplasmic stress responses. J Bacteriol **186**:4199-4208.
207. **Maxson M. E., and A. J. Darwin.** 2006. PspB and PspC of *Yersinia enterocolitica* are dual function proteins: regulators and effectors of the phage-shock-protein response. Mol Microbiol **59**:1610-1623.
208. **Ma Z., M. J. Faulkner, and J. D. Helmann.** 2012. Origins of specificity and cross-talk in metal ion sensing by *Bacillus subtilis* Fur. Mol Microbiol **86**:1144-1155.
209. **Ma Z., P. Chandrangsu, T. C. Helmann, A. Romsang, A. Gaballa, and J. D. Helmann.** 2014. Bacillithiol is a major buffer of the labile zinc pool in *Bacillus subtilis*. Mol Microbiol .
210. **McBroom A. J., A. P. Johnson, S. Vemulapalli, and M. J. Kuehn.** 2006. Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. J Bacteriol **188**:5385-5392.
211. **McCaig W. D., A. Koller, and D. G. Thanassi.** 2013. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. J Bacteriol **195**:1120-1132.
212. **McDevitt C. A., A. D. Ogunniyi, E. Valkov, M. C. Lawrence, B. Kobe, A. G. McEwan, and J. C. Paton.** 2011. A molecular mechanism for bacterial susceptibility to zinc. PLoS Pathog **7**:e1002357.
213. **McEwen J., and P. Silverman.** 1980. Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions. Proc Natl Acad Sci U S A **77**:513-517.
214. **McMahon K. J., M. E. Castelli, E. García Vescovi, and M. F. Feldman.** 2012. Biogenesis of outer membrane vesicles in *Serratia marcescens* is thermoregulated and can be induced by activation of the Rcs phosphorelay system. J Bacteriol **194**:3241-3249.
215. **Mellies J. L., K. Thomas, M. Turvey, N. R. Evans, J. Crane, E. Boedeker, and G. C. Benison.** 2012. Zinc-induced envelope stress diminishes type III secretion in enteropathogenic *Escherichia coli*. BMC Microbiol **12**:123.
216. **Mellies J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper.** 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Mol Microbiol **33**:296-306.

217. **Mellin J. R., M. Koutero, D. Dar, M. -A. Nahori, R. Sorek, and P. Cossart.** 2014. Riboswitches. Sequestration of a two-component response regulator by a riboswitch-regulated noncoding RNA. Science **345**:940-943.

218. **Michelitsch M. D., and J. S. Weissman.** 2000. A census of glutamine/asparagine-rich regions: Implications for their conserved function and the prediction of novel prions. Proc Natl Acad Sci U S A **97**:11910-11915.

219. **Miles S., B. M. Carpenter, H. Gancz, and D. S. Merrell.** 2010. *Helicobacter pylori* apo-Fur regulation appears unconserved across species. J Microbiol **48**:378-386.

220. **Mileykovskaya E., and W. Dowhan.** 1997. The Cpx two-component signal transduction pathway is activated in *Escherichia coli* mutant strains lacking phosphatidylethanolamine. J Bacteriol **179**:1029-1034.

221. **Missiakas D., M. P. Mayer, M. Lemaire, C. Georgopoulos, and S. Raina.** 1997. Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. Mol Microbiol **24**:355-371.

222. **Mogi T., H. Nakamura, and Y. Anraku.** 1994. Molecular structure of a heme-copper redox center of the *Escherichia coli* ubiquinol oxidase: evidence and model. J Biochem **116**:471-477.

223. **Morel F. M., R. J. Hudson, and N. M. Price.** 1991. Limitation of productivity by trace metals in the sea. Limnology and Oceanography **36**:1742-1755.

224. **Moschen A. R., V. Wieser, and H. Tilg.** 2012. Dietary Factors: Major Regulators of the Gut's Microbiota. Gut Liver **6**:411-416.

225. **Munson G. P., D. L. Lam, F. W. Outten, and T. V. O'Halloran.** 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J Bacteriol **182**:5864-5871.

226. **Mühlenhoff U., and R. Lill.** 2000. Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. Biochim Biophys Acta **1459**:370-382.

227. **Nakayama S., and H. Watanabe.** 1995. Involvement of *cpxA*, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of *Shigella sonnei virF* gene. J Bacteriol **177**:5062-5069.

228. **Nanninga N.** 1970. Ultrastructure of the cell envelope of *Escherichia coli* B after freeze-etching. J Bacteriol **101**:297-303.

229. **Nataro J. P., I. C. Scaletsky, J. B. Kaper, M. M. Levine, and L. R. Trabulsi.** 1985. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. Infect Immun **48**:378-383.

230. **Navarro C., L. F. Wu, and M. A. Mandrand-Berthelot.** 1993. The *nik* operon of *Escherichia coli* encodes a periplasmic binding-protein-dependent transport system for nickel. Mol Microbiol **9**:1181-1191.

231. **Nevesinjac A. Z., and T. L. Raivio.** 2005. The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic *Escherichia coli*. J Bacteriol **187**:672-686.

232. **Nicholas, D. J., Kobayashi, M., and P. W. Wilson.** 1962. Cobalt requirement for inorganic nitrogen metabolism in microorganisms. Proc Natl Acad Sci U S A **48**:1537-1542.

233. **Nies D. H.** 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev **27**:313-339.

234. **Nishino K., E. Nikaido, and A. Yamaguchi.** 2007. Regulation of multidrug efflux systems involved in multidrug and metal resistance of *Salmonella enterica* serovar Typhimurium. J Bacteriol **189**:9066-9075.

235. **Nishino K., T. Honda, and A. Yamaguchi.** 2005. Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system. J Bacteriol **187**:1763-1772.

236. **Nolan K. J., D. J. McGee, H. M. Mitchell, T. Kolesnikow, J. M. Harro, J. O'Rourke, J. E. Wilson, S. J. Danon, N. D. Moss, H. L. T. Mobley, and A. Lee.** 2002. In vivo behavior of a *Helicobacter pylori* SS1 nixA mutant with reduced urease activity. Infect Immun **70**:685-691.

237. **Noll M., K. Petrukhin, and S. Lutsenko.** 1998. Identification of a novel transcription regulator from *Proteus mirabilis*, PMTR, revealed a possible role of YJAI protein in balancing zinc in *Escherichia coli*. J Biol Chem **273**:21393-21401.

238. **Novick R. P., and C. Roth.** 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J Bacteriol **95**:1335-1342.

239. **Odermatt A., H. Suter, R. Krapf, and M. Solioz.** 1993. Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. J Biol Chem **268**:12775-12779.

240. **Ogunniyi A. D., L. K. Mahdi, M. P. Jennings, A. G. McEwan, C. A. McDevitt, M. B. Van der Hoek, C. J. Bagley, P. Hoffmann, K. A. Gould, and J. C. Paton.** 2010. Central role

of manganese in regulation of stress responses, physiology, and metabolism in *Streptococcus pneumoniae*. J Bacteriol **192**:4489-4497.

241. **Ollagnier-de-Choudens S., T. Mattioli, Y. Takahashi, and M. Fontecave.** 2001. Iron-Sulfur Cluster Assembly. J Biol Chem **276**:22604-22607.

242. **Onufryk C., M. -L. Crouch, F. C. Fang, and C. A. Gross.** 2005. Characterization of six lipoproteins in the sigmaE regulon. J Bacteriol **187**:4552-4561.

243. **Otto K., and T. J. Silhavy.** 2002. Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. Proc Natl Acad Sci U S A **99**:2287-2292.

244. **Ouellet H., Y. Ouellet, C. Richard, M. Labarre, B. Wittenberg, J. Wittenberg, and M. Guertin.** 2002. Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. Proc Natl Acad Sci U S A **99**:5902-5907.

245. **Outten C. E., F. W. Outten, and T. V. O'Halloran.** 1999. DNA Distortion Mechanism for Transcriptional Activation by ZntR, a Zn(II)-responsive MerR Homologue in *Escherichia coli*. J Biol Chem **274**:37517-37524.

246. **Outten F. W., D. L. Huffman, J. A. Hale, and T. V. O'Halloran.** 2001. The Independent *cue* and *cus* Systems Confer Copper Tolerance during Aerobic and Anaerobic Growth in *Escherichia coli*. J Biol Chem **276**:30670-30677.

247. **Outten F. W., O. Djaman, and G. Storz.** 2004. A *suf* operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. Mol Microbiol **52**:861-872.

248. **Padilla-Benavides T., A. M. George Thompson, M. M. McEvoy, and J. M. Argüello.** 2014. Mechanism of ATPase-mediated Cu+ Export and Delivery to Periplasmic Chaperones: the interaction of *Escherichia coli* CopA AND CusF. J Biol Chem **289**:20492-20501.

249. **Panina E. M., A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc Natl Acad Sci U S A **100**:9912-9917.

250. **Pasquali P., S. Ammendola, C. Pistoia, P. Petrucci, M. Tarantino, C. Valente, M. L. Marenzoni, G. Rotilio, and A. Battistoni.** 2008. Attenuated *Salmonella enterica* serovar Typhimurium lacking the ZnuABC transporter confers immune-based protection against challenge infections in mice. Vaccine **26**:3421-3426.

251. **Patzer S. I., and K. Hantke.** 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol Microbiol **28**:1199-1210.

252. **Patzer S. I., and K. Hantke.** 2000. The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. J Biol Chem **275**:24321-24332.

253. **Patzer S. I., and K. Hantke.** 2001. Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the *mntH* gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. J Bacteriol **183**:4806-4813.

254. **Pawlik M. -C., K. Hubert, B. Joseph, H. Claus, C. Schoen, and U. Vogel.** 2012. The zinc-responsive regulon of Neisseria meningitidis comprises 17 genes under control of a Zur element. J Bacteriol **194**:6594-6603.

255. **Pecqueur L., B. D'Autréaux, J. Dupuy, Y. Nicolet, L. Jacquamet, B. Brutscher, I. Michaud-Soret, and B. Bersch.** 2006. Structural changes of *Escherichia coli* ferric uptake regulator during metal-dependent dimerization and activation explored by NMR and X-ray crystallography. J Biol Chem **281**:21286-21295.

256. **Pennartz A., C. Généreux, C. Parquet, D. Mengin-Lecreulx, and B. Joris.** 2009. Substrate-induced inactivation of the *Escherichia coli* AmiD N-acetylmuramoyl-L-alanine amidase highlights a new strategy to inhibit this class of enzyme. Antimicrob Agents Chemother **53**:2991-2997.

257. **Perrin C., R. Briandet, G. Jubelin, P. Lejeune, M. -A. Mandrand-Berthelot, A. Rodrigue, and C. Dorel.** 2009. Nickel promotes biofilm formation by *Escherichia coli* K-12 strains that produce curli. Appl Environ Microbiol **75**:1723-1733.

258. **Pesciaroli M., F. Aloisio, S. Ammendola, C. Pistoia, P. Petrucci, M. Tarantino, M. Francia, A. Battistoni, and P. Pasquali.** 2011. An attenuated *Salmonella enterica* serovar Typhimurium strain lacking the ZnuABC transporter induces protection in a mouse intestinal model of *Salmonella infection*. Vaccine **29**:1783-1790.

259. **Petersen C., and L. B. Møller.** 2000. Control of copper homeostasis in *Escherichia coli* by a P-type ATPase, CopA, and a MerR-like transcriptional activator, CopR. Gene **261**:289-298.

260. **Petrarca P., S. Ammendola, P. Pasquali, and A. Battistoni.** 2010. The Zur-regulated ZinT protein is an auxiliary component of the high-affinity ZnuABC zinc transporter that facilitates metal recruitment during severe zinc shortage. J Bacteriol **192**:1553-1564.

261. **Piddock L. J. V.** 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol **4**:629-636.

262. **Pierce J. R., and C. F. Earhart.** 1986. *Escherichia coli* K-12 envelope proteins specifically required for ferrienterobactin uptake. J Bacteriol **166**:930-936.

263. **De Pina K., V. Desjardin, M. A. Mandrand-Berthelot, G. Giordano, and L. F. Wu.** 1999. Isolation and characterization of the *nikR* gene encoding a nickel-responsive regulator in *Escherichia coli*. J Bacteriol **181**:670-674.

264. **Pohl E., J. C. Haller, A. Mijovilovich, W. Meyer-Klaucke, E. Garman, and M. L. Vasil.** 2003. Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol Microbiol **47**:903-915.

265. **Pollack J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem Biophys Res Commun **38**:989-992.

266. **Pontel L. B., N. L. Scampoli, S. Porwollik, S. K. Checa, M. McClelland, and F. C. Soncini.** 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**:1659-1669.

267. **Posey J. E., and F. C. Gherardini.** 2000. Lack of a role for iron in the Lyme disease pathogen. Science **288**:1651-1653.

268. **Prasad A. S.** 2007. Zinc: mechanisms of host defense. J Nutr **137**:1345-1349.

269. **Price C. E., and A. J. Driessen.** 2010. Biogenesis of membrane bound respiratory complexes in *Escherichia coli*. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1803**:748-766.

270. **Price N. L., and T. L. Raivio.** 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J Bacteriol **191**:1798-1815.

271. **Prigent-Combaret C., E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, and C. Dorel.** 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. J Bacteriol **183**:7213-7223.

272. **Kihara, D.** 2011. Protein Function Detection for Omics Era. Springer. Dordrecht, New York.

273. **Quan S., L. Wang, E. V. Petrotchenko, K. A. Makepeace, S. Horowitz, J. Yang, Y. Zhang, C. H. Borchers, and J. C. Bardwell.** 2014. Super Spy variants implicate flexibility in chaperone action. Elife **3**:e01584.

274. **Quan S., P. Koldewey, T. Tapley, N. Kirsch, K. M. Ruane, J. Pfizenmaier, R. Shi, S. Hofmann, L. Foit, G. Ren, U. Jakob, Z. Xu, M. Cygler, and J. C. A. Bardwell.** 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat Struct Mol Biol **18**:262-269.

275. **Raffa R. G., and T. L. Raivio.** 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol Microbiol **45**:1599-1611.

276. **Raivio T. L., and T. J. Silhavy.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol **179**:7724-7733.

277. **Raivio T. L., and T. J. Silhavy.** 2001. Periplasmic stress and ECF sigma factors. Annual Reviews in Microbiology **55**:591-624.

278. **Raivio T. L., D. L. Popkin, and T. J. Silhavy.** 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. J Bacteriol **181**:5263-5272.

279. **Raivio T. L., M. W. Laird, J. C. Joly, and T. J. Silhavy.** 2000. Tethering of CpxP to the inner membrane prevents spheroplast induction of the cpx envelope stress response. Mol Microbiol **37**:1186-1197.

280. **Raivio T. L., S. K. D. Leblanc, and N. L. Price.** 2013. The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. J Bacteriol **195**:2755-2767.

281. **Ramer S. W., D. Bieber, and G. K. Schoolnik.** 1996. BfpB, an outer membrane lipoprotein required for the biogenesis of bundle-forming pili in enteropathogenic *Escherichia coli*. J Bacteriol **178**:6555-6563.

282. **Rensing C., B. Fan, R. Sharma, B. Mitra, and B. P. Rosen.** 2000. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci U S A **97**:652-656.

283. **Rensing C., B. Mitra, and B. P. Rosen.** 1997. The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. Proc Natl Acad Sci U S A **94**:14326-14331.

284. **Rigel N. W., and T. J. Silhavy.** 2012. Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria. Curr Opin Microbiol **15**:189-193.

285. **Roberts S. A., A. Weichsel, G. Grass, K. Thakali, J. T. Hazzard, G. Tollin, C. Rensing, and W. R. Montfort.** 2002. Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. Proc Natl Acad Sci U S A **99**:2766-2771.

286. **Rodrigue A., G. Effantin, and M. -A. Mandrand-Berthelot.** 2005. Identification of *rcnA* (*yohM*), a nickel and cobalt resistance gene in *Escherichia coli*. J Bacteriol **187**:2912-2916.
287. **Rosner J. L., and R. G. Martin.** 2013. Reduction of cellular stress by TolC-dependent efflux pumps in *Escherichia coli* indicated by BaeSR and CpxARP activation of *spy* in efflux mutants. J Bacteriol **195**:1042-1050.
288. **Rouvière P. E., and C. A. Gross.** 1996. SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. Genes Dev **10**:3170-3182.
289. **Rowland J. L., and M. Niederweis.** 2012. Resistance mechanisms of *Mycobacterium tuberculosis* against phagosomal copper overload. Tuberculosis (Edinburgh) **92**:202-210.
290. **Rowland J. L., and M. Niederweis.** 2013. A multicopper oxidase is required for copper resistance in *Mycobacterium tuberculosis*. J Bacteriol **195**:3724-3733.
291. **Saier M. H., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol **11**:841-847.
292. **Scherer J., and D. H. Nies.** 2009. CzcP is a novel efflux system contributing to transition metal resistance in *Cupriavidus metallidurans* CH34. Mol Microbiol **73**:601-621.
293. **Schmelcher M., F. Waldherr, and M. J. Loessner.** 2012. *Listeria* bacteriophage peptidoglycan hydrolases feature high thermoresistance and reveal increased activity after divalent metal cation substitution. Appl Microbiol Biotechnol **93**:633-643.
294. **Schmidt S. A., D. Bieber, S. W. Ramer, J. Hwang, C. Y. Wu, and G. Schoolnik.** 2001. Structure-function analysis of BfpB, a secretin-like protein encoded by the bundle-forming-pilus operon of enteropathogenic *Escherichia coli.* J Bacteriol **183**:4848-4859.
295. **Schoolnik G. K.** 1993. Intimin and the intimate attachment of bacteria to human cells. The Journal of Clinical Investigation **92**:1117-1118.
296. **Schröder J., N. Jochmann, D. A. Rodionov, and A. Tauch.** 2010. The Zur regulon of *Corynebacterium glutamicum* ATCC 13032. BMC Genomics **11**:12.
297. **Schulte M., D. Mattay, S. Kriegel, P. Hellwig, and T. Friedrich.** 2014. Inhibition of the *Escherichia coli* respiratory complex I by Zn2+. Biochemistry .

298. **Seiler C., and T. U. Berendonk.** 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol **3**:399.

299. **Sein-Echaluce V. C., A. González, M. Napolitano, I. Luque, F. Barja, M. L. Peleato, and M. F. Fillat.** 2014. Zur (FurB) is a key factor in the control of the oxidative stress response in *Anabaena* sp. PCC 7120. Environ Microbiol. EPub ahead of print DOI: 10.1111/1462-2920.12628.

300. **Seo S. W., D. Kim, H. Latif, E. J. O'Brien, R. Szubin, and B. O. Palsson.** 2014. Deciphering Fur transcriptional regulatory network highlights its complex role beyond iron metabolism in *Escherichia coli*. Nat Commun **5**:4910.

301. **Sham L. -T., E. K. Butler, M. D. Lebar, D. Kahne, T. G. Bernhardt, and N. Ruiz.** 2014. Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. Science **345**:220-222.

302. **Shen Q. -T., X. -C. Bai, L. -F. Chang, Y. Wu, H. -W. Wang, and S. -F. Sui.** 2009. Bowl-shaped oligomeric structures on membranes as DegP's new functional forms in protein quality control. Proc Natl Acad Sci U S A **106**:4858-4863.

303. **Shida T., H. Hattori, F. Ise, and J. Sekiguchi.** 2001. Mutational analysis of catalytic sites of the cell wall lytic N-acetylmuramoyl-L-alanine amidases CwlC and CwlV. J Biol Chem **276**:28140-28146.

304. **Shin J. -H., H. J. Jung, Y. J. An, Y. -B. Cho, S. -S. Cha, and J. -H. Roe.** 2011. Graded expression of zinc-responsive genes through two regulatory zinc-binding sites in Zur. Proc Natl Acad Sci U S A **108**:5045-5050.

305. **Silber K. R., K. C. Keiler, and R. T. Sauer.** 1992. Tsp: a tail-specific protease that selectively degrades proteins with nonpolar C termini. Proc Natl Acad Sci U S A **89**:295-299.

306. **Silhavy T. J., D. Kahne, and S. Walker.** 2010. The Bacterial Cell Envelope. Cold Spring Harbor Perspectives in Biology **2**:a000414.

307. **Simmonds S., and N. O. Toye.** 1967. The role of metal ions in the peptidase activity of *Escherichia coli* K-12. J Biol Chem **242**:2086-2093.

308. **Skare J. T., and K. Postle.** 1991. Evidence for a TonB-dependent energy transduction complex in *Escherichia coli*. Mol Microbiol **5**:2883-2890.

309. **Slamti L., and M. K. Waldor.** 2009. Genetic analysis of activation of the *Vibrio cholerae* Cpx pathway. J Bacteriol **191**:5044-5056.
310. **Slauch J. M., and T. J. Silhavy.** 1991. Genetic fusions as experimental tools. Methods Enzymol **204**:213-248.
311. **Sledjeski D. D., and S. Gottesman.** 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J Bacteriol **178**:1204-1206.
312. **Smith H. K., M. Shepherd, C. Monk, J. Green, and R. K. Poole.** 2011. The NO-responsive hemoglobins of *Campylobacter jejuni*: Concerted responses of two globins to NO and evidence in vitro for globin regulation by the transcription factor NssR. Nitric Oxide **25**:234-241.
313. **Snyder W. B., L. J. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy.** 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. J Bacteriol **177**:4216-4223.
314. **Sobota J. M., and J. A. Imlay.** 2011. Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. Proc Natl Acad Sci U S A **108**:5402-5407.
315. **Sohn J., R. A. Grant, and R. T. Sauer.** 2007. Allosteric activation of DegS, a stress sensor PDZ protease. Cell **131**:572-583.
316. **Sperandeo P., R. Cescutti, R. Villa, C. Di Benedetto, D. Candia, G. Dehò, and A. Polissi.** 2007. Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. J Bacteriol **189**:244-253.
317. **Spiess C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97**:339-347.
318. **Spiro S., R. E. Roberts, and J. R. Guest.** 1989. FNR-dependent repression of the *ndh* gene of *Escherichia coli* and metal ion requirement for FNR-regulated gene expression. Mol Microbiol **3**:601-608.
319. **Stafford S. L., N. J. Bokil, M. E. S. Achard, R. Kapetanovic, M. A. Schembri, A. G. McEwan, and M. J. Sweet.** 2013. Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper. Biosci Rep **33**.
320. **Stähler F. N., S. Odenbreit, R. Haas, J. Wilrich, A. H. M. Van Vliet, J. G. Kusters, M. Kist, and S. Bereswill.** 2006. The novel *Helicobacter pylori* CznABC metal efflux pump is

required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. Infect Immun **74**:3845-3852.

321. **Stephens D. L., M. D. Choe, and C. F. Earhart.** 1995. *Escherichia coli* periplasmic protein FepB binds ferrienterobactin. Microbiology **141 (Pt 7)**:1647-1654.

322. **Stohl E. A., E. M. Dale, A. K. Criss, and H. S. Seifert.** 2013. *Neisseria gonorrhoeae* metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H2O2- and neutrophil-mediated killing. MBio **4**.

323. **Storz G., Y. I. Wolf, and K. S. Ramamurthi.** 2014. Small proteins can no longer be ignored. Annu Rev Biochem **83**:753-777.

324. **Stout V., and S. Gottesman.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J Bacteriol **172**:659-669.

325. **Stoyanov J. V., J. L. Hobman, and N. L. Brown.** 2001. CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. Mol Microbiol **39**:502-511.

326. **Takahashi Y., and M. Nakamura.** 1999. Functional assignment of the ORF2-*iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 gene cluster involved in the assembly of Fe-S clusters in *Escherichia coli*. J Biochem **126**:917-926.

327. **Takahashi Y., and U. Tokumoto.** 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem **277**:28380-28383.

328. **Tam C., and D. Missiakas.** 2005. Changes in lipopolysaccharide structure induce the sigma(E)-dependent response of *Escherichia coli*. Mol Microbiol **55**:1403-1412.

329. **Tang D. -J., X. -J. Li, Y. -Q. He, J. -X. Feng, B. Chen, and J. -L. Tang.** 2005. The zinc uptake regulator Zur is essential for the full virulence of *Xanthomonas campestris* pv. campestris. Mol Plant Microbe Interact **18**:652-658.

330. **Tao K., S. -I. Narita, and H. Tokuda.** 2012. Defective lipoprotein sorting induces lolA expression through the Rcs stress response phosphorelay system. J Bacteriol **194**:3643-3650.

331. **Taylor D. L., X. R. Bina, L. Slamti, M. K. Waldor, and J. E. Bina.** 2014. Reciprocal regulation of resistance-nodulation-division efflux systems and the Cpx two-component system in *Vibrio cholerae*. Infect Immun **82**:2980-2991.

332. **Thede G. L., D. C. Arthur, R. A. Edwards, D. R. Buelow, J. L. Wong, T. L. Raivio, and J. N. M. Glover.** 2011. Structure of the periplasmic stress response protein CpxP. J Bacteriol **193**:2149-2157.

333. **Thompson K. M., V. A. Rhodius, and S. Gottesman.** 2007. SigmaE regulates and is regulated by a small RNA in *Escherichia coli.* J Bacteriol **189**:4243-4256.

334. **Thöny-Meyer L., P. James, and H. Hennecke.** 1991. From one gene to two proteins: the biogenesis of cytochromes b and c1 in *Bradyrhizobium japonicum*. Proc Natl Acad Sci U S A **88**:5001-5005.

335. **Tibazarwa C., S. Wuertz, M. Mergeay, L. Wyns, and D. van Der Lelie.** 2000. Regulation of the *cnr* cobalt and nickel resistance determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34. J Bacteriol **182**:1399-1409.

336. **Tokumoto U., and Y. Takahashi.** 2001. Genetic analysis of the isc operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. J Biochem **130**:63-71.

337. **Tottey S., S. A. M. Rondet, G. P. M. Borrelly, P. J. Robinson, P. R. Rich, and N. J. Robinson.** 2002. A Copper Metallochaperone for Photosynthesis and Respiration Reveals Metal-specific Targets, Interaction with an Importer, and Alternative Sites for Copper Acquisition. J Biol Chem **277**:5490-5497.

338. **Trepreau J., E. Girard, A. P. Maillard, E. de Rosny, I. Petit-Haertlein, R. Kahn, and J. Covès.** 2011. Structural basis for metal sensing by CnrX. J Mol Biol **408**:766-779.

339. **Troxell B., and X. F. Yang.** 2013. Metal-dependent gene regulation in the causative agent of Lyme disease. Front Cell Infect Microbiol **3**:79.

340. **Tschauner K., P. Hörnschemeyer, V. S. Müller, and S. Hunke.** 2014. Dynamic Interaction between the CpxA Sensor Kinase and the Periplasmic Accessory Protein CpxP Mediates Signal Recognition in E. coli. PLoS One **9**:e107383.

341. **Tsiboli P., D. Triantafillidou, F. Franceschi, and T. Choli-Papadopoulou.** 1998. Studies on the Zn-containing S14 ribosomal protein from *Thermus thermophilus*. Eur J Biochem **256**:136-141.

342. **Tsubaki M., T. Mogi, H. Hori, M. Sato-Watanabe, and Y. Anraku.** 1996. Infrared and EPR studies on cyanide binding to the heme-copper binuclear center of cytochrome bo-type ubiquinol oxidase from *Escherichia coli*. Release of a CuB-cyano complex in the partially reduced state. J Biol Chem **271**:4017-4022.

[NO STYLE for: Rae 1999].

344. **Vance C. K., and A. F. Miller.** 2001. Novel insights into the basis for Escherichia coli superoxide dismutase's metal ion specificity from Mn-substituted FeSOD and its very high E(m). Biochemistry **40**:13079-13087.

345. **Vega D. E., and K. D. Young.** 2014. Accumulation of periplasmic enterobactin impairs the growth and morphology of Escherichia coli tolC mutants. Mol Microbiol **91**:508-521.

346. **Vinogradov S. N., D. Hoogewijs, X. Bailly, R. Arredondo-Peter, J. Gough, S. Dewilde, L. Moens, and J. R. Vanfleteren.** 2006. A phylogenomic profile of globins. BMC Evolutionary Biology **6**:31.

347. **Vogel J., and K. Papenfort.** 2006. Small non-coding RNAs and the bacterial outer membrane. Curr Opin Microbiol **9**:605-611.

348. **Vogt S. L., A. Z. Nevesinjac, R. M. Humphries, M. S. Donnenberg, G. D. Armstrong, and T. L. Raivio.** 2010. The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic Escherichia coli bundle-forming pilus. Mol Microbiol **76**:1095-1110.

349. **Wagner D., J. Maser, I. Moric, N. Boechat, S. Vogt, B. Gicquel, B. Lai, J. -M. Reyrat, and L. Bermudez.** 2005. Changes of the phagosomal elemental concentrations by Mycobacterium tuberculosis Mramp. Microbiology **151**:323-332.

350. **Waldron K. J., and N. J. Robinson.** 2009. How do bacterial cells ensure that metalloproteins get the correct metal? Nat Rev Microbiol **7**:25-35.

351. **Wang C. C., and A. Newton.** 1971. An additional step in the transport of iron defined by the tonB locus of Escherichia coli. J Biol Chem **246**:2147-2151.

352. **Wang D., and C. A. Fierke.** 2013. The BaeSR regulon is involved in defense against zinc toxicity in E. coli. Metallomics **5**:372-383.

353. **Wang D., O. Hosteen, and C. A. Fierke.** 2012. ZntR-mediated transcription of zntA responds to nanomolar intracellular free zinc. J Inorg Biochem **111**:173-181.

354. **Wang D., S. S. Korban, and Y. Zhao.** 2009. The Rcs phosphorelay system is essential for pathogenicity in Erwinia amylovora. Mol Plant Pathol **10**:277-290.

355. **Wang K., O. Sitsel, G. Meloni, H. E. Autzen, M. Andersson, T. Klymchuk, A. M. Nielsen, D. C. Rees, P. Nissen, and P. Gourdon.** 2014. Structure and mechanism of Zn(2+)-transporting P-type ATPases. Nature .

356. **Wapnir R. A.** 2000. Zinc deficiency, malnutrition and the gastrointestinal tract. J Nutr **130**:1388S-1392S.

357. **Weidner U., S. Geier, A. Ptock, T. Friedrich, H. Leif, and H. Weiss.** 1993. The gene locus of the proton-translocating NADH: ubiquinone oxidoreductase in *Escherichia coli*. Organization of the 14 genes and relationship between the derived proteins and subunits of mitochondrial Complex I. J Mol Biol **233**:109-122.

358. **Weiner L., and P. Model.** 1994. Role of an *Escherichia coli* stress-response operon in stationary-phase survival. Proc Natl Acad Sci U S A **91**:2191-2195.

359. **Wei Y., and D. Fu.** 2005. Selective metal binding to a membrane-embedded aspartate in the Escherichia coli metal transporter YiiP (FieF). J Biol Chem **280**:33716-33724.

360. **Weski J., and M. Ehrmann.** 2012. Genetic analysis of 15 protein folding factors and proteases of the Escherichia coli cell envelope. J Bacteriol **194**:3225-3233.

361. **Wolfe A. J., N. Parikh, B. P. Lima, and B. Zemaitaitis.** 2008. Signal integration by the two-component signal transduction response regulator CpxR. J Bacteriol **190**:2314-2322.

362. **De Wulf P., O. Kwon, and E. C. Lin.** 1999. The CpxRA signal transduction system of *Escherichia coli*: growth-related autoactivation and control of unanticipated target operons. J Bacteriol **181**:6772-6778.

363. **Xu F. F., and J. A. Imlay.** 2012. Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify *Escherichia coli*. Appl Environ Microbiol **78**:3614-3621.

364. **Yamamoto K., and A. Ishihama.** 2005. Transcriptional response of *Escherichia coli* to external copper. Mol Microbiol **56**:215-227.

365. **Yamamoto K., H. Ogasawara, and A. Ishihama.** 2008. Involvement of multiple transcription factors for metal-induced *spy* gene expression in *Escherichia coli*. J Biotechnol **133**:196-200.

366. **Yang H. -H., J. B. Morrow, D. Grasso, R. T. Vinopal, and B. F. Smets.** 2006. Intestinal versus external growth conditions change the surficial properties in a collection of environmental *Escherichia coli* isolates. Environ Sci Technol **40**:6976-6982.

367. **Yan N., and Y. Shi.** 2007. Allosteric activation of a bacterial stress sensor. Cell **131**:441-443.

368. **Yatsunyk L. A., J. A. Easton, L. R. Kim, S. A. Sugarbaker, B. Bennett, R. M. Breece, I. I. Vorontsov, D. L. Tierney, M. W. Crowder, and A. C. Rosenzweig.** 2008. Structure and metal binding properties of ZnuA, a periplasmic zinc transporter from *Escherichia coli*. J Biol Inorg Chem **13**:271-288.

369. **Yeo W. -S., J. -H. Lee, K. -C. Lee, and J. -H. Roe.** 2006. IscR acts as an activator in response to oxidative stress for the *suf* operon encoding Fe-S assembly proteins. Mol Microbiol **61**:206-218.

370. **Zheng M., X. Wang, L. J. Templeton, D. R. Smulski, R. A. LaRossa, and G. Storz.** 2001. DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J Bacteriol **183**:4562-4570.

371. **Zhou X., R. Keller, R. Volkmer, N. Krauss, P. Scheerer, and S. Hunke.** 2011. Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein. J Biol Chem **286**:9805-9814.

372. **Zimmermann M., S. R. Udagedara, C. M. Sze, T. M. Ryan, G. J. Howlett, Z. Xiao, and A. G. Wedd.** 2012. PcoE--a metal sponge expressed to the periplasm of copper resistance Escherichia coli. Implication of its function role in copper resistance. J Inorg Biochem **115**:186-197.

373. **Zoll S., B. Pätzold, M. Schlag, F. Götz, H. Kalbacher, and T. Stehle.** 2010. Structural basis of cell wall cleavage by a staphylococcal autolysin. PLoS Pathog **6**:e1000807.

374. **Zückert W. R.** 2014. Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. Biochim Biophys Acta **1843**:1509-1516.