University of Alberta

Regulation of enteropathogenic *Escherichia coli* envelope protein expression by the Cpx response and small RNAs

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Microbiology & Biotechnology

Department of Biological Sciences

Fall 2013
Edmonton, Alberta

Abstract

Gram-negative bacteria are characterized by their complex cell envelope, which consists of the inner membrane, outer membrane, and intervening periplasmic space. Envelope-localized proteins play a critical role in many interactions of a bacterium with its environment, including uptake of nutrients, extrusion of waste, adhesion to surfaces, and motility. As such, regulation of envelope protein expression is crucial to the survival of Gram-negative organisms such as *Escherichia coli*. Two regulatory systems involved in controlling envelope protein expression are the Cpx envelope stress response and small non-coding RNAs (sRNAs). The Cpx response is believed to sense misfolding of inner membrane and periplasmic proteins; in response, Cpx increases the transcription of a suite of genes encoding envelope-localized protein folding and degrading factors. sRNAs, with assistance from the RNA chaperone protein Hfq, base-pair with target mRNAs to modulate their rate of translation and/or stability. The first goal of this thesis was to examine the regulatory effects of the Cpx response and sRNAs upon the expression of the bundle-forming pilus (BFP), an envelope-localized protein complex that mediates initial interaction of enteropathogenic *E. coli* (EPEC) with host cells. We found that the Cpx response affects BFP expression at multiple levels. Activation of the Cpx response represses transcription of the *bfp* gene cluster and prevents BFP expression, while inactivation of the Cpx response diminishes BFP expression at the post-translational level, as a result of decreased expression of periplasmic protein folding factors. The RNA chaperone Hfq also represses transcription of the *bfp* genes by destabilizing the *perA* transcript, which

encodes the major regulator of *bfp* transcription. A second goal of this work was to characterize the interactions between the Cpx response and sRNAs. I demonstrated that the Cpx response regulates the expression of four sRNA-encoding genes. Conversely, Hfq and sRNAs also affect activity of the Cpx response, as deletion of *hfq* in EPEC activates the Cpx pathway, while overexpression of the sRNA RprA diminishes Cpx activity. These results deepen our understanding of how regulatory systems attune envelope protein expression to environmental and physiological conditions, thereby contributing to the pathogenesis of EPEC and related organisms.

Acknowledgements

I would like to start by thanking my supervisor, Dr. Tracy Raivio. Thank you for welcoming me back into your lab and giving me so many opportunities over the course of my PhD. Somehow you have always known exactly how much guidance I needed. You have been a fantastic role model, both as a researcher and as a human being, and I have really enjoyed being in your lab.

Thanks to my committee members, Dr. Christine Szymanski and Dr. Stefan Pukatzki, for your helpful suggestions and for generally making this degree a positive experience. Thanks also to my thesis examiners, Dr. George Owttrim and Dr. Matthew Mulvey, for your time and effort.

Two people from the Department of Biological Sciences that I haven't thanked enough are Tom Hantos and Ben McDonald – between the two of you, you really do have all the answers!

To all of my labmates, past and present, thanks for making me look forward to coming into the lab each day. In one way or another, you have all contributed to this thesis, with your thoughtful suggestions or even just hearing me out over a coffee or beer. Nancy, Dawn, Roxana, Shannon, Julia, Nicole, Stefan, Suey, Junshu, Randi, Margarita, and Alex – I am happy to call each of you my friend.

Mom and Dad, I think it's pretty safe to say that I wouldn't have made it this far if not for your love, support, and encouragement – even if you sometimes told me that I work too hard! You have helped to keep everything in perspective. Kristi, I am proud to call you my sister. I'm glad that I've had the chance to get to know you as an adult over the past few years.

Last but not least: Ryan, you are the one who makes everything worthwhile. Thanks for making me laugh, giving me a hug when I needed one, and being there for me in a million different ways. I'm looking forward to the next stage of our adventure together.

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LIST OF SYMBOLS, NOMENCLATURE, AND ABBREVIATIONS

2CS: two-component system

A/E: attaching and effacing

AIEC: adherent-invasive *Escherichia coli*

Amk: amikacin

Amp: ampicillin

ATP: adenosine triphosphate

BAP: bacterial alkaline phosphatase

BFP: bundle-forming pilus/pili

Cam: chloramphenicol

cAMP: cyclic adenosine monophosphate

cps: counts per second

CRP: cAMP receptor protein

ddCt: delta-delta cycle threshold

DMEM: Dulbecco's modified Eagle medium

DNA: deoxyribonucleic acid

EAEC: enteroaggregative *Escherichia coli*

EAF: EPEC attachment factor

ECP: *Escherichia coli* common pilus

EHEC: enterohaemorrhagic *Escherichia coli*

EMSA: electrophoretic mobility shift assay

EPEC: enteropathogenic *Escherichia coli*

ER: endoplasmic reticulum

GMP: guanosine monophosphate

HK: histidine kinase

IM: inner membrane

IPTG: isopropyl β-D-thiogalactoside

Kan: kanamycin

Kdo: 3-deoxy-D-*manno*-oct-2-ulosonic acid

LA: localized adherence

LB: Luria-Bertani

LEE: locus of enterocyte effacement

LPS: lipopolysaccharide

MBP: maltose-binding protein

mRNA: messenger RNA

NSB: non-specific band

OD_{600}: optical density at 600 nm

OM: outer membrane

OMP: outer membrane protein

PAPI: poly(A) polymerase I

PCR: polymerase chain reaction

PDZ: post-synaptic density protein/*Drosophila* disk large tumour suppressor/zonula occludens 1

PMF: proton motive force

PNPase: polynucleotide phosphorylase

qRT-PCR: quantitative reverse transcriptase-PCR

RBS: ribosome binding site

RNA: ribonucleic acid

RNase: ribonuclease

rpm: rotations per minute

RR: response regulator

rRNA: ribosomal RNA

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Spc: spectinomycin

sRNA: small RNA

T3S: type III secretion

T3SS: type III secretion system

Tet: tetracycline

UPEC: uropathogenic *Escherichia coli*

UPR: unfolded protein response

UTR: untranslated region

VC: vector control

WT: wild-type

CHAPTER 1

General Introduction

A version of Section 1.3.1 has been published: Vogt, S.L. and T.L. Raivio. (2012) Just scratching the surface: an expanding view of the Cpx envelope stress response. *FEMS Microbiology Letters* **326(1):**2-11.

1.1 *Escherichia coli* and enteropathogenic *E. coli*

Escherichia coli is widely acknowledged as biology's premier model organism (73). First isolated from the stool of a healthy infant by German paediatrician Theodor Escherich in 1885, *E. coli* has since become an indispensible tool for the study of genetics, biochemistry, biotechnology, and systems biology. Several factors make this Gram-negative rod particularly suitable for laboratory studies, including its fast generation time, its ability to grow under aerobic conditions and on a variety of nutrients, and its genetic tractability (73).

E. coli can inhabit a variety of ecological niches. Many strains are commensal inhabitants of the mammalian large intestine; however, certain strains have adapted to a pathogenic lifestyle and there are even reports of *E. coli* strains becoming "naturalized" to abiotic habitats such as soil and sediments (83). Commensal *E. coli* strains are among the first microorganisms to colonize newborn humans after birth (188). Although *E. coli* is greatly outnumbered by anaerobic bacteria in the mature human gut, it is the most prevalent facultative aerobe in the human colon, being present in around 90% of adult humans (188). Commensal *E. coli* is adapted to live in the mucus layer covering the epithelial cells of the large intestine, as evidenced by its ability to efficiently metabolise several nutrients derived from mucus, such as gluconate (188).

Eight different pathovars of *E. coli* have been described that cause a variety of human diseases. Six of these cause gastrointestinal infections, while two are extraintestinal pathogens (30). Included in the group of diarrhoeagenic *E. coli* pathovars are enterohaemorrhagic *E. coli* (EHEC), which causes haemorrhagic colitis; enterotoxigenic *E. coli* and enteroaggregative *E. coli*, both of which cause traveller's diarrhoea; enteroinvasive *E. coli*, which causes bacillary dysentery; and diffusely adherent *E. coli* and enteropathogenic *E. coli* (EPEC), which both cause childhood diarrhoea (30). The extraintestinal pathovars include uropathogenic *E. coli* (UPEC), which causes cystitis and pyelonephritis;

and neonatal meningitis-associated *E. coli*, which causes both septicaemia and meningitis (30).

Of particular interest to this study is EPEC, which is a major cause of infant diarrhoea in developing countries (23). Unlike commensal *E. coli*, EPEC inhabits the human small intestine (30). The major adhesin responsible for initial attachment of typical EPEC strains to the intestinal epithelium is the bundle-forming pilus (BFP), which binds to *N*-acetyllactosamine-containing receptors on host cells (26, 80). BFP from adjacent EPEC cells also interact with each other, giving rise to the characteristic localized adherence phenotype, in which EPEC forms discrete clusters of cells called microcolonies on host tissues (41, 54). After initial attachment, EPEC cells use a type III secretion system (T3SS) to inject a variety of effector proteins into the host cytosol (23). One of these effectors, Tir, becomes localized to the host cell plasma membrane, where it acts as a receptor for the EPEC outer membrane protein intimin, allowing for a stable attachment known as intimate adherence (23). Tir and several other effectors also cause rearrangements of the host actin cytoskeleton, leading to effacement of microvilli and formation of a pedestal structure, referred to as an attaching and effacing (A/E) lesion (30). The process by which EPEC causes diarrhoea is poorly understood, but likely involves the combined action of various T3S effectors that disrupt tight junctions, alter ion transport in epithelial cells, and cause mislocalisation of aquaporins (30, 214). One prototypical EPEC strain for laboratory studies is E2348/69, which was isolated from an outbreak of infant diarrhoea in a British hospital in the 1970s (112). The long history of study of this pathogen, combined with the genetic tools developed in non-pathogenic strains of *E. coli*, have made it an excellent model for studying the regulation of bacterial pathogenesis.

1.2 The Gram-Negative Envelope

In Gram-negative bacteria, the envelope represents the first point of contact with the environment. The envelope performs a variety of functions that

are crucial to cell survival, including maintenance of cell integrity, transport of nutrients and waste, attachment to abiotic and host surfaces, and motility. In this section, I will give a brief overview of each compartment of the *E. coli* envelope, including the inner membrane (IM), periplasm, outer membrane (OM), and cell surface (diagrammed in Figure 1-1).

1.2.1 The inner membrane

The principal components of the inner membrane are phospholipids and proteins. The zwitterionic phospholipid phosphatidylethanolamine comprises approximately 75 to 80% of the phospholipid content of the IM; the negatively charged phospholipids phosphatidylglycerol and cardiolipin account for approximately 20% and 5%, respectively (31). There are three main categories of IM proteins: integral IM proteins, which span the IM with one or more hydrophobic α-helices; lipoproteins, which are anchored in the periplasmic face of the IM by a lipid modification at their N-termini; and peripheral membrane proteins, which are soluble cytoplasmic proteins that do not span the IM (115). IM proteins account for approximately 25% of the genes encoded in the *E. coli* genome (115). Among the many functions carried out by IM proteins are transport of small molecules, electron transport necessary for aerobic respiration, lipid biosynthesis, and protein translocation from the cytoplasm into the periplasm by the Sec and Tat complexes (169).

1.2.2 The periplasm

The periplasm is an aqueous layer approximately 50 nm wide located between the IM and the OM, containing solutes, soluble proteins, and the peptidoglycan layer of the cell wall (200). Although the periplasm was previously considered to be a viscous, gel-like layer, more recent measurements have concluded that the viscosity and therefore the density of proteins in the periplasm is comparable to that of the cytoplasm (141, 184). Periplasmic proteins include a variety of solute-binding transport proteins, hydrolytic enzymes, and protein folding chaperones (169). Periplasmic chaperones operate under unique

constraints, such as the lack of ATP and the oxidizing environment. As a consequence of the latter, many envelope proteins contain disulphide bonds that stabilize their tertiary structure. Disulphide oxidoreductases located in the periplasm catalyse the formation and isomerisation of disulphide bonds in order to promote proper protein folding (84). Other protein quality-control factors located in the periplasm include peptidyl-prolyl *cis-trans* isomerases (e.g. PpiA and SurA), molecular chaperones (e.g. Spy and Skp), and ATP-independent proteases (e.g. DegP) (128).

The peptidoglycan layer consists of polymers of *N*-acetylglucosamine and *N*-acetylmuramic acid linked by pentapeptide bridges to form a mesh (217). The primary function of the cell wall is to protect the cell from turgor pressure caused by low environmental osmolarity.

1.2.3 The outer membrane

The most important feature of the OM is likely its selective permeability, which allows it to prevent the entry of toxic hydrophobic and hydrophilic molecules (146). The main contributor to this formidable permeability barrier is the lipopolysaccharide (LPS) of the outer leaflet of the OM. LPS consists of the lipid A glycolipid, a relatively conserved core oligosaccharide, and a repeating polysaccharide known as the O-antigen. Notably, many laboratory strains of *E. coli* are "rough mutants", meaning that they contain mutations that prevent synthesis of the O-antigen (169). The inner leaflet of the OM is composed of phospholipids, giving rise to an unusual asymmetric membrane (169).

The OM also contains numerous proteins to assist in the acquisition of nutrients and the expulsion of waste. These outer membrane proteins (OMPs) typically form β-barrels that span the OM (146, 169). Some OMPs form channels that are relatively non-selective in their permeability to small hydrophilic molecules; these are termed porins (146). Other OMPs form channels that are specific transporters for certain compounds, or else they may act as enzymes or adhesins (169). The OM is also the site of the majority of lipoproteins in *E. coli* (149). These proteins, found at the periplasmic face of the OM, play roles in a

variety of processes, including the insertion of LPS, β-barrel OMPs, and other lipoproteins into the OM (149).

1.2.4 Cell-surface structures

E. coli is capable of synthesizing a variety of surface-exposed multiprotein complexes. Many of these are anchored in the OM, but some span the entire envelope, including the IM, periplasm, and OM. Included in this category are pili, flagella, and secretion systems (Figure 1-2).

Pili, also known as fimbriae, are non-flagellar proteinaceous fibres extending outward from the bacterial cell surface. These appendages may perform a variety of functions, including attachment to biotic or abiotic surfaces, motility, and uptake of DNA (48). Strains of *E. coli* produce numerous different types of pili, but three have been particularly well studied: type 1 pili, curli, and type IV pili (Figure 1-2). Type 1 or chaperone-usher pili are the most common type of pilus (48). All components of the type 1 pilus are transported across the IM by the Sec apparatus. The pilus fibre consists mainly of a single type of pilin subunit, which may be capped with a short tip complex consisting of one or more alternative pilins. These tip proteins often play a role in binding to and invading host cells (48). Type 1 pili are characterized by their periplasmic chaperones, which guide pilin subunits to the OM while assisting them in folding into a structure suitable for polymerization, and their OM usher proteins, which form both a platform for pilus polymerization and a pore through which pilins are transported to the cell surface (189).

Curli are a more recently identified type of pilus, common among enterobacteria (reviewed in 9). Much like type 1 pili, all curli components are translocated into the periplasm through the Sec complex. A major and a minor subunit comprise the curli fibre; these curlins are functional amyloid proteins, with each subunit being a stable, five-stranded β-sheet. These subunits are translocated to the cell surface through a curli-specific OM pore. Additional periplasmic and cell-surface proteins assist in the translocation and nucleation of the curli fibre.

Type IV pili are unique in that they form a continuous structure spanning both the IM and the OM (reviewed in 189). The pilus filament is composed mainly or entirely of a single pilin subunit, which consists of a hydrophobic N-terminus and a C-terminal globular domain. Prepilins are inserted into the IM by the Sec apparatus. After cleavage of the leader sequence by a dedicated prepilin peptidase, mature pilin monomers are polymerized into a filament, with the N-terminus remaining buried within the core of the pilus filament and the globular domain exposed. The energy for assembly is supplied by a cytoplasmic ATPase. Another interesting feature of type IV pili is that they can be both extended and retracted. When pili are retracted, the hydrophobic N-terminus of the pilin subunits is thought to "melt" back into the IM. Retraction of type IV pili requires a second, separate cytoplasmic ATPase. Type IV pili also contain an IM scaffold and an OM pore-forming protein called a secretin, as well as a variety of periplasmic and membrane-associated proteins of undefined function. Several *E. coli* pathovars utilize type IV pili for adherence to host tissues (53, 55, 218). Although the genome of non-pathogenic *E. coli* K-12 contains a type IV pilus biogenesis gene cluster, this strain has never been shown to elaborate a type IV pilus (171).

Flagella are the major appendage used for bacterial locomotion. The flagellar structure can be broken down into three major components: the engine, the propeller, and the joint connecting them (25). The engine consists of the basal body – the series of rings and rods in the IM, OM, and periplasm that forms the structural foundation of the flagellum – and the motor, which utilizes the proton motive force (PMF) to power rotation of the flagellum. The propeller is a long, rigid, hollow tube composed of thousands of monomers of the flagellin protein, covered by a capping protein. Finally, another set of proteins comprises a flexible hook that links the basal body to the flagellin filament. The flagellum is assembled following an inside-out principle (18, 43). Briefly, the IM and cytoplasmic ring structures are assembled first, followed by the motor complex. Using this structural base, a secretion system is assembled in the IM. Most of the

remaining components of the flagellum (including the periplasmic rod, the hook, and the capped filament) are secreted through this flagellar secretion system.

A related surface structure that is produced by EPEC but not non-pathogenic *E. coli* is the T3SS. T3SSs are evolutionarily related to flagella, but are utilized for translocation of proteins into eukaryotic cells (18). The T3SS, which is composed of over 20 proteins, forms a continuous channel for protein secretion from the bacterial cytosol through three biological membranes into the cytosol of the host cell. The secretion process is believed to be powered by both ATP hydrolysis, for the unfolding of effectors and delivery to the T3SS, and the PMF, for protein translocation (18). The effector proteins modulate host cell properties to suit the needs of the bacterium. As with the flagellum, the IM and OM components of the T3SS are assembled by protein translocation through the Sec complex; however, the periplasmic rod, extracellular needle, needle tip complex, and proteins composing the translocation pore in the eukaryotic membrane are all secreted via the T3SS itself (18).

Given the many critical functions performed by the envelope, it is imperative that Gram-negative organisms like *E. coli* have mechanisms to monitor the status of this compartment and correct any problems that may arise. Two types of regulatory systems involved in this process are envelope stress responses (described in Section 1.3) and small regulatory RNAs (described in Section 1.4).

1.3 Envelope Stress Responses

Envelope stress responses can be described as signal transduction pathways that sense damage to the envelope and mediate an adaptive response. In *E. coli*, five such envelope stress responses are known: the Cpx, σ^E, Bae, Rcs, and Psp responses (reviewed in 116). The Bae (bacterial adaptive response) pathway plays an important role in removing toxic metal compounds and plant secondary metabolites from the periplasm via increased expression of multidrug efflux pumps (71, 109, 142). The Rcs (regulator of capsular synthesis) pathway plays an important role in biofilm formation by controlling the expression of surface

structures such as capsule and flagella in response to damage to the OM and peptidoglycan layer (103, 120). The Psp (phage shock protein) response is involved in maintenance of the PMF in response to loss of IM integrity (89). The final two envelope stress responses – Cpx and σ^E – are described in greater detail below.

1.3.1 The Cpx response

The Cpx system was first discovered when mutations in the chromosomal *cpxA* (conjugative pilus expression) locus were found to reduce expression of the F-plasmid conjugative pilus in *E. coli* (126). Several years later, CpxA was identified by sequence analysis as a two-component system (2CS) sensor protein (147), with *cpxR*, the gene encoded immediately upstream of *cpxA*, demonstrated to encode its cognate response regulator (RR) (40, 159). In the 1990s, a series of studies established the view of Cpx as a novel envelope stress response. Mutations in *cpxA* were found to suppress the toxicity of secreted LamB-LacZ-PhoA fusion proteins, suggesting that activation of the Cpx system alleviates envelope protein misfolding (27). In support of this idea, several envelope-localized protein folding and degrading factors were found to be Cpx-regulated, including the periplasmic protease and chaperone DegP (35), the disulphide bond oxidoreductase DsbA (32, 153), and the peptidyl-prolyl isomerase PpiA (153). Other studies identified a number of signals capable of inducing the Cpx response. These include alkaline pH (33, 143), alterations to the composition of the IM (34, 130) and the expression of UPEC Pap pilus subunits in the absence of their cognate chaperone (91). All of these inducing cues are believed to have the common feature of generating misfolded periplasmic and/or IM proteins. From these results arose a model in which accumulation of misfolded envelope proteins activates the Cpx response, leading to the upregulation of a suite of periplasmic chaperones and proteases that refold or degrade these misfolded proteins, thereby ameliorating the envelope stress.

Although these studies highlighted the importance of the Cpx response in *E. coli*'s ability to survive potentially lethal envelope protein misfolding, recent

work has emphasized that this is only one facet of the Cpx system's cellular role. Below, I describe more recent studies that have broadened our view of the sensing mechanisms, regulon members, and physiological functions of the Cpx response.

1.3.1.1 Signal sensing

At the heart of the Cpx response is a 2CS consisting of the histidine kinase (HK) CpxA and the RR CpxR. In the presence of an inducing signal, the IM-localized CpxA first autophosphorylates and then transfers the phosphate group to CpxR, allowing CpxR to act as a transcription factor to alter gene expression (159). In the absence of an inducing signal, CpxA acts as a phosphatase to maintain CpxR in an inactive state (159). There is a growing recognition that signal sensing by 2CSs is not accomplished solely by the HK input domain; in fact, many 2CSs integrate a number of inducing signals using various domains of both the HK and the RR, as well as auxiliary sensing proteins (16). In the case of the Cpx system, at least four proteins in different cellular compartments participate in signal sensing: the OM lipoprotein NlpE, the periplasmic protein CpxP, the IM HK CpxA and the cytoplasmic RR CpxR (Figure 1-3).

NlpE (new lipoprotein E) was first identified as a multicopy suppressor of the toxicity of the envelope-localized LamB-LacZ-PhoA fusion protein (183), with suppression being dependent on activation of the Cpx response (35). The physiological role of NlpE was not well understood until several years later, when Otto & Silhavy (150) demonstrated that this protein is required for Cpx induction in response to adhesion to a hydrophobic surface. However, NlpE does not appear to be involved in sensing a variety of known Cpx-sensed envelope stresses, such as alkaline pH or Pap subunit overexpression, because *nlpE* mutants retain their ability to activate the Cpx response in the presence of these cues (39).

More recent studies have shed some light into the mechanism by which NlpE activates the Cpx response. Mutant NlpE constructs that are IM-localized, but not those localized to the periplasm, retain their Cpx-inducing capacity, showing that membrane association is critical for NlpE's signalling function (134). X-ray crystallography of NlpE revealed that it forms a two-barrel structure, with the N-terminal barrel anchored in the OM (72). Two possibilities for how NlpE,

an OM lipoprotein, could potentially interact with CpxA in the IM have been proposed (72). One possibility is that the N-terminal domain, which is inherently unstable, could unfold during surface adhesion, allowing the C-terminus of NlpE to directly contact the IM. Alternatively or in addition, when the periplasmic protein folding machinery is overloaded, NlpE might not fold properly, preventing recognition by the Lol transport machinery and therefore causing mislocalization of NlpE to the IM, thereby inducing the Cpx response.

There are hints that NlpE may be responsible for sensing other signals in addition to surface adhesion. *nlpE* was also identified in a screen for copper-sensitive *E. coli* mutants (64). Intriguingly, the N-terminus of NlpE contains a CXXC motif that may be able to chelate copper ions (72). NlpE also contains motifs with homology to the lipid-binding protein lipocalin, as well as an oligonucleotide/oligosaccharide-binding fold (72). Therefore, NlpE could conceivably have the ability to detect a variety of envelope constituents, including lipids, LPS or peptidoglycan components. Furthermore, NlpE may not be the only auxiliary lipoprotein capable of inducing the Cpx response, as overexpression of the lipoproteins OsmB, Pal, NlpA and, in particular, YafY also increases expression of a *degP-lacZ* fusion (134). Whether induction of the Cpx response by these lipoproteins has a physiological role, and if so, what the cues sensed by these other lipoproteins are remain to be identified.

A second auxiliary regulator of CpxA is the periplasmic protein CpxP, which inhibits Cpx pathway activity when overexpressed (160). Although direct evidence is still lacking, it is believed that this inhibition is mediated by protein–protein interaction between CpxP and the periplasmic domain of CpxA. In support of this hypothesis, inhibition by CpxP is lost when the periplasmic domain of CpxA is mutated (160). Furthermore, the addition of CpxP to an *in vitro* reconstituted CpxA-CpxR system decreases the rate of CpxA autophosphorylation (45). The recent crystal structure of CpxP revealed a bowl-shaped dimer, with each protomer forming a long, bent and hooked hairpin (190, 223). The concave surface of the dimer is positively charged and has been proposed to interact with acidic residues present in the CpxA periplasmic domain

(223). One study failed to find any interaction *in vitro* between *Vibrio parahaemolyticus* CpxP and the CpxA periplasmic domain (99); however, given the significant sequence differences between *V. parahaemolyticus* CpxA and *E. coli* CpxA, it is possible that an interaction does occur between the *E. coli* proteins that is not conserved in *V. parahaemolyticus*, or that additional factors such as membranes are required for the interaction to occur.

In the presence of envelope stress, the inhibitory function of CpxP is inactivated (39). Under these conditions, CpxP may be titrated away from CpxA through binding to misfolded proteins like pilins (82). CpxP also becomes a substrate for the DegP protease under Cpx-inducing conditions (15, 82). Proteolysis of CpxP is an important component of the Cpx response, as the Cpx pathway cannot be fully activated in a *degP* mutant (15). Interestingly, there is no change in the dimerization state of CpxP and only minor alterations in its conformation at alkaline pH, an inducing condition, suggesting that Cpx-inducing conditions may affect CpxP's ability to interact with partners like CpxA without causing large rearrangements in its structure (190).

The role of CpxP in signal sensing is poorly understood. CpxP is not responsible for detecting known Cpx-specific envelope stresses, because *cpxP* mutants retain their ability to sense NlpE overexpression, alkaline pH, PapE and PapG overexpression, and other stresses (39, 160). CpxP could therefore be responsible for fine-tuning Cpx activation, by preventing inappropriate induction of CpxA and allowing rapid shut-off of the Cpx response once envelope stress is relieved (160). Alternatively, CpxP could be capable of sensing a signal that has not yet been identified. It is interesting to note that CpxP has structural homology to periplasmic metal-binding proteins such as CnrX and ZraP, and that zinc ions were found in the CpxP crystal structure (190). The role of CpxP in metal ion sensing therefore merits further research. The crystal structure of CpxP is also similar to the recently solved structure of Spy, a periplasmic protein that is positively regulated by the Cpx response (98, 158). Despite the structural similarity, Spy does not share CpxP's ability to inhibit Cpx pathway activation (15, 161); rather, Spy functions as an ATP-independent periplasmic chaperone

(158). As might be expected from the structural similarity, CpxP also displays a modest chaperone activity, in addition to its signalling role (158, 223).

The HK CpxA represents a major signal integration point. The periplasmic domain of CpxA is required for both induction by NlpE (159) and inhibition by CpxP (160). Mutations in the periplasmic domain of CpxA also prevent detection of envelope stresses such as alkaline pH, PapE and PapG overexpression, and envelope perturbation by EDTA (39), all of which are sensed independently of CpxP and NlpE. It is therefore possible that CpxA can directly sense some feature of misfolded envelope proteins, the nature of which has not been identified. There is some specificity to the signal, as the overexpression of only certain Pap pilins activates the Cpx response, and Cpx activation does not correlate directly with pilin aggregation (110). In support of CpxA's ability to directly sense misfolded proteins, the MalE219 mutant protein is capable of increasing the rate of phosphotransfer from CpxA to CpxR in an *in vitro* assay (95). However, in most cases, it is formally possible that CpxA-dependent signal sensation could involve another, currently unknown auxiliary protein(s).

The function of conserved residues in the CpxA periplasmic domain has recently been analysed using alanine substitution mutations (Malpica and Raivio, in preparation). Strikingly, virtually all of the substitutions with a mutant phenotype led to increased Cpx pathway activity, even under noninducing conditions. These results suggest that the Cpx response is activated by default, with mutations leading to a loss of phosphatase function and/or elevated kinase activity and therefore increased Cpx pathway activity. It is possible that misfolded proteins could interact with some of the inhibitory residues in the CpxA periplasmic domain to allow CpxA to adopt an activated conformation. Alternatively, these residues could interact with CpxP or other, currently unidentified inhibitory proteins. The removal of these inhibitory interactions in the presence of activation signals could then be responsible for induction of the pathway.

Finally, cytoplasmic or growth signals can be integrated into the Cpx pathway downstream of CpxA, through CpxR. The expression of *cpxRA* is

activated at the onset of stationary phase (216), and in *E. coli* strain MC4100, this growth-related activation is CpxR-dependent but CpxA-independent (39). CpxR can also be activated independently of CpxA when cells are grown in the presence of excess carbon, such as glucose or pyruvate (213). This is believed to occur via the Pta-AckA pathway, which generates acetyl phosphate from acetyl-CoA (213). Acetyl phosphate itself can phosphorylate CpxR *in vitro* (153, 159) and under particular growth conditions *in vivo* (213). Additionally, other indirect products of the Pta-AckA pathway can influence the CpxR-dependent transcription of *cpxP* (213), with acetylation of residue K298 in the α subunit of RNA polymerase playing a role in this activation (113). Although the mechanism is not fully understood, it is clear that CpxR is capable of sensing signals related to growth and central metabolism without the involvement of CpxA.

1.3.1.2 Cpx regulon

The list of target genes regulated by CpxR has also undergone a recent expansion. Although genes associated with envelope protein folding and degradation constitute the most strongly regulated members of the Cpx regulon (156), a recent microarray analysis shows that the Cpx regulon may contain several hundred genes (162), clearly indicating a broader cellular role. The newly identified Cpx regulon members fall into several functional categories, including envelope protein complexes, IM proteins, peptidoglycan metabolic enzymes and other cellular regulators (Figure 1-3).

Although the first identified Cpx regulon members were all positively regulated by CpxR, microarray analysis reveals that the Cpx regulon contains approximately equal numbers of upregulated and downregulated genes (17, 162). One category of downregulated genes is those involved with the biogenesis of envelope-localized protein complexes such as pili and flagella. The mechanisms by which this downregulation is achieved, however, are diverse. Mutations in *cpxA* that constitutively activate the Cpx response render cells incapable of elaborating conjugal F-pili (126, 178). This downregulation is mediated at the level of protein stability, through degradation of the transcriptional activator TraJ

by the Cpx-regulated protease HslVU (59, 104). On the other hand, CpxR downregulates expression of the curli fimbriae both directly and indirectly. CpxR directly represses expression of the *csgBA* operon, encoding the major curlin subunit CsgA. Further repression of the *csgBA* operon is achieved indirectly through the CpxR-mediated inhibition of expression of the *csgDEFG* operon, which encodes the major transcriptional activator of curli expression, CsgD (42, 92, 148, 157). Flagellar motility of *E. coli* K-12 is also decreased by the Cpx response (216). Regulation of motility appears to occur at several levels. CpxR directly represses expression of the *motABcheAW*, *tsr* and *aer* genes, encoding components of the flagellar motor and chemotaxis and aerotaxis proteins (215, 216). Microarray results also suggest that expression of the flagellar master regulator FlhC is downregulated in response to overexpression of NlpE (162). Although the downregulation of various pili, flagella and additional virulence-related envelope structures (discussed in Section 1.3.1.3) by the Cpx response is clear, the rationale for regulation of these genes is uncertain. Downregulation of nonessential protein complexes may relieve the burden on the envelope protein folding machinery when misfolded proteins are already abundant (116). Alternatively or in addition, the repression of these energy-intensive structures may help to conserve finite cellular resources during times of stress (216).

There is also a growing appreciation of the connection between the Cpx response and IM proteins. Many of the originally identified Cpx-inducing cues, such as Pap pilus subunit and NlpE overexpression, result in the aggregation of misfolded proteins at the periplasmic face of the IM (91, 134). Additionally, Shimohata *et al*. (175) showed that the Cpx response is activated by mutation of the IM protease-encoding gene *ftsH*, and that in response, CpxR upregulates expression of *htpX*, encoding another IM protease. These results suggest that the Cpx response can sense abnormalities of integral IM proteins caused by the lack of FtsH and respond by regulating IM proteolysis. In support of a role for the Cpx response in regulating IM proteolysis, another recently characterized Cpx-regulated IM protein is YccA, which aids cell survival when protein translocation is stalled by preventing FtsH-mediated proteolysis of the Sec complex (199).

Microarray analysis of the genes affected by overexpression of NlpE revealed an enrichment for IM proteins (162). Included among these IM proteins are numerous transporters for a variety of substrates, such as fatty acids, amino acids and ions, most of which were downregulated (162). Together, these observations may suggest that the function of the Cpx response is tightly linked to the status of the IM and/or its protein content. Because many of the Cpx-regulated IM proteins identified by microarrays have currently unknown functions (17, 162), the cellular impact of Cpx regulation of IM proteins is yet to be fully understood.

An additional envelope constituent that appears to be affected by the activation of the Cpx response is the peptidoglycan of the cell wall. Weatherspoon-Griffin *et al*. (212) have recently shown that CpxR directly activates expression of *amiA* and *amiC*, genes encoding two *N*-acetylmuramoyl-L-alanine amidases that cleave peptide crossbridges from *N*-acetylmuramic acid residues to allow daughter cell separation during cell division. Interestingly, *amiA* and *amiC* mutants are characterized by increased OM permeability (85, 212), suggesting that CpxR regulation of these genes may function to improve the integrity of the cell envelope. A similar role may be played by the Cpx-regulated protein YcfS, which is an L,D-transpeptidase that links peptidoglycan to the OM lipoprotein Lpp (119, 156, 219). A number of other proteins with known or predicted roles in peptidoglycan metabolism are upregulated by the overexpression of NlpE (162), which may indicate peptidoglycan remodelling during the Cpx response.

Another factor likely contributing to the relatively large size of the Cpx regulon is that several other cellular regulators appear to be under the control of CpxR. Surprisingly, CpxR negatively and directly regulates the expression of the *rpoErseABC* operon, which encodes the alternative sigma factor σ^E (described in more detail in Section 1.3.2) (156, 215). The cellular benefit of downregulating another envelope stress response is unknown, but could suggest that some σ^E regulon members perform functions that are detrimental under Cpx-inducing conditions (156). CpxR also interfaces with the EnvZ/OmpR 2CS, in this case via positive regulation of the small, IM-localized protein MzrA (52). MzrA and EnvZ

physically interact via their periplasmic domains (51). This interaction increases the expression of genes in the OmpR regulon in an EnvZ- and OmpR-dependent manner, presumably by either increasing EnvZ phosphorylation of OmpR or decreasing EnvZ phosphatase activity or both (52). Positive regulation of MzrA therefore allows CpxAR to communicate with EnvZ-OmpR without cross-phosphorylation by noncognate HK-RR pairs, which has been shown to be kinetically unfavourable (58, 180). Another regulatory protein that is positively regulated by CpxR is YdeH, a diguanylate cyclase capable of synthesizing the signalling molecule cyclic di-GMP (90, 156, 219). YdeH both inhibits motility and promotes biofilm formation (10, 90). These connections with other cellular regulatory networks therefore allow the Cpx response to affect a variety of complex bacterial behaviours.

1.3.1.3 Role of the Cpx response in pathogenesis

Because many structures critical for bacterial virulence reside in the envelope, it is unsurprising that the Cpx response affects the ability of numerous Gram-negative pathogens to infect their hosts. Early results suggested that the Cpx response might enhance virulence by increasing the expression of periplasmic protein folding factors such as DsbA that are required for the assembly of cell-surface structures like pili (86, 151, 222). Other Cpx regulon members appear to contribute to cell-surface structure expression as well; for example, both DegP and CpxP are required for efficient elaboration of the EPEC type IV bundle-forming pilus (BFP) (78, 207). In accordance with these findings, inactivation of the Cpx response adversely affects assembly of some pili. When the UPEC Pap pilus genes are expressed in *E. coli* K-12, mutation of *cpxR* results in the production of shorter pili and a higher proportion of cells that do not express any pili because of phase variation (79). Likewise, expression of the BFP pilin bundlin and adherence to cultured human cells is reduced in an EPEC *cpxR* mutant (145).

Studies in several other organisms revealed that the Cpx response has important virulence-related functions beyond its role in pilus elaboration

(Table 1-1). In *Shigella spp*., the Cpx response controls the expression of two key virulence regulators, VirF and InvE (133, 143, 144). The expression of *virF*, which encodes a positive regulator of type III secretion genes, is enhanced by the direct binding of CpxR to its promoter (144). In an interesting example of post-transcriptional regulation by the Cpx response, the protein levels of InvE, but not its mRNA abundance, are decreased in a *cpxA* mutant of *Shigella sonnei*, in which the Cpx response is presumably constitutively activated (133). In *Legionella pneumophila*, CpxR has been shown to positively regulate the transcription of numerous components of the Icm/Dot type IV secretion system and its substrates, including the chaperone IcmR (49); the structural subunits IcmV, IcmW, DotA and LvgA (3, 203); and a host of newly identified Icm/Dot translocated substrates (3). Curiously, mutations in either *cpxR* or *cpxA* have no effect upon *L. pneumophila* intracellular growth within macrophages or amoebae (49). The benefit of Cpx regulation of type IV secretion in *L. pneumophila* therefore remains to be determined. A deletion of the *cpxRA* operon in UPEC was recently shown to impair colonization of the mouse bladder and also reduced virulence in a zebrafish infection model (38). Although the mechanisms by which Cpx promotes virulence in UPEC are not yet fully understood, the *ΔcpxRA* mutant was found to have a slightly reduced ability to adhere to and invade cultured cells, along with a decreased serum resistance (38).

In contrast to these results, recent studies have suggested that in many pathogens, activation of the Cpx response is detrimental to virulence (Table 1-1). In several organisms, mutations in *cpxA*, which in many cases result in an accumulation of phosphorylated CpxR (213; Malpica and Raivio, in preparation), have been found to decrease expression of adhesins and adherence to host cells. For example, expression of the EPEC BFP, the UPEC Pap pilus and invasin, a nonfimbrial adhesin produced by *Yersinia pseudotuberculosis*, is decreased in *cpxA* mutant strains (20, 70, 207). In addition, a *Salmonella enterica* serovar Typhimurium *cpxA* mutant has defects in host cell adherence, although the specific adhesin affected in this strain was not determined (77). The Cpx response therefore appears to have a conserved role in the repression of adhesive structures.

Expression of several virulence-associated protein secretion systems is also reduced by mutations in *cpxA*, including the EPEC and *Yersinia enterocolitica* type III secretion systems and the *Haemophilus ducreyi* LspB-LspA2 two-partner secretion system (19, 101, 117). Accordingly, the *S.* Typhimurium, *H. ducreyi*, and EPEC *cpxA* mutants were also found to be less virulent in infection models (77, 111, 187). As suggested earlier, this repression of adhesive structures and secretion systems by the Cpx response may be a pre-emptive mechanism to prevent further envelope protein misfolding. Alternatively, it is possible that the Cpx response plays a critical role in the life cycle of these pathogens by repressing expression of adhesive cell-surface structures to allow for detachment from the site of infection and transmission to new hosts or infection sites. These possibilities remain to be investigated.

One model system that shows promise in revealing the role of the Cpx response in bacterium–host interactions involves the organism *Xenorhabdus nematophila*. *X. nematophila* associates mutualistically with the entomopathogenic nematode *Steinernema carpocapsae*; the bacterium and the nematode cooperatively kill a variety of insect hosts (22). Interestingly, inactivation of the Cpx response reduces the ability of *X. nematophila* to both colonize its nematode host and successfully infect an insect host (67). Subsequent studies determined that the nematode colonization defect of the *cpxR* mutant likely results from diminished expression of the envelope-localized colonization factors NilA, NilB and NilC (68), while the virulence defect could be the result of insufficient expression of the virulence-related transcriptional regulator LrhA (69). It therefore appears that the Cpx response has important functions in multiple stages of the *X. nematophila* life cycle. Further studies in this pathogen and others will undoubtedly improve our understanding of the role of the Cpx response in bacterium–host interactions.

It is now clear that the Cpx envelope stress response represents more than simply a means to detect and repair misfolded periplasmic proteins. A variety of signals can enter the Cpx signalling pathway at multiple points, with NlpE sensing adhesion, CpxA possibly sensing misfolded envelope proteins, and CpxR sensing

growth and metabolism. A variety of target genes are regulated by phosphorylated CpxR, including those encoding envelope protein complexes, IM proteins, peptidoglycan metabolic enzymes and other regulators. Finally, the Cpx response regulates virulence processes in numerous pathogens (Table 1-1). Most of these inducing cues and regulatory targets still pertain to the cell envelope, validating the original characterization of CpxAR as an envelope stress response; however, the Cpx response also promotes envelope function in diverse ways not previously recognized (summarized in Figure 1-3).

1.3.2 The σ^E response

A complementary envelope stress response is mediated by the alternative sigma factor σ^E (Figure 1-4). Whereas the Cpx response appears to sense and respond to misfolding of IM and periplasmic proteins, the σ^E response is attuned to the status of the OM (116). Conditions leading to induction of the σ^E response include both general agents of envelope disruption, such as ethanol and heat, and specific defects in OM biogenesis, such as overexpression of OMPs and deletion of genes encoding periplasmic chaperones and LPS biosynthesis enzymes (127, 131, 168). The importance of the envelope maintenance functions provided by the σ^E response is underscored by the fact that *rpoE*, the gene encoding σ^E, is essential in *E. coli* (102).

1.3.2.1 Signalling

Unlike CpxR, whose activity is regulated by phosphorylation, activity of σ^E is regulated by protein sequestration (reviewed in 7). In the absence of envelope stress, σ^E is bound by its anti-sigma factor, the integral IM protein RseA (132). The interaction with RseA prevents σ^E from associating with core RNA polymerase and initiating transcription. The interaction between RseA and σ^E is reinforced by the periplasmic regulatory protein RseB, which binds to the periplasmic domain of RseA and protects it from proteolysis (132).

The process of σ^E activation is well characterized and involves two molecular signals (Figure 1-4 ;7, 114). The first signal is the presence of

misfolded OMPs in the periplasm. This signal is sensed by the IM protease DegS, whose PDZ domain interacts with a YxF peptide motif that is common in the C-terminus of β-barrel OMPs (210). In properly folded OMPs, the YxF motif is buried between two β-strands; however, the motif becomes accessible when the OMP is improperly folded (28). Binding of the YxF peptide to the DegS PDZ domain activates the protease domain, causing DegS to cleave the periplasmic domain of RseA (210). The truncated IM and cytoplasmic fragment of RseA then becomes a substrate for cleavage by a second protease, RseP, which cleaves RseA in its transmembrane domain (2, 94). The cytoplasmic domain of RseA is further degraded by the ClpXP complex (46), thereby releasing free σ^E into the cytoplasm, where it can associate with core RNA polymerase and transcribe its regulon.

The second signal required for activation of σ^E is related to the presence of LPS biosynthesis intermediates in the periplasm (114). This signal is sensed by RseB. Lipid A compounds containing the phosphorylated *N*-acetylglucosamine disaccharide and two N-linked acyl chains bind to RseB, causing it to dissociate from RseA (114). Importantly, both the misfolded OMP signal sensed by DegS and the lipid A signal sensed by RseB must be present in order for RseA cleavage and σ^E activation to occur. Because RseB protects RseA from proteolysis, inactivation of RseB must occur before DegS can cleave RseA, even in the presence of misfolded OMPs (21, 114). This dual signal requirement likely prevents spurious activation of the σ^E response by the transient presence of inducing cues in the periplasm.

1.3.2.2 Regulon

Consistent with OM biogenesis problems leading to induction of the σ^E response, the core σ^E regulon consists of genes whose products promote biosynthesis and assembly of two of the major constituents of the OM, β-barrel OMPs and LPS (167). Some of the first σ^E regulon members to be identified encode periplasmic proteases and chaperones, such as the protease/chaperone DegP, the peptidyl-prolyl isomerases SurA and FkpA, and the periplasmic

chaperone Skp (32, 37, 44). All of these proteins play an important role in the folding of OMPs, with SurA believed to be the primary OMP chaperone, and Skp and DegP playing a supporting role in folding OMPs during times of stress (182). The five genes encoding components of the BamABCDE β-barrel OMP insertion complex are also transcribed by σ^E (37, 166, 167). Together, the upregulation of chaperones and the Bam assembly complex during the σ^E response increases the OMP biogenesis capacity of the cell.

The σ^E regulon is also involved in biogenesis of major OM lipids, including both LPS and phospholipids. Genes encoding several lipid A biosynthesis enzymes (*lpxA*, *lpxB*, and *lpxD*) are transcribed by σ^E (167). After the complete LPS molecule has been synthesized at the periplasmic face of the IM, it must be transported across the periplasm and inserted into the OM by the Lpt complex (194). Several of the *lpt* genes, including the *lptA-lptB* operon and *lptD*, are also members of the σ^E regulon (12, 37, 186). *plsB*, which encodes an enzyme involved in the early stages of phospholipid biosynthesis, is also σ^E-regulated (167), demonstrating that σ^E promotes synthesis of lipids contained in both leaflets of the OM.

Finally, several regulatory proteins and RNAs are also part of the σ^E regulon. There are σ^E promoters located upstream of the *rpoErseABC* operon and also internally upstream of *rseA* (167, 168). This endows the σ^E response with both positive and negative feedback – when the response is activated, increased transcription of *rpoE* gives rise to an even higher level of active σ^E within the cytoplasm; however, concomitant expression of *rseA* ensures that the response can be rapidly shut off when the stress is alleviated. The σ^E response also interfaces with cytoplasmic stress responses via increased expression of *rpoH*, encoding the cytoplasmic heat shock sigma factor (44). Two other critical members of the σ^E regulon are the small RNAs (sRNAs) RybB and MicA (87, 192, 196; see Section 1.4 for a description of sRNA mechanism of action). Together, these sRNAs post-transcriptionally repress the expression of more than 30 mRNAs, including every major porin and numerous minor OMPs (56). Downregulation of *omp* transcripts reduces the accumulation of pre-OMPs in the

periplasm and eases the burden on the OMP folding and assembly machinery. The importance of this aspect of the σ^E response is demonstrated by the finding that the lethality of σ^E depletion can be suppressed by overexpression of either *rybB* or *micA* (56).

As a whole, the σ^E response can be viewed as an adaptation to perturbations to the OM (Figure 1-4). When the response is activated due to the misfolding of OMPs and accumulation of LPS biosynthesis intermediates, a suite of genes is transcribed that promotes assembly of OMPs, LPS, and probably also phospholipids. At the same time, *omp* mRNA translation is reduced, helping to restore homeostasis to the envelope.

1.4 Regulation of Envelope Structures by Small RNAs

Although the five envelope stress responses described in Section 1.3 have major effects on gene expression at the transcriptional level, many envelope protein-encoding genes are also regulated post-transcriptionally, often by sRNAs. This section will describe how sRNAs, along with the RNA chaperone Hfq and cellular RNases, control expression of a variety of envelope proteins.

1.4.1 Small RNAs

There has been a growing appreciation in the past decade for the role of sRNAs in regulating gene expression in bacteria. Currently, approximately 80 sRNAs have been described in *E. coli*, but deep-sequencing studies suggest that several hundred more remain to be identified (176). There are several basic criteria that define sRNAs (57):

- sRNAs are typically short molecules, in the range of 50 to 300 nucleotides.
- sRNAs function as distinct molecules, rather than as part of a larger mRNA. Some mRNAs contain untranslated regions (UTRs) that alter the translation of their own message in response to external conditions such as

temperature or the presence of a metabolite; these are *cis*-acting and are referred to as riboswitches (172). sRNAs, in contrast, act in *trans*.

- sRNAs mediate their regulatory function as RNA molecules, without being translated into proteins. Several cases of sRNAs containing short ORFs are known (such as SgrS; 209); however, in these cases, the regulatory function is performed by the RNA molecule rather than by the protein.
- sRNAs are expressed. Numerous putative sRNAs have been identified by genomic searches, but they cannot be considered sRNAs until they have been experimentally detected, normally by Northern blotting.

There are several general mechanisms by which sRNAs can exert their effects (211). One major class of sRNA includes those that regulate the activity of a protein. Two well-known examples of this type of sRNA are CsrB and CsrC in *E. coli*. These sRNAs bind to and antagonize the activity of CsrA, an RNA-binding protein that prevents the translation of mRNAs involved in carbon utilization (193).

The second mechanism of sRNA action, which will be the focus of the rest of this section, is direct base-pairing with mRNAs. The base-pairing of an sRNA with an mRNA can have different effects upon the mRNA target (Figure 1-5). The most common effect is repression of translation, which occurs when the sRNA binds to and obscures the ribosome-binding site (RBS) of the mRNA. Recent studies have demonstrated that the region of base-pairing does not necessarily have to overlap the RBS in order to inhibit translation. Two sRNAs known to bind upstream of the RBS are IstR-1 and GcvB (36, 174). In the case of IstR-1, the region of base-pairing overlaps a "ribosome standby site" that is required for efficient translation of the target, *tisB* (36). Alternatively, the sRNA can bind within the coding sequence of the mRNA. Bouvier and colleagues (11) have identified a window of five codons downstream of the AUG initiation codon within which binding of an sRNA will effectively inhibit translation initiation.

In contrast, some sRNAs activate translation of their target mRNAs (Figure 1-5). This effect can occur when the mRNA contains a secondary

structure that normally prevents the ribosome from accessing the RBS, which is the case with the *rpoS* mRNA. Several sRNAs, including DsrA, RprA, and ArcZ, can bind to the stem-loop structure in the *rpoS* mRNA, thereby making the RBS accessible for translation (121, 122, 124). Finally, the binding of the sRNA to the mRNA frequently results in degradation of both molecules by RNases (Figure 1-5). sRNAs that regulate their targets by this mechanism can bind to virtually any region of the mRNA; for example, the MicC sRNA base-pairs with codons 23 to 26 of the *ompD* mRNA in *Salmonella*, well downstream of the translation initiation site (152). The role of RNases in sRNA-mediated regulation will be discussed in more detail in Section 1.4.2.

1.4.2 Hfq and RNases

Several proteins play an important role in the ability of base-pairing sRNAs to regulate their targets. One key player in sRNA-based regulation is the RNA chaperone protein Hfq. Hfq was originally identified as a host factor required for replication of the RNA bacteriophage Qβ (47). When an *E. coli hfq* mutant was generated decades later, pleiotropic mutant phenotypes were discovered, including decreased growth and sensitivity to osmolarity and UV light (195). Subsequent studies revealed that some of the phenotypes of the *hfq* mutant can be attributed to inefficient translation of the *rpoS* mRNA, which encodes the stationary phase and general stress sigma factor σ^S (14, 140). Hfq is now known to be a highly abundant and phylogenetically conserved protein, being found in about half of all sequenced Gram-positive and Gram-negative bacteria with approximately 50,000 to 60,000 copies per *E. coli* cell (13).

The discovery that Hfq is involved in translation of the *rpoS* mRNA led to investigations into its ability to post-transcriptionally regulate gene expression, particularly in association with sRNAs. In general, Hfq is believed to play a more important role in establishing an sRNA-mRNA duplex than in subsequent translational inhibition or activation. *In vitro* studies have shown that, once the sRNA-mRNA duplex has been formed, the presence of Hfq is no longer required in order for the sRNA to repress or activate translation of the target mRNA (123,

185). There are three models for how Hfq could increase the ability of sRNAs to duplex with mRNAs (205). First, binding of both RNA species to Hfq could increase their local concentration, thereby increasing the probability of successful base-pairing. Second, in some cases, Hfq assists the sRNA and mRNA to form a more stable complex than they do in its absence (185). Third, Hfq may remodel the secondary structure of one or both RNAs to remove structures that inhibit intermolecular base-pairing.

Another important role for Hfq *in vivo* is to protect sRNAs from RNase-mediated degradation. In part, this stabilizing effect may be due to competition between Hfq and RNase E for binding to A/U-rich regions in RNAs (201). A study by Urban and Vogel (198) found that all 10 examined sRNAs had a significantly decreased effect on their target mRNAs in an *hfq* mutant; additionally, eight of the sRNAs were destabilized in this strain.

There is some evidence that Hfq functions as a post-transcriptional regulator independently of its interactions with sRNAs. The Hfq protein is able to repress translation of the *ompA* mRNA *in vitro* (208). However, the subsequent discovery of the MicA sRNA that represses *ompA* has drawn into question whether Hfq would act on the transcript without the assistance of an sRNA *in vivo* (165, 197). Both *in vitro* and *in vivo*, Hfq was found to stimulate the polyadenylation of the *rpsO* ribosomal protein mRNA (65), thereby enhancing its exonucleolytic degradation. Additionally, dozens of mRNAs can be co-purified with Hfq from *E. coli* (221) and *S.* Typhimurium (181); it is currently unclear whether these transcripts are all targeted by sRNAs or whether some of them may represent direct targets of Hfq.

In addition to Hfq, RNases also have a large impact on the ability of sRNAs to regulate their targets. The process of RNA degradation in *E. coli* has been reviewed by Viegas and Arraiano (201). Typically, bacterial mRNAs and sRNAs are stabilized by secondary structure at their 3' ends, often in the form of a ρ-independent terminator. The process of RNA degradation is initiated by an endonuclease, which generates new 3' ends lacking secondary structure that can be easily degraded by 3'-to-5' exonucleases. In order to degrade any remaining

RNA fragments containing 3' secondary structure, the enzyme poly(A) polymerase I (PAPI) generates a poly(A) tail at the 3' end of the RNA. This tail provides a toehold for exonucleases, allowing them to degrade through the region of secondary structure. Alternatively, exonucleases may be assisted by a helicase, which unwinds secondary structures in RNAs. All of these functions may be performed by the complex known as the "degradosome" (201). In this complex, the essential endonuclease RNase E acts as a scaffold for the exonuclease polynucleotide phosphorylase (PNPase), the helicase RhlB, and the glycolytic enzyme enolase.

RNase E plays a major role in sRNA degradation in *E. coli*. RNase E affects sRNA stability in two ways: (i) it degrades sRNAs in the absence of Hfq, and (ii) it mediates coupled degradation of sRNAs along with their mRNA targets (108, 125). This intimate link between RNase E and sRNAs could be, at least in part, due to an interaction between Hfq and the C-terminal scaffold of RNase E; this interaction allows Hfq to deliver sRNA-mRNA duplexes to RNase E for degradation (81).

With regard to the mechanism by which sRNAs induce degradation of target mRNAs by RNase E, one model depends on inhibition of translation; in this model, if the sRNA prevents the mRNA from being translated, RNase recognition sites that are normally masked by translating ribosomes are revealed and the mRNA is degraded (108). However, this model does not apply to all mRNAs that are destabilized by sRNAs; in some cases, inhibition of translation (for example, with antibiotics) is insufficient to induce mRNA degradation (139, 155). Additionally, some sRNAs induce degradation of their targets without repressing translation, such as previously mentioned sRNA MicC, which base-pairs with the *Salmonella ompD* mRNA well inside its open reading frame (152). In this case, MicC's 5' monophosphate allosterically activates RNase E to cleave the *ompD* transcript (6).

Recent data indicate that PNPase is also an important player in sRNA regulation. In exponentially growing cells, deletion of the *pnp* gene encoding PNPase causes numerous sRNAs to be degraded in an RNase E-dependent

fashion (5, 106). Under these conditions, PNPase may prevent sRNAs from being prematurely degraded by RNase E prior to pairing with target mRNAs (106). However, in stationary phase, PNPase activity is reversed – mutations in *pnp* stabilize sRNAs (4, 202). The stabilizing effect of *pnp* mutations is particularly striking in an *hfq* mutant background, suggesting that PNPase may function to degrade sRNAs that are not bound to Hfq (5).

It is clear that sRNAs do not act alone in regulating the expression of their target mRNAs. The presence of RNA-binding proteins such as Hfq, RNase E, and PNPase is required for most sRNAs to affect their targets.

1.4.3 sRNAs regulating OMP expression

Among the sRNAs whose function is known, a large fraction – approximately one third to one half – regulates the expression of OMPs (62; Figure 1-6). Since most of these sRNAs negatively regulate *omp* expression, many OMPs are overexpressed in *hfq* mutants, leading to chronic activation of the σ^E envelope stress response (63, 192). As discussed in Section 1.3.2.2, two sRNAs – RybB and MicA – are members of the σ^E regulon. Together, they repress the expression of at least 14 OMPs and lipoproteins, including every major porin in *E. coli* (56). A major function of these sRNAs appears to be preventing the synthesis of excess OMPs. During periods of envelope stress, this reduction in the load of OMPs entering the periplasm would provide an opportunity for the protein folding and degrading machinery to remove any OMPs that are already misfolded.

In addition to σ^E, another regulatory system controlling the expression of *omp*-regulating sRNAs is the EnvZ/OmpR 2CS (Figure 1-6). The EnvZ HK is stimulated by environmental cues including high osmolarity, acidic pH, and the lipophilic local anaesthetic procaine (154, 163, 170). Since both osmotic stress and procaine disrupt the integrity of bacterial membranes (163), it is believed that EnvZ may monitor some aspect of membrane integrity or function. The best-studied regulatory targets of the OmpR RR are the porin-encoding genes *ompC* and *ompF* (1, 154). OmpC and OmpF are both abundant, non-specific porins with

a high degree of sequence homology; the major difference between these two porins is that OmpF contains a slightly larger pore than OmpC (146). As such, OmpF expression is maximal and OmpC expression is minimal in environments with low nutrient and toxin concentrations, while the reverse is true in environments with high nutrient and toxin concentrations (146). This osmoregulation is at least partially achieved by phosphorylated OmpR. Under conditions of low osmolarity, the cellular concentration of phosphorylated OmpR is low; phospho-OmpR therefore binds only to its high affinity sites upstream of *ompF*, activating its transcription (154, 220). When osmolarity is high, the concentration of phospho-OmpR is high; as a result, the transcription factor binds to its low affinity sites upstream of *ompF* and *ompC*, thereby increasing transcription of *ompC* but repressing transcription of *ompF* (154, 220). Additional EnvZ/OmpR regulon members include *csgD*, the transcriptional regulator of curli expression, which is activated by OmpR; *flhDC*, the flagellar master regulator genes, which are repressed by OmpR; and four sRNAs – *micF*, *micC*, *omrA*, and *omrB* (24, 29, 60, 157, 177).

The sRNAs MicF and MicC each regulate at least one OMP – MicF represses expression of *ompF* and *phoE*, while MicC represses *ompC* expression (24, 74, 135). Interestingly, EnvZ/OmpR regulates expression of these two sRNAs inversely. *micF* expression is activated by high levels of OmpR~P, while *micC* expression is repressed under these conditions (24, 29). As such, the purpose of sRNA regulation by the EnvZ/OmpR 2CS does not appear to be bulk repression of porin synthesis; by repressing *micC* expression, OmpR actually increases translation of the *ompC* mRNA. Instead, MicF and MicC might complement the transcriptional regulation of *ompF* and *ompC* by EnvZ/OmpR. These two sRNAs participate in a regulatory network motif known as a feedfoward loop, in which a regulator (EnvZ/OmpR) both directly and indirectly regulates expression of a target gene (*ompF* and *ompC*) by directly binding to its promoter and also by regulating the expression of a second regulator (MicF and MicC), which itself regulates the target. Although the role of these feedforward loops in *ompF* and *ompC* regulation has not been experimentally investigated,

they could expand the range of attainable OmpF/OmpC ratios (8). Under EnvZ-activating conditions, increased expression of *micF* could reduce leaky expression of *ompF*, and reduced expression of *micC* could increase the translation of the *ompC* mRNA, thereby enhancing the transcriptional repression of *ompF* and activation of *ompC*.

The two other EnvZ/OmpR-regulated sRNAs, OmrA and OmrB, are encoded by adjacent genes that have a high degree of sequence homology, particularly at their 5' and 3' ends (60). Given the similarity in sequence, it is unsurprising that these two sRNAs share almost all of the same target genes, including several minor OMPs (*ompT*, *fecA*, *fepA*, and *cirA*), *ompR*, *csgD*, and *flhD* (60, 61, 75, 107). Many of the OMPs repressed by OmrA and OmrB are TonB-coupled OM receptors. The benefit of reducing expression of these genes under EnvZ/OmpR-activating conditions is currently unknown. Perhaps these OMPs are important under low osmolarity conditions but are less important or harmful under high osmolarity conditions (60). Alternatively, activation of the EnvZ HK as a result of the high osmolarity conditions encountered during host colonization could reduce expression of these OMPs and prevent them from becoming targets for the host immune response (60). OmrA and OmrB also exert negative feedback regulation on their own regulator, *ompR*; this negative feedback loop might limit accumulation of OmpR~P or reduce cell-cell variability in OmpR levels (8, 61).

In addition to the σ^E- and EnvZ/OmpR-regulated sRNAs, there are still more sRNAs that regulate the expression of OMPs (Figure 1-6). These include the CRP/cAMP-activated sRNA CyaR, which represses expression of the porin *ompX*, and the sRNA ChiX, which represses expression of the chitobiose porin ChiP (88, 105, 164). What advantages does regulating OMP expression with sRNAs provide for bacterial cells? In addition to attuning porin expression to the osmolarity of the environment, sRNAs allow bacteria to repress expression of porins that could be targeted by the host immune system or bacteriophage (206). Guillier *et al.* (62) have noted that many OMP-regulating sRNA genes are located adjacent to non-cognate porin-encoding genes (i.e. porins that are not regulated by

the sRNA in question). This genetic arrangement frequently results in the coordinated expression of the sRNA and the porin. The coordinated upregulation of one porin and sRNA-mediated downregulation of another porin may reflect a need for the cell to balance total porin synthesis in order to prevent destabilization of the OM and/or overloading of the OMP biogenesis machinery. Another explanation for post-transcriptional regulation is that highly abundant porins such as OmpA require strong promoters and translation signals to ensure that they are adequately expressed when the cell is growing (62). sRNAs represent an ideal way to reduce synthesis of a highly abundant and stable mRNA under non-favourable conditions. Finally, sRNAs are faster and less energetically expensive to synthesize than regulatory proteins, which may be a significant advantage to the cell during periods of stress.

1.4.4 sRNAs regulating other envelope components

In contrast to sRNA regulation of OMPs, which has been recognized since the 1980s and thoroughly investigated during the past decade, sRNA regulation of other envelope components is an emerging area of research. Recent studies suggest that sRNAs also regulate the expression of flagella, curli, LPS, and secretion systems in *E. coli*.

Early indications that sRNAs may regulate the expression of flagella in *E. coli* came from the observations that *Δhfq* mutants of several pathogenic strains of *E. coli* (UPEC, EHEC, and adherent-invasive *E. coli*) had reduced motility in low-agar medium (66, 97, 179). More recently, specific sRNAs that regulate motility have been identified. The *flhDC* transcript, encoding the master regulator of flagellar genes, appears to be an sRNA signalling hub (Figure 1-6). Translation of *flhDC* is positively regulated by one sRNA (McaS) and negatively regulated by four others (OxyS, OmrA, OmrB, and ArcZ) (107, 191). Other effects of sRNAs on motility are likely yet to be discovered. The sRNA MicA increases motility, yet has no effect on *flhDC* translation, suggesting that it acts on a different flagellar or chemotaxis gene (107). Moreover, the effects of ArcZ on motility do not appear to be limited to repression of *flhDC*, as overexpression of ArcZ still

reduces motility in a strain with a point mutation in the *flhDC* 5' UTR that abolishes its interaction with ArcZ (107).

Another cell surface structure whose expression was recently shown to be regulated by numerous sRNAs is the curli fimbriae (Figure 1-6). As with flagella, sRNA regulation is mediated through effects on the transcriptional regulator of curli genes, CsgD. Translation of the *csgD* mRNA is repressed by at least five sRNAs – OmrA, OmrB, McaS, RprA, and GcvB (75, 93, 129, 191). Since expression of these five sRNAs is regulated by a variety of different transcription factors, translation of *csgD* and production of curli can be fine-tuned in response to a variety of environmental and physiological conditions. Currently, *csgD* is the only pilus-related gene whose expression is known to be regulated by specific *E. coli* sRNAs. However, other studies suggest that the type 1 pilus might also be subject to sRNA regulation. A defect in type 1 piliation was observed during microscopy studies of an adherent-invasive *E. coli Δhfq* mutant (179), and downregulation of several type 1 pilus *fim* genes was observed upon overexpression of OmrA and OmrB (60). Further studies will be needed to clarify the mechanism of sRNA regulation of type 1 pilus expression.

sRNAs can also influence the structure of LPS. Moon and Gottesman identified *eptB*, encoding a phosphoethanolamine transferase that modifies one of the Kdo sugars of LPS, as a negatively regulated target of the sRNA MgrR (136). As anticipated, deletion of the *mgrR* gene increases the proportion of LPS molecules carrying this phosphoethanolamine modification (138). The *ΔmgrR* mutant is also more resistant to the cationic antimicrobial peptide polymyxin B (136), suggesting that MgrR reduces resistance to antimicrobial peptides. This was a surprising finding because expression of *mgrR* is positively regulated by the PhoQ/P 2CS, which is generally thought to increase resistance to antimicrobial peptides. One possible explanation for this apparent discrepancy is that the EptB-mediated phosphoethanolamine modification may interfere with other LPS-modifying enzymes induced by PhoQ/P (136). More recently, the *eptB* mRNA was also shown to be regulated by a second sRNA, ArcZ, which is expressed under aerobic growth (137, 138). Additionally, there is some evidence suggesting

that *waaR*, encoding a glucosyltransferase involved in LPS outer core synthesis, may be targeted by the σ^E-regulated sRNA RybB (96), but further experiments are needed to validate this regulatory interaction. Given the large number of enzymes involved in LPS biosynthesis and modification, it seems likely that more instances of regulation by sRNAs will emerge with further research.

Finally, expression of T3SSs in pathogenic *E. coli* may also be controlled by sRNAs. Studies in both EHEC and EPEC demonstrated that T3S is elevated in *Δhfq* mutants (66, 173). Hfq appears to affect the expression of the master regulator of T3S, Ler, in several ways. Shakhnovich and colleagues demonstrated that expression of a *ler-lacZ* translational reporter is enhanced in the EHEC *Δhfq* mutant (173), while Hansen and Kaper showed that Hfq reduces stability of the *grlRA* transcript, encoding two regulators of *ler* transcription (66). The specific sRNA(s) regulating expression of *grlRA* and *ler* have yet to be identified.

As with OMPs, the expression of surface structures such as flagella, pili, LPS, and secretion systems needs to be finely attuned to environmental conditions to ensure host colonization and stress survival. Complex transcriptional regulation of these genes has been recognized for decades, but only in the past few years has post-transcriptional regulation been thoroughly studied. Undoubtedly, the studies described above are only the tip of the iceberg of sRNA regulation of surface structures.

1.5 Objectives

There is abundant evidence that both the Cpx envelope stress response and sRNAs control the expression of cell surface structures, but the specific mechanisms by which they regulate the expression of virulence genes, such as the type IV bundle-forming pilus in EPEC, are poorly understood. In addition, no studies have yet examined whether there are any regulatory interactions between these two types of regulators.

The overall goal of this thesis was to examine regulatory interactions between the Cpx envelope stress response, sRNAs, and BFP expression. The specific objectives were:

1. Cpx regulation of BFP expression:
    a. To examine whether the decreased BFP expression in an EPEC *cpxR* mutant, previously reported by Nevesinjac and Raivio (145), can be attributed to transcriptional or post-transcriptional effects of Cpx pathway inactivation;
    b. To determine whether activation of the Cpx response in EPEC affects BFP expression, and if so, whether effects are transcriptional or post-transcriptional;
2. Effects of an *Δhfq* mutation upon envelope stress in EPEC:
    a. To assess whether inactivation of Hfq (and therefore the majority of sRNAs) causes envelope stress that activates the Cpx pathway, similar to the σ^E activation previously reported in *E. coli Δhfq* mutants (59);
    b. To examine whether Hfq (likely in conjunction with sRNAs) regulates BFP expression in EPEC;
3. Regulatory connections between Cpx and sRNAs:
    a. To determine whether the Cpx 2CS regulates the expression of any sRNAs; and
    b. To assess whether any sRNAs directly or indirectly affect Cpx pathway activity.

1.6 Tables and Figures

Table 1-1. Virulence phenotypes associated with Cpx response mutations.

Organism	Virulence phenotype	Reference
Cpx response promotes virulence		
Enteropathogenic *Escherichia coli*	*cpxR* null mutant has decreased elaboration of bundle-forming pilus and decreased adherence to cultured cells	145
Shigella spp.	*cpxR* null mutation abolishes expression of T3S transcriptional regulator VirF; *cpxA* deletion post-transcriptionally reduces expression of InvE regulator	133, 144
Legionella pneumophila	CpxR directly activates expression of several Icm/Dot type IV secretion system components, as well as some secreted substrates	3, 49, 203
Uropathogenic *Escherichia coli*	*ΔcpxRA* mutant has reduced colonization in a mouse bladder model, reduced virulence in a zebrafish model, decreased ability to adhere to and invade cultured cells, and diminished serum resistance	38
Xenorhabdus nematophila	*cpxR* mutant has reduced virulence in the insect host *Manduca sexta*, likely related to its decreased growth rate *in insecta*, increased stimulation of antimicrobial peptide production and reduced expression of the pathogenesis regulator LrhA	67, 69
Cpx response inhibits virulence		
Enteropathogenic *Escherichia coli*	Activation of Cpx response inhibits bundle-forming pilus expression, type III secretion, and motility; *cpxA** constitutively active mutation attenuates virulence in *Galleria mellonella* infection model	111, 117, 118, 207
Salmonella enterica serovar Typhimurium	*cpxA** constitutively active mutation inhibits adherence to cultured cells and reduces virulence in mice	77
Yersinia pseudotuberculosis	*cpxA* mutation inhibits type III secretion and adherence to host cells, via downregulation of the adhesin invasin	19, 20
Legionella pneumophila	CpxR inhibits expression of several Icm/Dot type IV-secreted substrates	3

Haemophilus ducreyi	Cpx inhibits expression of several virulence determinants, including LspA2 and DsrA; *ΔcpxA* pathway-activating mutation renders *H. ducreyi* avirulent in human volunteers	50, 100, 101, 187

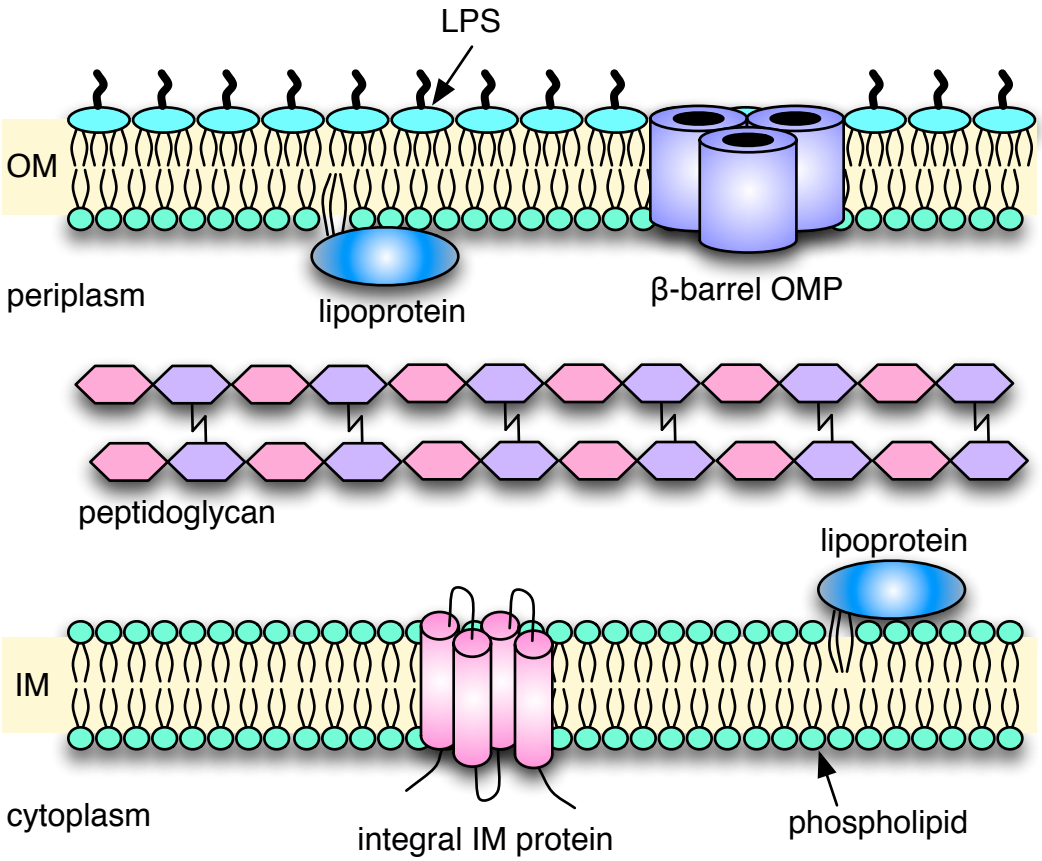

Figure 1-1. Overview of the *Escherichia coli* envelope. The outer membrane (OM) is composed of an outer leaflet of lipopolysaccharide (LPS) and an inner leaflet of phospholipids. Outer membrane proteins (OMPs) are typically trimeric β-barrel porins. The periplasmic space contains the peptidoglycan of the cell wall. The inner membrane (IM) is composed of phospholipids and integral IM proteins that span the IM with hydrophobic α-helices. Lipoproteins can be found anchored in both the inner leaflet of the OM and the outer leaflet of the IM. Figure adapted from (169).

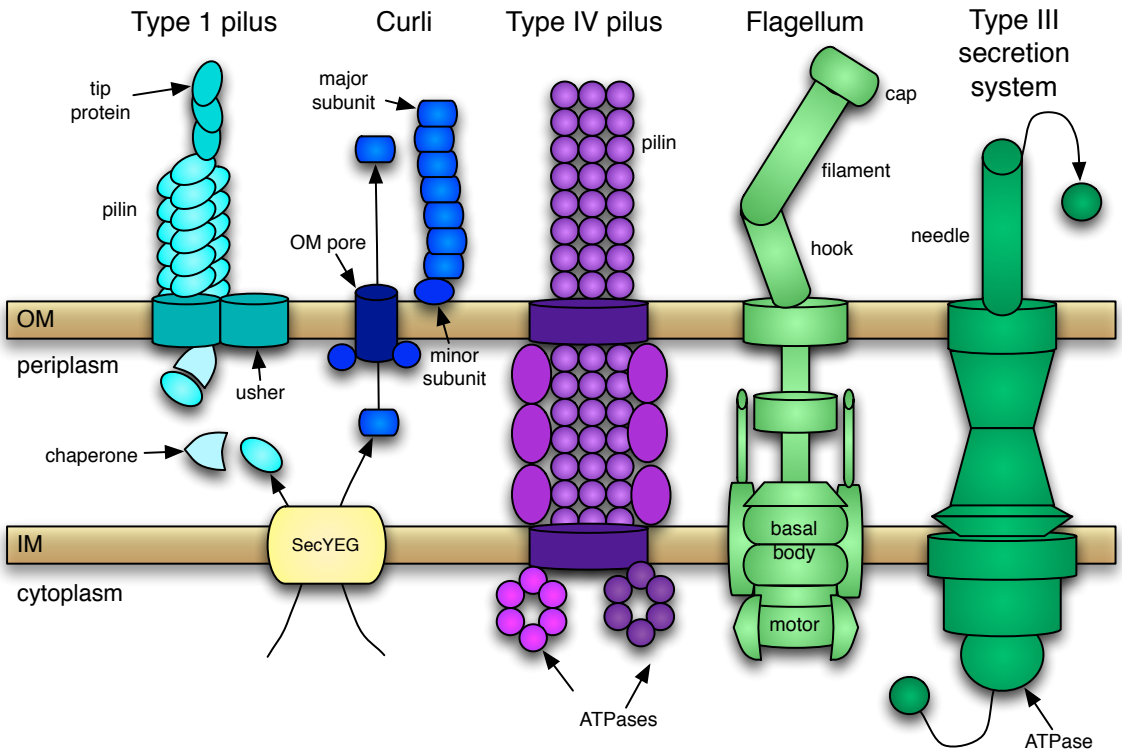

Figure 1-2. Cell-surface protein structures in *Escherichia coli*. Cell surface structures depicted include the type 1 pilus, curli, type IV pilus, flagellum, and type III secretion system. See the text for a description of each structure. OM, outer membrane; IM, inner membrane; SecYEG, general secretion complex. Figure adapted from (25, 48).

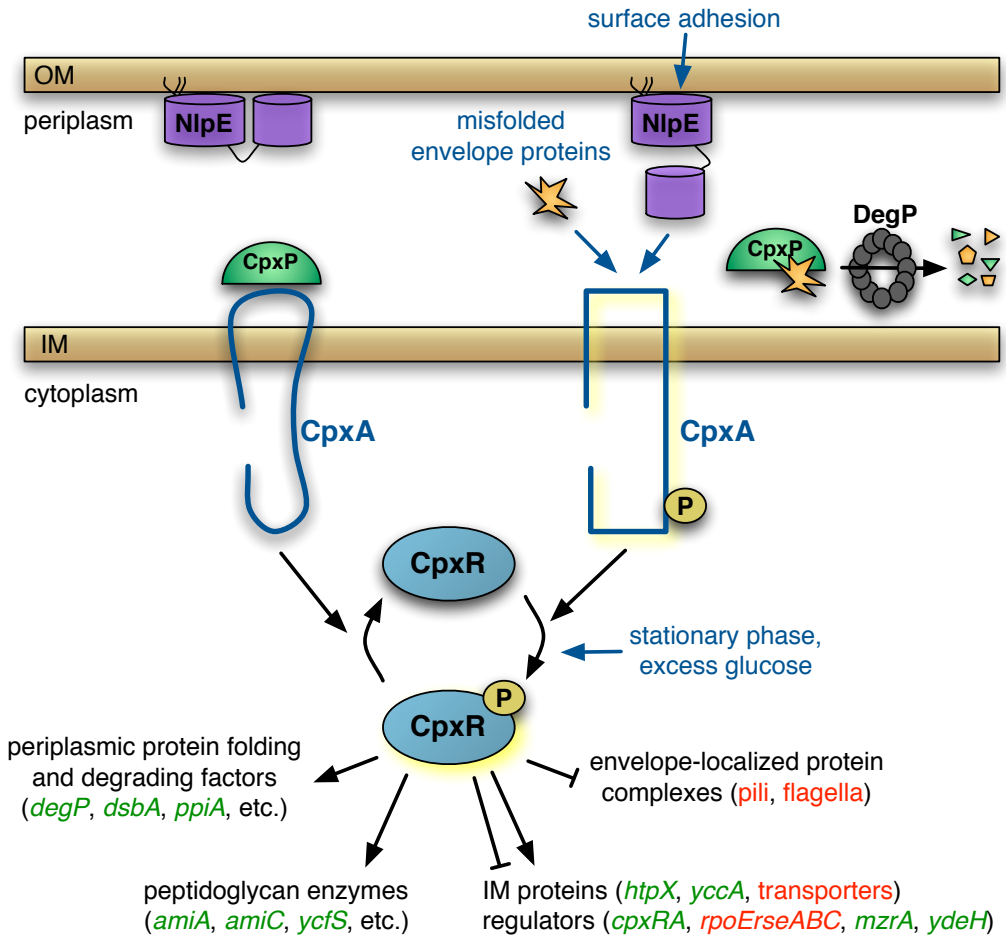

Figure 1-3. Overview of the Cpx pathway in *Escherichia coli*. Under non-inducing conditions (left side of diagram), the CpxA HK acts as a phosphatase on the RR CpxR, keeping it dephosphorylated and therefore inert. CpxP inhibits activation of CpxA, possibly through a direct interaction. Inducing cues enter the Cpx pathway at several points (indicated by blue arrows): surface adhesion is sensed by the OM lipoprotein NlpE, misfolded envelope proteins may be sensed directly by CpxA, and growth/metabolic cues are sensed by CpxR. Under inducing conditions (right side of diagram), the inhibitory molecule CpxP is degraded by DegP, CpxA acts as an autokinase and a CpxR kinase, and phosphorylated CpxR binds to DNA to regulate transcription. Target genes upregulated by CpxR-P (indicated by green text) include those encoding periplasmic protein folding and degrading factors, peptidoglycan metabolic enzymes, and some IM proteins and regulators. Targets downregulated by CpxR-P (indicated by red text) include envelope-localized protein complexes and other IM proteins and regulators.

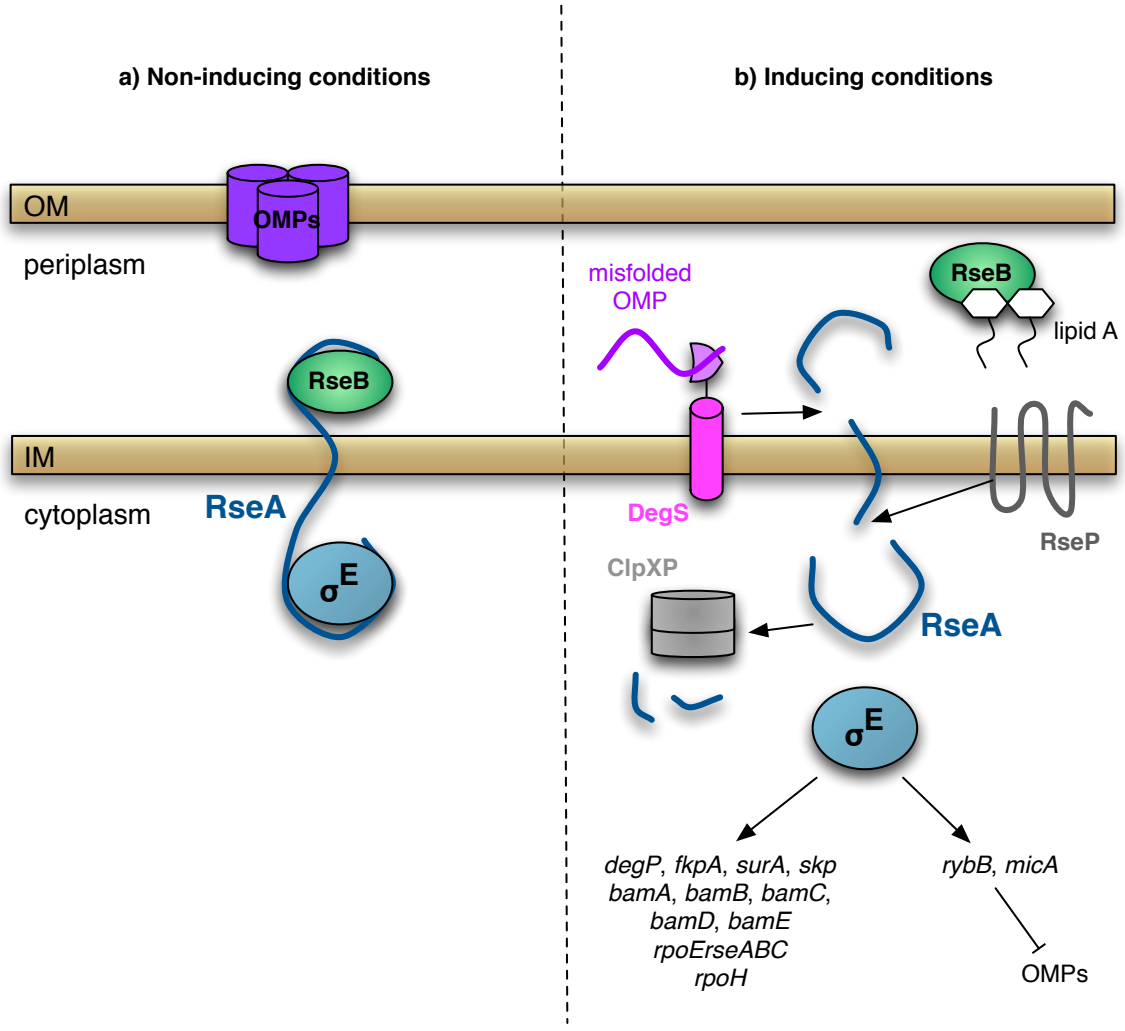

Figure 1-4. Overview of the σ^E pathway in *Escherichia coli*. a) Under non-inducing conditions, σ^E is sequestered at the IM by the anti-sigma factor RseA. The periplasmic regulator RseB also interacts with RseA, protecting it from proteolysis. **b)** Induction of the σ^E response requires the presence of both misfolded OMPs and off-pathway LPS biosynthesis intermediates. Lipid A derivatives interact with RseB, releasing it from its interaction with RseA. Misfolded OMPs interact with the PDZ domain of the protease DegS, activating the protease domain and causing DegS to cleave the periplasmic region of RseA. The truncated RseA is then cleaved by a second protease, RseP, in its transmembrane region. The remaining cytoplasmic portion of RseA is degraded by the ClpXP protease, thereby releasing σ^E into the cytoplasm to interact with RNA polymerase and transcribe its regulon. Genes transcribed by σ^E include those encoding periplasmic chaperones and proteases, the Bam OMP assembly complex, σ^E itself (autoregulation), the heat shock sigma factor σ^H, and the small RNAs RybB and MicA, which post-transcriptionally repress the expression of a variety of OMPs. Figure adapted from (7).

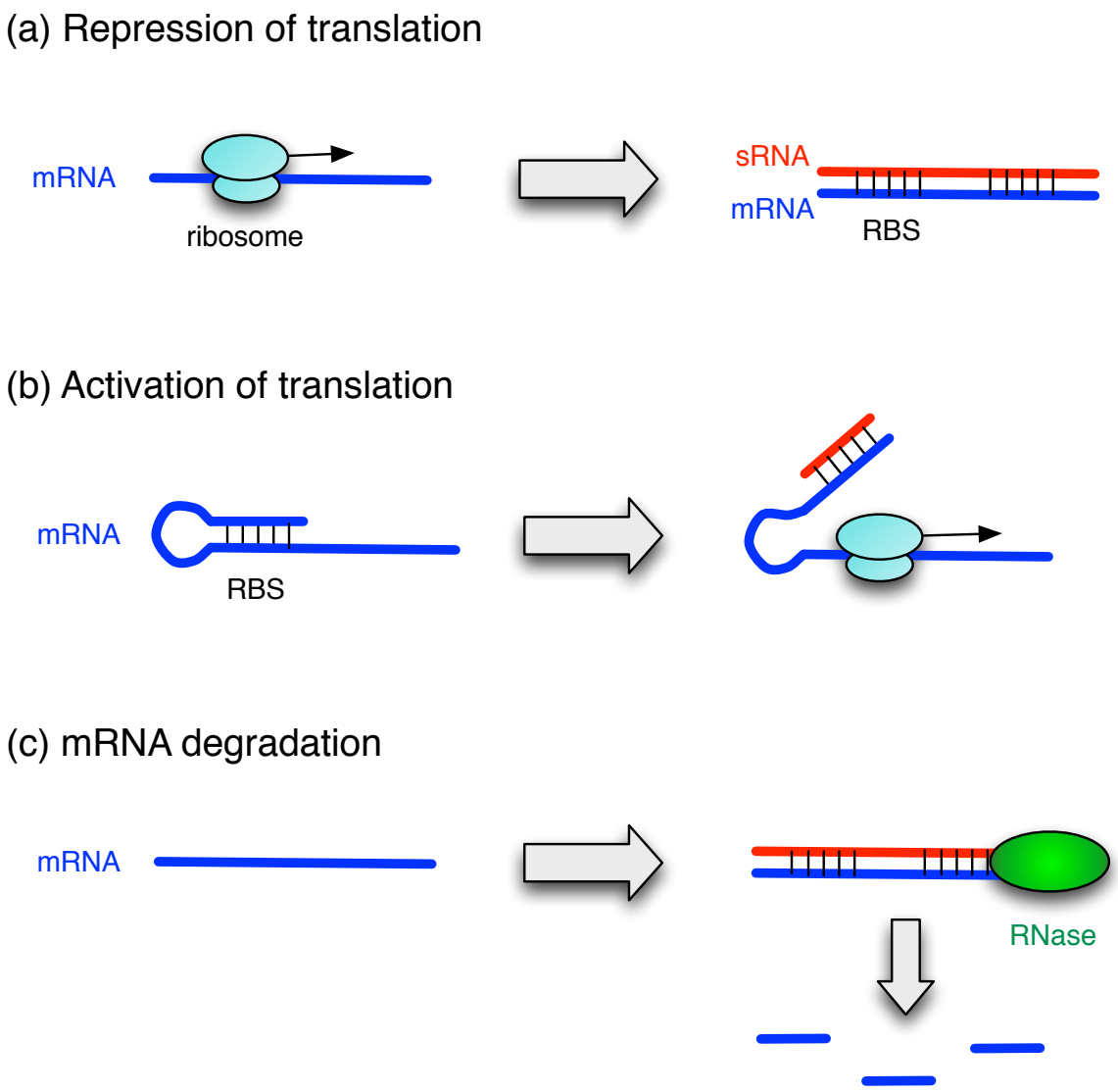

Figure 1-5. Mechanisms of target regulation by base-pairing sRNAs. a) The binding of the sRNA to the mRNA obscures its RBS, thereby preventing translation. **b)** The mRNA contains an inhibitory stem-loop that prevents ribosome binding; the sRNA binds to and removes this inhibitory structure, thereby allowing translation to occur. **c)** The sRNA in complex with the mRNA increases the susceptibility of the mRNA to degradation by RNases. RBS, ribosome binding site. Figure modified from (76).

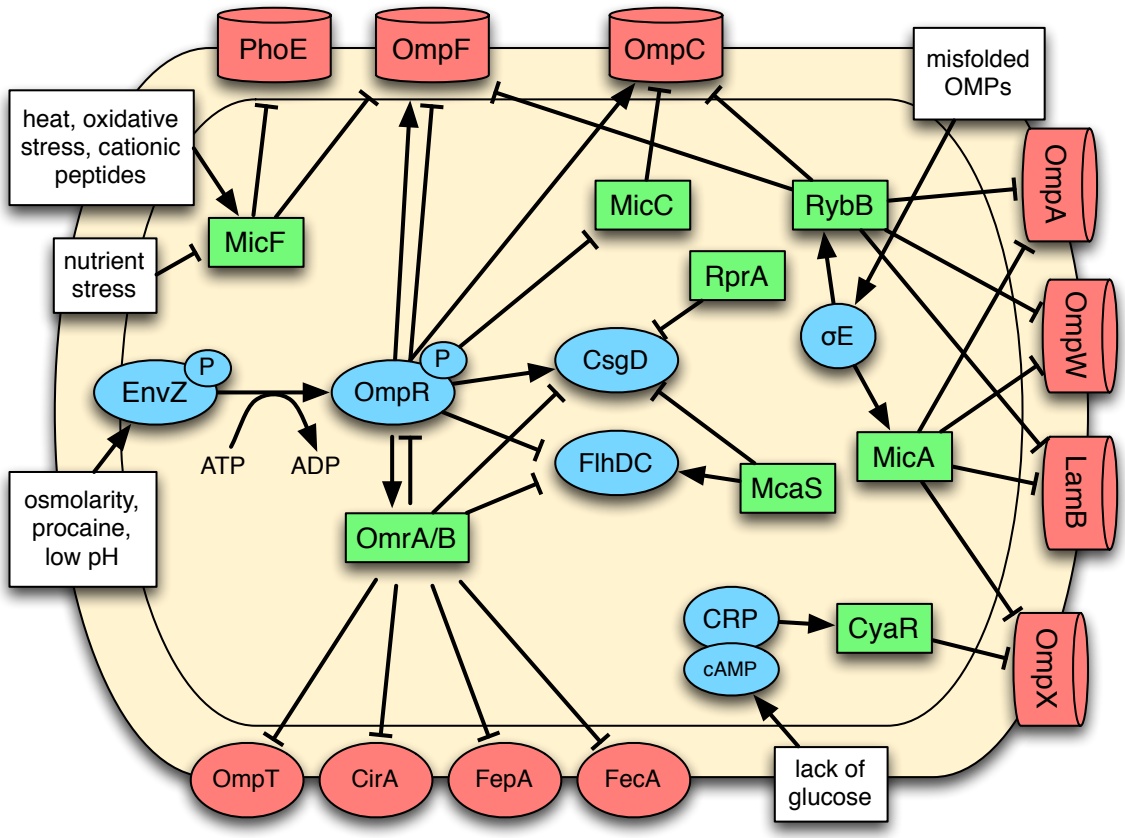

Figure 1-6. Model of the sRNA-OMP regulatory network in *Escherichia coli*. Details can be found in the text. For clarity, some targets of MicA and RybB as well as some sRNAs regulating *csgD* and *flhDC* have been omitted. Regulatory proteins are shown in blue, target proteins are shown in pink, sRNAs are shown in green, and environmental cues are shown in white boxes. Arrows denote positive regulation and lines with bars denote negative regulation. Phosphorylated OmpR is shown to both activate and inhibit OmpF because it can have either effect depending on osmolarity (see text). Figure modified from (204).

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CHAPTER 2

The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus

A version of this chapter has been published: Vogt, S.L., A.Z. Nevesinjac, R.M. Humphries, M.S. Donnenberg, G.D. Armstrong, and T.L. Raivio. (2010) *Molecular Microbiology* **76(5):**1095-1110.

Note: The strains listed as "ALNXXX" in Table 2.1 of this chapter were constructed by Anna Nevesinjac.

2.1 Introduction

The Gram-negative bacterial envelope is the cell's first point of contact with the external environment, and also contains many structures crucial for survival in diverse habitats. Organisms such as *Escherichia coli* have therefore evolved numerous regulatory systems capable of detecting and responding effectively to envelope stress. One such system is the Cpx signal transduction pathway, which is composed of the inner membrane (IM) histidine kinase CpxA and the cytoplasmic response regulator CpxR. In accordance with the behaviour of other two-component systems, CpxA autophosphorylates upon detecting an inducing cue (55). Subsequently, CpxA phosphorylates CpxR, thereby enabling the response regulator to bind to DNA and modify gene transcription (55). In the absence of envelope stress, CpxR is maintained in an inactive state by the phosphatase activity of CpxA (55). One unorthodox aspect of the Cpx system is that a third component, a small periplasmic protein called CpxP, inhibits Cpx pathway activation under non-inducing conditions (56). Since mutation of the CpxA periplasmic sensing domain results in the loss of CpxP-mediated inhibition, it is believed that this inhibition occurs through a direct interaction between the two proteins (56).

Although the precise molecular nature of the envelope perturbation that activates the Cpx response has not been determined, a number of inducing cues have been identified. Known activators of the Cpx response include alkaline pH (14), membrane composition alterations (15, 44) and overproduction of pilus component proteins such as PapE, PapG and BfpA (33, 46). Since all of these inducing cues are expected to generate misfolded envelope proteins, one potential physiological role of the Cpx pathway is to monitor periplasmic protein folding (39). An additional Cpx-activating signal is the overexpression of the outer membrane (OM) lipoprotein NlpE (67). NlpE is required for induction of the Cpx response when *E. coli* cells adhere to hydrophobic surfaces (47), but does not play a role in Cpx sensing of stresses such as alkaline pH or overexpression of Pap pilus subunits (18). Therefore, the Cpx pathway may detect bacterial adhesion to

surfaces in addition to sensing envelope stress.

Once the Cpx response has been activated, CpxR upregulates the expression of numerous genes whose products are involved in envelope protein folding, ameliorating the envelope stress. One of the first identified Cpx regulon members is the periplasmic enzyme DegP (16), which possesses both protease and chaperone functions and therefore is important for envelope protein quality control (70). Other Cpx-regulated proteins include DsbA, the primary disulphide bond oxidoreductase in the *E. coli* periplasm (13, 24, 48) and PpiA (also known as RotA), which catalyses *cis-trans* peptide bond isomerization around proline residues in periplasmic proteins (48). Phosphorylated CpxR also activates transcription of the *cpxRA* and *cpxP* operons, thereby endowing the system with both positive and negative feedback mechanisms (14, 56). Interestingly, the Cpx pathway inhibitor CpxP also plays a stress-combative role, by facilitating degradation of misfolded P-pilus proteins by DegP (31).

Phosphorylated CpxR is also capable of repressing target genes, many of which encode bacterial cell surface appendages. Included in this category are the flagellar motor, chemotaxis and aerotaxis genes *motAB-cheAW*, *tsr* and *aer* (50, 74, 75); the *csgBA* and *csgDEFG* operons, encoding components of the curli fimbriae and its transcriptional regulator (19, 51); and the *pap* genes encoding the uropathogenic *E. coli* P-pilus (25). The Cpx-mediated repression of these genes encoding envelope-localized structures may reflect the benefit of reducing non-essential envelope protein traffic during periods of envelope stress (39). Recent evidence indicates that the Cpx pathway is frequently involved in regulating structures required for virulence in pathogenic Gram-negative bacteria (reviewed by 54), including enteropathogenic *E. coli* (EPEC).

Enteropathogenic *E. coli* is a common cause of acute diarrhoea among infants and young children in developing countries, and has been associated with occasional disease outbreaks in daycares and hospitals in industrialized nations (9). EPEC pathogenesis is thought to proceed by three major steps: (i) initial adherence to epithelial cells of the small intestine, (ii) signal transduction via a type III secretion system (T3SS), and (iii) intimate adherence, which is associated

with enterocyte effacement and the formation of a pedestal beneath the bacterial cell (9). Several adhesins, including flagella, EspA filaments and the *E. coli* common pilus (10, 59), have been proposed to play a role in the initial attachment of EPEC to epithelial cells. However, in typical EPEC strains, the bundle-forming pilus (BFP) is likely the predominant adhesin (10). The primary BFP pilin, bundlin, binds to *N*-acetyllactosamine-like receptors on human cells (29), and BFP filaments from adjacent bacterial cells can also intertwine in rope-like bundles (22). These properties of BFP give rise to EPEC's characteristic localized adherence (LA) phenotype, where bacteria adhere to tissue culture cells in discrete microcolonies (61).

As type IV pili, BFP comprise a complex of proteins that spans all cellular compartments. The extracellular pilus filament contains mainly, and possibly only, polymerized bundlin residues. The pilus is extruded through a donut-like complex of the secretin BfpB, a major component of the OM subassembly of the BFP (17, 58, 62). The periplasmic component BfpU performs an unknown, yet essential, role in BFP elaboration (63). The pilus filament is anchored to the IM by a scaffold composed of the polytopic protein BfpE (58), which interacts with the additional IM component BfpC (12). Finally, two cytoplasmic ATPases are associated with the pilus: BfpD provides the energy required for extension of the pilus filament (2), while BfpF permits retraction of the BFP (1). BfpF may enhance bacterial transmission by permitting disaggregation of individual bacterial cells from microcolonies, allowing the released cells to colonize other areas of the intestine or to be shed by the host (4, 36).

All of the genes encoding BFP components are located in a single 14-gene cluster on the large EPEC attachment factor (EAF) plasmid (68, 71). Non-pathogenic *E. coli* K-12 strains are capable of elaborating BFP when this *bfp* gene cluster is expressed from an inducible promoter (71). The native *bfp* gene cluster in EPEC is highly regulated at the transcriptional level. *bfpA* transcription is increased by the presence of calcium ions and decreased by ammonium ions; transcription is maximal during exponential phase and at a temperature of 37°C (52). Moreover, *bfpA* expression requires the transcriptional activator PerA

(BfpT), which is also encoded on the EAF plasmid (72). Regulation of *perA* expression is similarly complex, being enhanced by the EPEC quorum sensing cascade (66, 69), the Pst phosphate-specific transport system (21), and PerA binding to its own promoter (41). *perA* transcription is repressed by ammonium ions, temperatures above or below 37°C, and the acid resistance regulator GadX (41, 64). These observations suggest that PerA assimilates various environmental signals in order to ensure that BFP are elaborated only under favourable conditions.

Recently, our laboratory has begun to examine the contribution of the Cpx pathway to EPEC virulence gene regulation. MacRitchie *et al*. (40) demonstrated that mutational inactivation of the Cpx pathway has little effect on EPEC T3S; however, activation of the pathway inhibits T3S, at least in part by repressing transcription of translocator and effector genes. Furthermore, we observed that an EPEC *cpxR* mutant has reduced expression of bundlin compared with wild-type EPEC and is defective in the BFP-mediated process of LA (46). In the current study, we examined the basis for the decreased bundlin expression of *cpxR* null EPEC. Although we found that transcription of *bfpA* was not significantly altered in this strain, we determined that several Cpx-regulated folding factors are required for proper BFP biogenesis, suggesting that the *cpxR* mutant has a reduced ability to properly fold BFP protein components. In light of the seemingly conflicting observations that the Cpx pathway positively influences BFP biogenesis but negatively regulates T3S, we also investigated the effect of Cpx pathway activation upon the BFP. We found that, similarly to the T3SS, BFP expression is repressed at the transcriptional level during the Cpx response, demonstrating for the first time that the Cpx response can mediate either positive or negative effects upon a single cell-surface structure, depending on the level of pathway activity.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in the course of this study are listed in Table 2-1. EPEC strains were routinely cultured in LB broth containing the appropriate antibiotics at 37°C with shaking at 225 r.p.m. Strains harbouring *degP* or *cpxA24* mutations were routinely cultured at 30°C with shaking, except when performing assays to detect BFP expression (whole-cell lysates and luminescence assays). Under these circumstances, all strains were grown at 37°C to induce maximal BFP expression. When required, isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen) was added to a concentration of 0.1 mM, unless otherwise indicated. Antibiotics (all from Sigma) were added as necessary to the following concentrations: amikacin (Amk), 3 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}; chloramphenicol (Cam), 25 μg ml^{-1}; and kanamycin (Kan), 50 μg ml^{-1}.

2.2.2 Strain construction

To construct strain SV76, plasmid pKDS302, which contains the fourteen-gene *bfp* cluster expressed from P*trc* (71), was electroporated into strain JPN15, a derivative of E2348/69 that was spontaneously cured of the *bfp*-encoding EAF plasmid (32). The suicide plasmid pRE112*cpxA24* was subsequently transferred into the transformed strain by biparental mating as previously described (40). The presence of the *cpxA24* mutation in this strain was verified by PCR amplification of *cpxA*, showing a deletion of approximately 100 bp, and by confirmation of growth on media containing 3 μg ml^{-1} amikacin. Strain SV75 was constructed in a similar manner, except that pRE112*cpxA24* was conjugated into UMD916.

The *bfpFcpxR*::cam double mutant strain SV82 was constructed by conjugating the suicide plasmid pRE118*cpxR*::cam (40) into strain UMD916 and selecting for chloramphenicol- and sucrose-resistant colonies. Presence of the *cpxR* insertion mutation was verified by PCR analysis (not shown) and Western blotting (Figure 2-9C).

2.2.3 Construction of *bfpA–lux* and *perA–lux* reporter plasmids

To construct the *bfpA–lux* reporter plasmid pJW23, the *bfpA* promoter region was amplified from E2348/69 using the primers ProBFPA-*Eco*RI (5′-GTGAATTCTGCAGGGGAATAATGTTGTTC-3′) and ProBFPA-*Kpn*I (5′-GGGGTACCCCAAGCACCATTGCAGATT-3′) (underlining denotes restriction enzyme tag). The resulting PCR product was purified and digested with the restriction enzymes *Eco*RI and *Kpn*I, then ligated into the multiple cloning site upstream of the promoterless *luxCDABE* operon in pJW15 (40), using standard techniques. A similar procedure was followed to generate the *perA–lux* reporter plasmid pJW22, except using the primers proPER-L (5′-CGGAATTCTACTCACTTAGCCGCGTGTC-3′) and proPER-R2 (5′-GGGGTACCTTAACAATAACGCTAAATTCTCCTC-3′) and the restriction enzymes *Eco*RI and *Bam*HI.

2.2.4 Western blot analysis

Whole-cell lysates for Western blot analysis were generally prepared by subculturing EPEC strains in DMEM: Nutrient Mixture F-12 (DMEM/F12, Gibco) containing 0.1 M Tris (pH 7.4) as previously described (46), with cultures grown to an OD_{600} of 0.5–0.6. The strains carrying pKDS302 or its vector control p*trc*99A were subcultured in LB containing 1 mM IPTG. Electrophoresis and Western blotting were performed as previously described (56). Blots were incubated with primary antisera at the following concentrations: anti-bundlin (20), 1:5000; anti-BfpB (17), 1:10 000; anti-BfpC (12), 1:1000; anti-BfpD, 1:2000; or anti-MBP-CpxR, 1:10 000 (56). BfpD antiserum was raised in guinea pigs against BfpD protein, purified as described (12). The secondary anti-rabbit (or for BfpD anti-guinea pig) immunoglobulin G-alkaline phosphatase conjugates (Sigma) were used at a concentration of 1:25 000. Blots were developed with the chemiluminescent Immun-Star AP Substrate Pack (Bio-Rad) according to manufacturer's directions.

2.2.5 Autoaggregation assay

Autoaggregation assays were performed as previously described (46). Most EPEC strains were subcultured in DMEM/F12 containing 0.1 M Tris (pH 7.4) for these assays, except those strains carrying pKDS302 or p*trc*99A, which were subcultured in LB containing 1 mM IPTG. Assays were performed in triplicate at least two times.

2.2.6 Localized adherence assay

The ability of EPEC strains to exhibit LA to HEp-2 tissue culture cells was assessed as previously described (73), with bacteria incubated on host cells for 1 h. Experiments were performed two times in triplicate.

2.2.7 Luminescence assay

Activity of the *bfpA–lux* and *perA–lux* reporters was assessed as previously described (40), with EPEC strains subcultured in DMEM/F12 containing 0.1 M Tris (pH 7.4) at 37°C with aeration. Data presented here represent growth for 4 h post subculture unless otherwise stated. Assays were performed at least two times in quintuplicate.

2.3 Results

2.3.1 Cpx-regulated periplasmic protein folding and degrading factors are required for normal BFP biogenesis

One potential explanation for the reduced BFP expression of an EPEC *cpxR* null mutant (46) is that this strain produces insufficient amounts of periplasmic protein folding factors to ensure proper folding of BFP component proteins. This hypothesis seemed particularly plausible given that Zhang and Donnenberg (1996) previously demonstrated that DsbA is required for stability of the BFP major pilin, bundlin. To examine the role of Cpx-regulated protein folding and degrading factors in BFP biogenesis, we constructed EPEC mutants with null alleles of the genes *dsbA*, *degP*, *cpxP* and *ppiA*, all of which are

positively regulated by the Cpx response in EPEC as well as in *E. coli* K-12 (57).

To analyse BFP protein levels in the Cpx regulon mutants, whole-cell lysates were collected from wild-type and mutant EPEC strains grown under conditions favourable for BFP expression (see Section 2.2.4). Western blotting revealed that the expression of bundlin is eliminated in the *dsbA* mutant, as expected; bundlin levels are also reduced in the *degP* and *cpxP* mutants (Figure 2-1A). Mutation of *ppiA*, on the other hand, did not reduce bundlin levels (Figure 2-1A). Interestingly, the *dsbA*, *degP* and *cpxP* mutations affected not only the abundance of the pilin monomer, but also the expression of other BFP proteins spanning multiple cellular compartments (Figure 2-1A). Production of the OM secretin BfpB, the IM component BfpC and the cytoplasmic ATPase BfpD was reduced in these strains, suggesting that not just the pilin protein but the entire BFP apparatus is influenced by the activity of the Cpx pathway. Expression of the BFP proteins could be restored in the *degP*, *cpxP* and *dsbA* mutants by complementation *in trans* (data not shown). Since the expression of bundlin was reduced but not abolished in several Cpx regulon mutants, we examined the functionality of the BFP expressed by these EPEC strains using autoaggregation and LA assays. The autoaggregation assay measures the ability of EPEC strains to form aggregates when grown in liquid culture, a phenotype that is correlated with BFP elaboration (1). All of the Cpx regulon mutants, including strains lacking *degP*, *ppiA*, *cpxP* and *dsbA*, had a reduced capacity to aggregate under these assay conditions compared with the wild-type strain E2348/69 (Figure 2-1B). Among these strains, only the *ppiA* mutant was able to aggregate significantly better than the *bfp*-negative control strain JPN15, in accordance with the higher levels of BFP protein synthesis in this strain (Figure 2-1A).

Localized adherence assays were performed to assess whether the Cpx regulon mutants were compromised in their ability to adhere to host cells. The *dsbA* mutant was not examined for LA, since it was previously demonstrated that LA is abolished in the absence of DsbA (76). After 1 h of interaction between bacteria and host cells, the *degP* mutant had formed significantly fewer microcolonies than the wild-type control E2348/69, adhering to only 33.4% as

many host cells as did the wild-type strain ($P < 0.0001$, Fisher's exact test). The *cpxP* mutant was slightly but reproducibly impaired in LA as well, adhering to 84.9% as many host cells as E2348/69 ($P < 0.0001$, Fisher's exact test). The *ppiA* mutant, however, was able to adhere at wild-type levels (101% of control, $P > 0.05$).

Mutation of *dsbA* clearly had the largest influence on BFP protein levels (Figure 2-1A) and LA (76) among the Cpx regulon members tested. It was therefore possible that the BFP expression defect of the *cpxR* mutant might be entirely attributable to decreased expression of *dsbA*. To test this hypothesis, we overexpressed *dsbA* from an inducible promoter in both the *dsbA* and *cpxR* mutants. Western blotting to detect two BFP components (bundlin and BfpB) revealed that, although pDsbA substantially increased BFP protein synthesis in the *dsbA* mutant, the overexpression of *dsbA* did not increase BFP expression in the *cpxR* mutant (Figure 2-2). The BFP expression defect of the *cpxR* mutant therefore cannot be entirely explained by its decreased DsbA expression.

Overall, these results demonstrate that Cpx-regulated periplasmic protein folding and degrading factors, including DsbA, DegP, CpxP and possibly others yet to be identified, are required for normal elaboration of the EPEC BFP.

2.3.2 Transcription of *bfpA* is not significantly affected by mutation of *cpxR*

Since phosphorylated CpxR is capable of modulating transcription of target genes, we wished to assess whether transcription of the *bfp* operon or the gene encoding its transcriptional regulator, *perA*, might also be altered in the EPEC *cpxR* mutant. To measure transcription, we constructed *bfpA–lux* and *perA–lux* transcriptional fusions using the previously described reporter plasmid pJW15 (40). These fusions were designed to contain all of the upstream regulatory elements known to be important for regulation of *bfpA* and *perA* transcription (41, 52). We validated the use of these fusions to measure *bfpA* and *perA* transcription by comparing their activity under different culture and strain conditions to the activity of previously published *bfpA-cat* and *perA-cat* fusions (41, 52). Both the *bfpA–lux* and *perA–lux* reporters were (i) expressed at higher levels in Dulbecco's

modified Eagle's medium (DMEM) than in Luria–Bertani (LB), (ii) expressed at considerably lower levels in EPEC lacking *perA* than in wild-type EPEC, and (iii) repressed by the addition of 20 mM ammonium sulphate to the DMEM growth medium (data not shown).

To assess the effect of a *cpxR* null mutation upon *bfpA* and *perA* transcription, each reporter was transformed into EPEC wild-type and *cpxR* mutant strains. Bacteria were subcultured into DMEM, and the activity of the reporters was monitored every 2 h up to 8 h post subculture. No consistent difference in the activity of either reporter could be detected between wild-type and *cpxR* null strains at any time point ($P > 0.05$, unpaired *t*-tests) (Figure 2-3). In conjunction with the results described above, these data suggest that reduced transcription of the *bfp* gene cluster cannot explain decreased BFP elaboration in the EPEC *cpxR* mutant, while a diminished level of folding factors, such as DegP, CpxP and DsbA, could account for this difference.

2.3.3 Activation of the Cpx response inhibits BFP elaboration

In order to determine whether Cpx pathway activation would reduce BFP expression as is the case with EPEC T3S, we compared BFP synthesis in wild-type EPEC with that of an EPEC *cpxA24* mutant (40). The *cpxA24* mutation, arising from a deletion of approximately 30 amino acids in the periplasmic sensing domain of CpxA, results in constitutive activation of the Cpx response regardless of the presence or absence of inducing cues (55). Strikingly, the BFP component proteins bundlin, BfpB, BfpC and BfpD were undetectable in the *cpxA24* mutant EPEC (Figure 2-4A). Further experiments revealed that this strain exhibited significantly reduced autoaggregation ($P < 0.0001$, unpaired *t*-test) (Figure 2-4B); as expected based on the Western blot results, the ability of the *cpxA24* mutant to aggregate was comparable to that of the *bfp*$^-$ control strain JPN15 (not shown). Furthermore, the *cpxA24* mutant was incapable of forming microcolonies on epithelial cells during the LA assay, while the wild-type strain E2348/69 formed microcolonies on 83.5% of epithelial cells under the conditions

used in this assay. These results demonstrate that the *cpxA24* constitutively active mutation dramatically inhibits BFP expression.

To confirm that the inhibition of BFP synthesis could be reproduced with different methods of Cpx pathway activation, we also examined the effect of overexpressing the lipoprotein NlpE. This inducing cue has the advantage of being relatively specific to the Cpx pathway, unlike more general cues such as alkaline pH, and also allows us to activate the wild-type Cpx two-component system. Compared with the vector control, wild-type EPEC overexpressing NlpE produced reduced amounts of the BFP components bundlin, BfpB, BfpC and BfpD (Figure 2-5). Although NlpE overexpression resulted in a less pronounced phenotype than mutation of *cpxA*, these results confirmed that activation of the Cpx response inhibits BFP elaboration.

2.3.4 Cpx pathway activation primarily affects BFP at the transcriptional level

To begin to uncover the mechanism of BFP inhibition in the *cpxA24* mutant, we examined expression of the *bfpA–lux* and *perA–lux* transcriptional reporters in this strain compared with wild-type EPEC. The activity of both reporters was reduced in the *cpxA24* strain (Figure 2-6A). The activity of the *bfpA–lux* reporter was reduced about 20-fold relative to the wild-type strain, and this reduction was consistent regardless of the growth phase of the cultures (data not shown). The *perA–lux* reporter was also expressed at lower levels in the *cpxA24* mutant, but the reduction was milder (only about twofold at the time point shown in Figure 2-6A), and was not observed after 6 h or longer post subculture (data not shown). In agreement with the Western blotting results (Figures 2-4 and 2-5), overexpression of NlpE also reduced *perA–lux* and *bfpA–lux* activity (Figure 2-6B).

The strongly decreased transcription of *perA* and *bfpA* in the *cpxA24* mutant suggested that the defect in BFP expression in this strain may be entirely the result of reduced transcription of the *bfp* operon. To separate transcriptional and post-transcriptional effects of the Cpx pathway, we generated a pair of strains

in which the *bfp* gene cluster is expressed from a promoter that is not regulated by the Cpx pathway. This was accomplished by transforming the plasmid pKDS302, containing the entire *bfp* gene cluster under the control of an IPTG-inducible promoter (71), into the pEAF plasmid-cured strain JPN15. Although pEAF contains a total of 115 protein-coding genes (6), no genes other than the *bfp* gene cluster and the *per* operon are known or suspected to have any role in either BFP expression or the Cpx response. This information, along with the ability of JPN15 (pKDS302) to express functional BFP (see Figure 2-7), validates the use of this strain in further experiments.

The *cpxA24* mutation was introduced into JPN15 (pKDS302) to give rise to a strain in which the activated Cpx pathway cannot influence BFP expression at the transcriptional level. Western blotting revealed that in this strain background, the *cpxA24* mutation no longer diminished expression of bundlin and BfpB (Figure 2-7A). Activation of the Cpx response in this strain was verified by an increased ability to grow on media containing amikacin (data not shown) and an increased cellular level of CpxR (Figure 2-7A), resulting from autoactivation of *cpxRA* gene transcription (56). Moreover, the autoaggregation assay demonstrated that both the wild-type and *cpxA24* mutant JPN15 (pKDS302) strains are equally capable of expressing functional BFP on the cell surface (Figure 2-7B). These results indicate that there is little or no inhibition of BFP expression at the post-transcriptional level in the *cpxA24* mutant.

Since the defect in BFP expression in the *cpxA24* mutant seemed to be mainly due to decreased transcription, and because expression of *perA* in this strain was repressed, we wished to determine whether BFP expression could be restored to the *cpxA24* mutant by expression of *perA* from an inducible promoter. The *perA* overexpression plasmid pCS-A (41) was transformed into the *cpxA24* mutant, and BFP expression was monitored by Western blotting. Although bundlin and BfpB could not be detected in the *cpxA24* mutant carrying the vector control plasmid, expression of these proteins was restored to near-wild-type levels in the *cpxA24* mutant overexpressing *perA* (Figure 2-8). The ability of a transcriptional activator to restore BFP expression in the *cpxA24* mutant further

supports the notion that the defect in BFP elaboration in this strain can largely be attributed to decreased transcription from the *bfpA* promoter.

2.3.5 Phenotypes of *bfpF cpx* double mutants

Experiments conducted thus far suggested that the *cpxA24* mutation leads to a drastic decrease in pilus assembly, mainly as a result of decreased *bfp* gene transcription, while the *cpxR* mutation does not affect *bfp* transcription, but rather the ability to synthesize a functional pilus. To corroborate these observations, we examined the effect of *cpx* mutations in *bfpF* mutant EPEC, which lacks the ATPase that powers retraction of the BFP (1). Studies with type IV pilus retraction double mutant strains have previously been used in other species to separate mutations that prevent pilus assembly from those that simply destabilize the pilus and therefore cause pili to be rapidly retracted (8). When the first category of mutation is introduced into a pilus retraction mutant, functional pili still cannot be synthesized. However, when the pilus-destabilizing class of mutation is introduced into a pilus retraction mutant, piliation is restored, since even structurally abnormal pili are trapped on the cell surface. We therefore hypothesized that introducing the *cpxA24* mutation into the *bfpF* mutant would strongly reduce BFP elaboration, much like in wild-type EPEC, since *bfp* gene expression, and therefore BFP assembly should be blocked. In contrast, we predicted that introducing a *cpxR* null allele may not affect BFP elaboration in the *bfpF* mutant, since this mutation does not appear to completely block BFP assembly in an otherwise wild-type strain, as evidenced by the reduced but not abolished ability of this strain to undergo LA (46).

The *cpxA24* and *cpxR*::cam alleles were introduced into the *bfpF* mutant UMD916 as described in Section 2.2.2, and the mutations were confirmed by PCR analysis and anti-CpxR Western blotting, which revealed increased CpxR expression in the *cpxA24* mutant and, as expected, a loss of CpxR in the *cpxR*::cam strain (Figure 2-9A and C). We then examined BFP synthesis in the resulting double mutants by Western blotting and autoaggregation assays. Introducing the *cpxA24* mutation into UMD916 strongly reduced BFP synthesis.

Bundlin and BfpB could not be detected in this strain by Western blotting (Figure 2-9A), and its ability to autoaggregate was reduced to a level comparable to that of the *cpxA24* single mutant (Figure 2-9B). Interestingly, introducing the *cpxR::cam* null allele into UMD916 did not appreciably decrease BFP expression, as assessed by Western blotting (Figure 2-9C), nor did this mutation significantly reduce autoaggregation of UMD916 ($P > 0.05$, unpaired *t*-test) (Figure 2-9D). The phenotypes of the *bfpF* double mutants are therefore consistent with those observed in previous experiments, indicating a defect in BFP assembly (by virtue of a defect in expression of the *bfp* genes) in the *cpxA24* mutant, but no assembly defect in the *cpxR* mutant (i.e. BFP can still be made in this mutant, albeit not as well as in wild-type strains). Rather, the decreased autoaggregation and LA of the *cpxR* single mutant (46) can likely be attributed to unstable pili that are prone to retraction.

2.4 Discussion

In this study, we investigated the mechanism by which the Cpx envelope stress response regulates EPEC BFP expression. Previously, we demonstrated that EPEC lacking a functional Cpx pathway, due to mutation of *cpxR*, exhibits reduced expression of bundlin and diminished LA to tissue culture cells (46). In the current study, we found that this decreased BFP synthesis cannot be attributed to reduced transcription of the *bfp* gene cluster in the *cpxR* mutant (Figure 2-3). On the other hand, we found that mutating the Cpx-regulated periplasmic proteins DsbA, DegP and CpxP reduced both BFP protein accumulation and BFP-mediated processes like bacterial aggregation (Figure 2-1). Therefore, the decreased BFP synthesis in the EPEC *cpxR* mutant can most likely be explained by insufficient expression of one or more factors crucial for the proper folding of BFP components. Importantly, BFP protein expression could not be increased in the *cpxR* mutant simply by overexpressing the disulphide bond oxidoreductase DsbA (Figure 2-2). This finding indicates that the BFP expression defect in the *cpxR* mutant is not simply the product of insufficient DsbA, which was previously

shown to be necessary for bundlin stability (76). The increased piliation of the *cpxR bfpF* double mutant compared with the *cpxR* single mutant (Figure 2-9) supports the assertion that the *cpxR* mutant is fully capable of expressing the *bfp* genes and synthesizing all required BFP proteins. However, the *cpxR* mutant likely assembles a structurally defective pilus that is prone to pilus retraction; this pilus instability is most likely the result of decreased expression of folding factors like DsbA, DegP and CpxP. We believe that the adherence defects of the *cpxR* mutant are more likely the result of pilus retraction rather than shedding of BFP into the culture medium, since we have never been able to detect bundlin in *cpxR* culture supernatants by Western blotting nor have we observed any BFP, attached to cells or sheared off, in the *cpxR* mutant by transmission electron microscopy (46; data not shown).

Since our previous work showed that activating the EPEC Cpx response inhibits type III secretion, we also examined the effect of Cpx pathway activation upon BFP expression. Strikingly, we were unable to detect BFP proteins in the EPEC *cpxA24* mutant (Figure 2-4), in which the Cpx response is constitutively active due to a mutation in the periplasmic sensing domain of CpxA. A similar, though less severe, BFP repression phenotype was observed when the wild-type Cpx pathway was activated by overexpressing NlpE (Figure 2-5). We believe this inhibition of BFP expression is achieved primarily at the transcriptional level. Transcription of *bfpA* was reduced approximately 20-fold in the *cpxA24* mutant (Figure 2-6), and introducing the *cpxA24* mutation into a strain in which the *bfp* gene cluster is expressed from an inducible promoter did not impair BFP synthesis (Figure 2-7), demonstrating that the Cpx response does not cause any significant post-transcriptional repression of BFP expression. Our hypothesis is also consistent with the observation that BFP expression was restored to the *cpxA24* mutant by overexpressing the *bfp* transcriptional activator PerA (Figure 2-8), which raises the possibility that the Cpx response could influence *bfp* transcription through PerA (discussed in more detail below). In contrast to the results obtained with the *bfpF cpxR* double mutant, BFP synthesis was not restored in a *bfpF cpxA24* double mutant (Figure 2-9), again suggesting that the

cpxA24 mutant is incapable of BFP synthesis rather than synthesizing a defective pilus. The Cpx pathway therefore appears to mediate both transcriptional and post-transcriptional effects upon the BFP; the transcriptional effects are negative and are evident only when the pathway is activated, as in the *cpxA24* mutant. On the other hand, the post-transcriptional effects, which are likely mediated by interaction between Cpx-regulated periplasmic protein folding factors and BFP protein components, are positive and occur even when the pathway is basally active, such as in wild-type cells. This is the first report that reconciles the ability of the Cpx pathway to act at multiple regulatory levels to mediate both positive and negative effects upon a single cell-surface structure.

2.4.1 Role of Cpx-regulated protein folding factors in BFP biogenesis

In this work, we confirmed the importance of DsbA in BFP synthesis, as well as implicating two additional Cpx regulon members in this process: DegP and CpxP. DsbA is the major catalyst of disulphide bond formation in the *E. coli* periplasm (24) and is required for stability of bundlin (76). The C-terminal disulphide bond formed by DsbA in bundlin is a conserved feature of all Gram-negative type IV pilin proteins (11), which implies that disulphide bond oxidoreductases may be essential for type IV pilus biogenesis in a variety of pathogens. Recent studies have shown that DsbA homologues are required for proper folding of the OM secretin PilQ, a component of the *Neisseria meningitidis* type IV pilus (65), and that EPEC DsbA is required for stability of the OM secretin EscC, which forms part of the T3S complex (43). Thus, DsbA may also be required for disulphide bond formation in BFP components other than the pilin itself; indeed, all of the BFP proteins we examined were undetectable or noticeably less abundant in the *dsbA* mutant (Figure 2-1A).

Although its phenotype was less dramatic than that of the *dsbA* mutant, the EPEC *degP* mutant also expressed reduced levels of BFP proteins (Figure 2-1A), which correlated with decreased abilities to autoaggregate (Figure 2-1B) and to adhere to epithelial cells. These data are consistent with those obtained by Humphries *et al.* (2010), who found that bundlin expression was delayed in a

degP mutant compared with wild-type EPEC. This defect was attributed to the loss of the chaperone activity of DegP. To our knowledge, this is the first example of a role for DegP as a chaperone in facilitating pilus assembly.

The expression of BFP proteins was also reduced in the *cpxP* mutant (Figure 2-1A). The role of CpxP in BFP biogenesis, however, remains unclear. CpxP has two distinct cellular functions – it acts both as a repressor of Cpx pathway activation (56) and as an accessory factor to the protease DegP (31). Since we also showed that activating the Cpx pathway results in decreased BFP expression (Figures 2-4 and 2-5), it is possible that the reduced BFP expression in the *cpxP* mutant is simply the result of the Cpx pathway being more active in this strain. Another possibility is that CpxP promotes the folding of BFP substrate proteins. In addition to facilitating proteolysis of misfolded Pap pilins by DegP (31), CpxP has recently been shown to possess weak chaperone activity *in vitro* (53). Future *in vitro* studies of CpxP's chaperone function will hopefully clarify whether and how CpxP interacts with BFP components such as bundlin.

Interestingly, we observed that the EPEC mutants expressing a reduced level of bundlin, including the *dsbA*, *degP* and *cpxP* mutants, also expressed lower levels of other BFP proteins contained within different cellular compartments (Figure 2-1). This observation extends to the protein BfpD, whose cytoplasmic location precludes a direct interaction with periplasmic folding factors. One possible explanation is that reduced bundlin expression or stability results in proteolysis of other BFP components, or perhaps feedback repression of transcription. However, non-polar mutations in *bfpA* have no effect on the stability of other BFP proteins (58), nor do they decrease transcription from the *bfpA* promoter (S.L. Vogt and T.L. Raivio, unpublished observations). These data support the idea that Cpx-regulated proteins such as DsbA and DegP are required not only for the folding of bundlin, but likely also assist in the folding of additional BFP proteins as well. Most of the BFP proteins are required for the stability of at least one other BFP component, and often several; in fact, mutating the IM scaffolding protein BfpE destabilizes all of the BFP components to some extent (58). Since BfpE contains a large C-terminal periplasmic domain (5), this

protein may require Cpx regulon members for proper folding and stability, which could account for destabilization of the entire pilus in the *dsbA*, *degP* and *cpxP* mutants. Alternatively, Cpx-regulated folding factors may play a more indirect role in BFP assembly, by facilitating the folding of another envelope protein that is essential for this process.

2.4.2 Transcriptional regulation of *bfp* by CpxR

In addition to these post-transcriptional effects mediated by Cpx-regulated folding factors, we also investigated how the Cpx pathway affects *bfp* transcription. Using a *bfpA–lux* reporter, we found that activating the Cpx response results in decreased *bfpA* transcription (Figure 2-6). CpxR therefore acts as a transcriptional repressor of the *bfp* operon, through either direct or indirect means. Given this result, we might have expected that *bfpA* transcription would be elevated in the *cpxR* null mutant. In contrast, we found no difference in *bfpA* transcription between wild-type and *cpxR* mutant strains (Figure 2-3). These results suggest that the Cpx pathway affects *bfpA* transcription only when there is a high concentration of phosphorylated CpxR in the cell, such as in the *cpxA24* mutant (55), which could be the result of a low-affinity binding site for CpxR upstream of *bfpA* or one of its regulators.

Although *bfpA* transcription is attuned to numerous environmental parameters (52), only PerA has thus far been identified as a direct transcriptional regulator of the *bfp* operon (30, 72). Numerous environmental signals and genetic regulators feed into transcriptional regulation of *perA* (21, 41, 64, 69). PerA, in turn, directly activates expression of the *bfp* operon and indirectly activates transcription of type III secretion genes via PerC and Ler (49). We favour the hypothesis that CpxR also mediates its transcriptional repression of the *bfp* operon at least partially via repression of *perA*. We observed that a *perA–lux* reporter is expressed at lower levels when the Cpx pathway is activated (Figure 2-6). We also found that we could restore BFP synthesis in the *cpxA24* mutant by overexpressing *perA* (Figure 2-8). Additionally, we could not detect any CpxR consensus binding sequences upstream of *bfpA* either visually or using the online

tool Virtual Footprint (45). Finally, during time-course experiments examining *bfpA–lux* activity when the Cpx response was induced by overexpressing NlpE, we found that repression of the *bfpA–lux* reporter occurred only 3 h or later after inducing NlpE overexpression (data not shown). In contrast, the expression of genes that are known to be directly regulated by CpxR is altered within 30 min of NlpE overexpression (57). The slow kinetics of *bfpA* repression is suggestive of indirect regulation. At this time, we cannot say whether CpxR directly represses transcription of *perA*, represses transcription of *perA* through another regulator, or perhaps affects *bfpA* through another, yet to be identified regulator, thereby possibly inducing negative feedback on *perA* expression. Direct repression of *perA* seems least likely because, as with the *bfpA* promoter, we could not find a CpxR consensus sequence upstream of *perA*, and the kinetics of *perA* repression after NlpE overexpression are comparably slow. Another intriguing possibility is that CpxR regulates PerA post-transcriptionally, for example by upregulating a cytoplasmic protease that degrades PerA, thereby also reducing *perA* transcription by preventing PerA autoactivation (41). Further studies will hopefully elucidate the molecular mechanism(s) by which the Cpx pathway affects this important regulator of EPEC virulence.

2.4.3 Regulation of pilus expression by the Cpx response

In addition to its role in regulating BFP expression, the Cpx response also modulates the expression of several other types of pili in *E. coli* strains. The Cpx response represses transcription of the structural and regulatory genes for synthesis of curli, adhesive structures involved in surface attachment and biofilm formation (3), via direct binding of CpxR~P to the promoter regions of the relevant genes (19, 34, 51). Additionally, when the Cpx pathway is activated, transcription of the *pap* structural genes, encoding the chaperone-usher-type Pap pili of uropathogenic *E. coli*, is reduced through a mechanism involving the inhibition of Lrp-mediated phase variation (25). Conversely, and parallel to results obtained here with BFP, *cpxR* mutations decrease Pap pilus elaboration even when the *pap* genes are expressed from an inducible promoter, an effect that

has been attributed to diminished expression of periplasmic protein folding and degrading factors (28). Finally, similarly to curli and the Pap pilus, Cpx-mediated inhibition of elaboration of the conjugal F-pilus also occurs by transcriptional repression of the *tra* operon encoding F-pilus structural components (60). In this case, however, the action of CpxR is indirect. Activation of the Cpx pathway results in upregulation of the cytoplasmic protease/chaperone pair HslVU (37), leading to degradation of the *tra* operon activator TraJ (23, 37). Therefore, Cpx-regulated protein folding and degrading factors seem to promote the elaboration of several pilus types, while activating the Cpx response represses transcription of pili-encoding genes. This finding may point to the importance of preventing pilus component accumulation in the periplasm when the cell is attempting to recover from a period of envelope stress and folding factors required for pilus assembly would be limiting.

2.4.4 Significance of the Cpx pathway to EPEC pathogenesis

The results presented in this report demonstrate that the activated Cpx response strongly inhibits the expression of BFP, which are believed to be a major adhesin responsible for EPEC early adherence (10, 29). These findings raise the question of whether induction of the Cpx response is likely to occur during EPEC colonization of the human intestine. Humphries *et al.* (2010) have demonstrated that the Cpx response is actually downregulated by BFP retraction induced by the receptor analogue LacNAc-BSA. This observation suggests that the Cpx response is unlikely to be triggered by the binding of EPEC to the host epithelium. Perhaps activation of the Cpx pathway is more likely to occur during the transmission phase of the EPEC life cycle, when bacteria can be faced with a variety of stresses in the abiotic environment and when expression of pili may be disadvantageous. However, our results predict that a basal level of Cpx pathway activity is likely important during the early stages of infection, since BFP are not fully expressed and appear to be prone to retraction in the absence of CpxR.

This work, in combination with that of MacRitchie *et al*. (40), shows that activating the Cpx response inhibits the expression of two major EPEC virulence

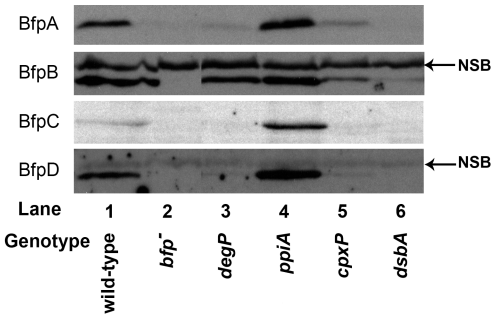
determinants, BFP and the type III secretion system. At least part of this inhibitory effect may be the result of decreased expression of *perA* (Figure 2-6), the master regulator of virulence in EPEC. Activation of the Cpx response appears to also repress virulence processes in the related enteric pathogen *Salmonella enterica* serovar Typhimurium (26). As such, a chemical inducer of the Cpx envelope stress response could represent a valuable therapeutic tool, potentially capable of preventing intestinal colonization by numerous human pathogens.

2.5 Tables and Figures

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

Strain or plasmid	Description	Source or reference
Bacterial strains		
E2348/69	Prototypical EPEC O127:H6 strain	38
JPN15	Spontaneous pEAF-cured derivative of E2348/69	32
ALN88	E2348/69 *cpxR*::Kan^R	46
ALN188	E2348/69 *degP*::Kan^R	This study
ALN190	E2348/69 *ppiA*::Kan^R	This study
ALN194	E2348/69 *cpxP*::Kan^R	This study
ALN195	E2348/69 *cpxA24* (Amk^R)	40
ALN234	E2348/69 *cpxR*::Cam^R	40
TR1121	E2348/69 *dsbA*::Kan^R	This study
SV76	JPN15 *cpxA24* (pKDS302) (Amk^R Amp^R)	This study
UMD916	E2348/69 *bfpF*::Kan^R	1
SV75	UMD916 *cpxA24* (Kan^R Amk^R)	This study
SV82	UMD916 *cpxR*::Cam^R (Kan^R)	This study
Plasmids		
p*trc*99A	High copy-number expression vector with IPTG-inducible promoter (Amp^R)	Pharmacia
pDsbA	p*trc*99A-based *dsbA* overexpression vector (Amp^R)	7
pJW22	*perA* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	40; this study
pJW23	*bfpA* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	40; this study
pCA24N	High copy-number expression vector with IPTG-inducible promoter (Cam^R)	35
pCA-*nlpE*	pCA24N-based *nlpE* overexpression vector (Cam^R)	35
pKDS302	p*trc*99A containing the *bfpA-L* gene cluster expressed from an IPTG-inducible promoter (Amp^R)	71
pMPM-K3	Low copy-number cloning vector derived from pACYC184 and pBluescript (Kan^R)	42
pCS-A	pMPM-K3-derived *perA* overexpression plasmid (Kan^R)	41

a)



b)

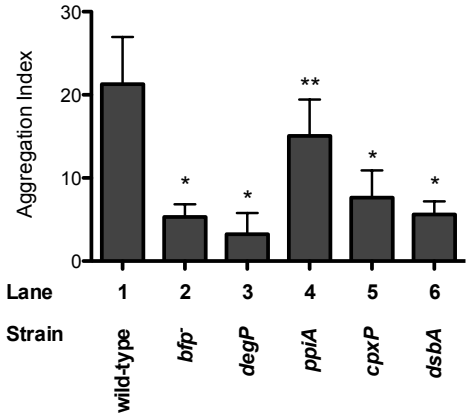


Figure 2-1. BFP expression is reduced in EPEC mutants lacking *dsbA*, *degP* and *cpxP*. a) Western analysis of bundlin (BfpA), BfpB, BfpC and BfpD expression in wild-type and mutant EPEC strains: lane 1, wild-type (E2348/69); lane 2, *bfp*$^-$ strain (JPN15); lane 3, *degP*::kan (ALN188); lane 4, *ppiA*::kan (ALN190); lane 5, *cpxP*::kan (ALN194); lane 6, *dsbA*::kan (TR1121). Whole-cell lysates were collected from EPEC grown in DMEM/F12 as described in Section 2.2.4. Samples were collected from each strain at least three times; one representative blot is shown. Arrows denote non-specific bands (NSB). **b)** Results of autoaggregation assay performed on EPEC Cpx regulon mutants. Autoaggregation assays were performed as described in Section 2.2.5; the overall average and standard deviation resulting from two separate experiments performed in triplicate are shown. One asterisk (*) denotes a value significantly different from positive control E2348/69; two asterisks (**) denote a value significantly different from both E2348/69 and negative control JPN15 (one-way ANOVA with Bonferroni's multiple comparison test; $P < 0.05$).

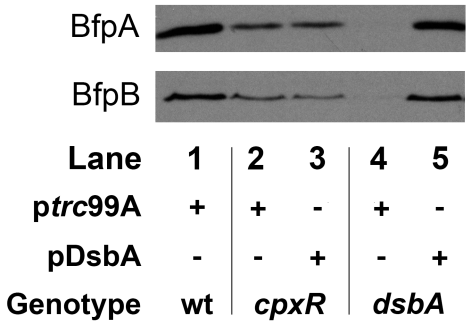


Figure 2-2. Overexpression of *dsbA* in an EPEC *cpxR* mutant does not restore BFP synthesis. Western blotting was used to detect expression of the proteins bundlin (BfpA) and BfpB in the wild-type strain E2348/69 (p*trc*99A) (lane 1), the *cpxR* mutant ALN88 (p*trc*99A) (lane 2), ALN88 (pDsbA) (lane 3), the *dsbA* mutant TR1121 (p*trc*99A) (lane 4) and TR1121 (pDsbA) (lane 5). Whole-cell lysates were collected from EPEC grown in DMEM/F12 without IPTG as described in Section 2.2.4. Samples were collected from each strain at least three times; one representative blot is shown.

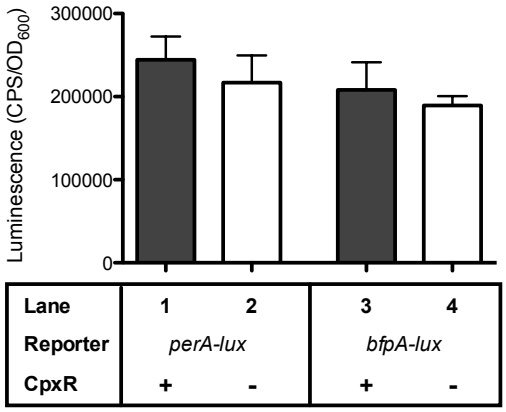


Figure 2-3. Transcription of *bfpA* and *perA* is not affected by a *cpxR* null mutation. After overnight growth in LB, wild-type and *cpxR* mutant EPEC strains transformed with the *bfpA–lux* and *perA–lux* reporter plasmids were subcultured 1:100 into DMEM/F12 as described in Section 2.2.7. Luminescence (cps, counts per second) and optical density of the culture (OD_{600}) were measured every 2 h. The normalized luminescence was calculated by subtracting the luminescence reading of a medium blank from the luminescence of the culture sample, then dividing that value by the OD_{600} of the culture, reduced by the OD_{600} of the blank. Experiments were performed in quintuplicate at least twice; the mean and standard deviation from the 4 h reading from one experiment are shown.

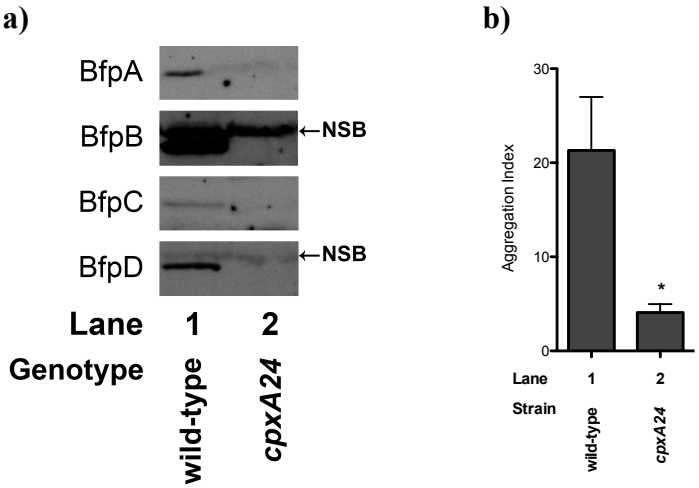


Figure 2-4. BFP elaboration is inhibited by the *cpxA24* gain-of-function mutation. a) Western analysis of bundlin (BfpA), BfpB, BfpC and BfpD expression in wild-type strain E2348/69 (lane 1) and *cpxA24* mutant EPEC strain ALN195 (lane 2). Whole-cell lysates were collected from EPEC grown in DMEM/F12 as described in Section 2.2.4. Samples were collected from each strain at least three times; one representative blot is shown. Arrows denote non-specific bands (NSB). **b)** Results of autoaggregation assay performed on wild-type (lane 1) and *cpxA24* mutant (lane 2) EPEC. Autoaggregation assays were performed as described in Section 2.2.5; the overall average and standard deviation resulting from two separate experiments performed in triplicate are shown. The asterisk (*) denotes a value significantly different from positive control E2348/69 ($P < 0.0001$, unpaired *t*-test).

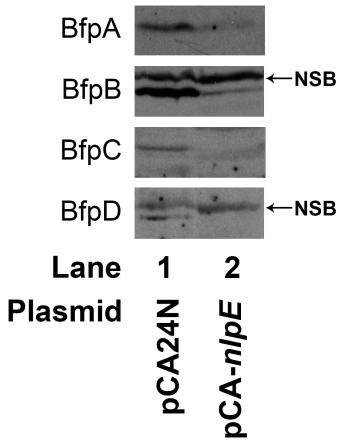
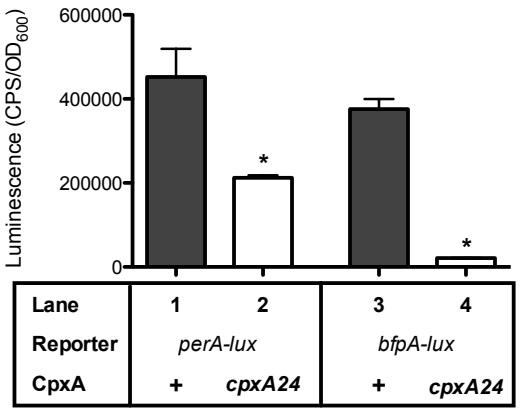


Figure 2-5. BFP expression is reduced when the Cpx response is activated by overexpressing NlpE. Western analysis of bundlin (BfpA), BfpB, BfpC and BfpD expression in wild-type strain E2348/69 harbouring the vector control pCA24N (lane 1) or the overexpression plasmid pCA-*nlpE* (lane 2). Whole-cell lysates were collected from EPEC grown in DMEM/F12 as described in Section 2.2.4. Subcultures were grown for 2 h before being induced with 1 mM IPTG, followed by an additional 3 h incubation at 37°C. Samples were collected from each strain at least three times; one representative blot is shown. Arrows denote non-specific bands (NSB).

a)



b)

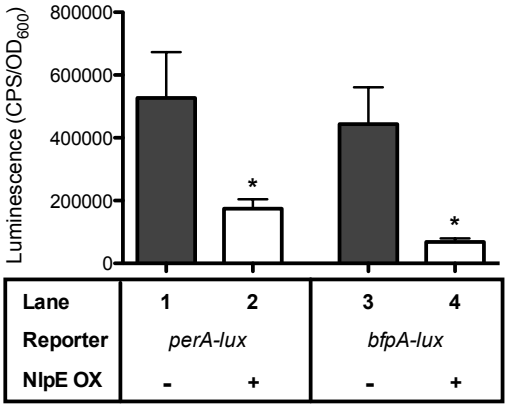


Figure 2-6. Transcription of *bfpA* and *perA* is repressed during the Cpx response. EPEC strains harbouring *bfpA–lux* and *perA–lux* reporters were subcultured in DMEM/F12 as described in Section 2.2.7. Normalized luminescence, in units of counts per second (cps) per optical density of the culture (OD_{600}) was measured hourly and calculated as described in the Figure 2-3 caption. Experiments were performed in quintuplicate at least twice; the mean and standard deviation from one representative experiment are shown. An asterisk (*) denotes significant difference from the wild-type or vector control ($P < 0.05$, unpaired *t*-test). **a)** Comparison of *bfpA–lux* and *perA–lux* activity in wild-type strain E2348/69 and *cpxA24* mutant ALN195. Results presented are from 4 h post subculture. **b)** Comparison of *bfpA–lux* and *perA–lux* activity in wild-type EPEC harbouring the vector control pCA24N or the overexpression plasmid pCA-*nlpE*. Strains were subcultured into DMEM/F12 for 2 h, then induced with 0.1 mM IPTG. Results presented are from 5 h post induction.

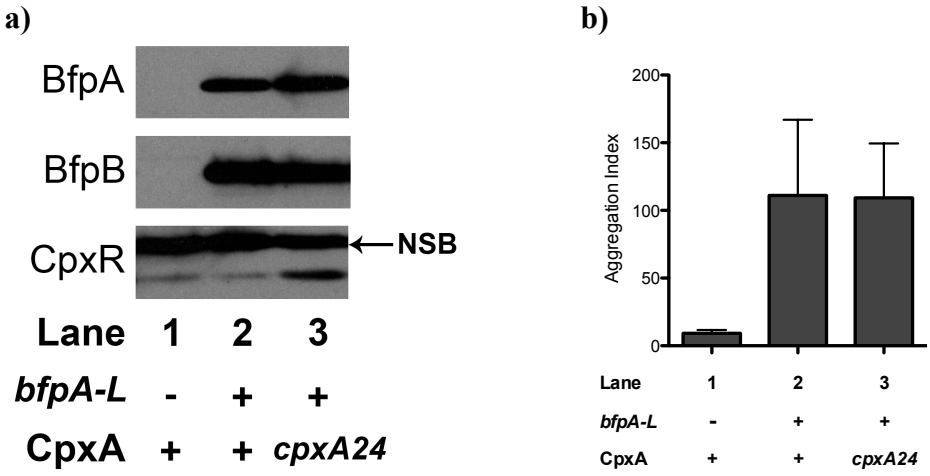


Figure 2-7. The *cpxA24* mutation does not affect BFP elaboration when the *bfp* genes are expressed from an inducible promoter. **a)** Western analysis of bundlin (BfpA), BfpB and CpxR expression in the negative control JPN15 (p*trc*99A) (lane 1), the positive control JPN15 (pKDS302), which expresses the *bfpA-L* operon (lane 2), and its isogenic *cpxA24* mutant SV76 (lane 3). Whole-cell lysates were collected from EPEC grown in LB containing 1 mM IPTG as described in Section 2.2.4. Samples were collected from each strain at least two times; one representative blot is shown. Arrows denote non-specific bands (NSB). **b)** Results of autoaggregation assay performed on JPN15 (p*trc*99A) (lane 1), JPN15 (pKDS302) (lane 2) and SV76 (lane 3). Autoaggregation assays were performed as described in Section 2.2.5; the overall average and standard deviation resulting from three separate experiments performed in triplicate are shown.

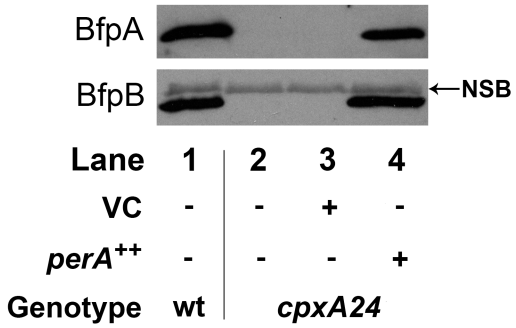


Figure 2-8. Overexpression of *perA* in an EPEC *cpxA24* mutant restores BFP synthesis. Western blotting was used to detect expression of the proteins bundlin (BfpA) and BfpB in the wild-type strain E2348/69 (lane 1), the *cpxA24* mutant ALN195 (lane 2), the vector control (VC) strain ALN195 (pMPM-K3) (lane 3) and the *perA*-overexpressing strain ALN195 (pCS-A) (lane 4). Whole-cell lysates were collected from EPEC grown in DMEM/F12 with 50 μM IPTG as described in Section 2.2.4. Samples were collected from each strain at least twice; one representative blot is shown. The arrow denotes a non-specific band (NSB).

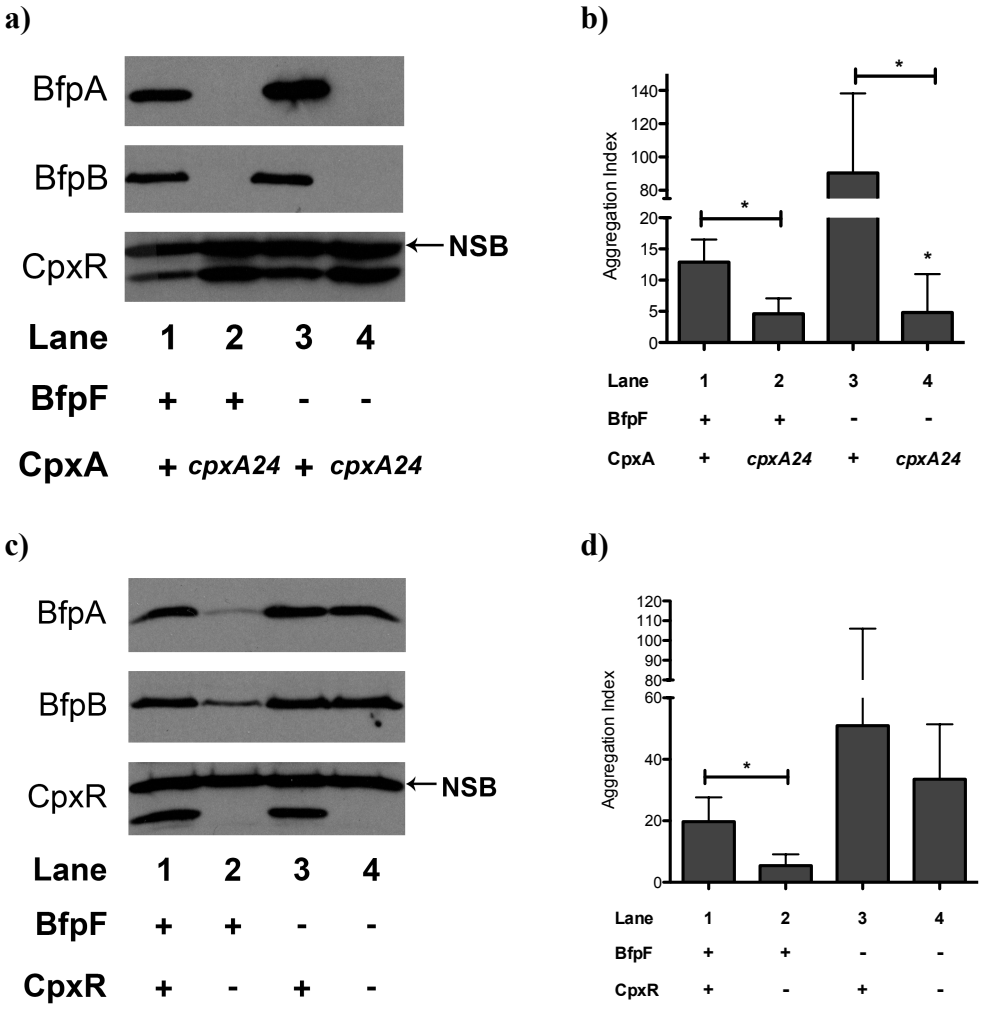


Figure 2-9. The *cpxA24* mutation, but not the *cpxR*::cam mutation, reduces BFP elaboration in pilus retraction-defective EPEC. a) and c) Western analysis of bundlin (BfpA), BfpB and CpxR expression in wild-type and mutant EPEC strains. Whole-cell lysates were collected from EPEC grown in DMEM/F12 as described in Section 2.2.4. Samples were collected from each strain at least two times; one representative blot is shown. Arrows denote non-specific bands (NSB). **b) and d)** Results of autoaggregation assays, which were performed as described in Section 2.2.5; the overall average and standard deviation resulting from three separate experiments performed in triplicate, are shown. The asterisk (*) denotes a value significantly different from the relevant *cpx*$^+$ control strain ($P < 0.05$, unpaired *t*-test). Shown in (a) and (b) are wild-type strain E2348/69 (lane 1), *cpxA24* mutant ALN195 (lane 2), *bfpF* mutant UMD916 (lane 3) and *bfpF cpxA24* double mutant SV75 (lane 4). Shown in (c) and (d) are E2348/69 (lane 1), *cpxR* mutant ALN234 (lane 2), UMD916 (lane 3) and *bfpF cpxR* double mutant SV82 (lane 4).

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CHAPTER 3

Hfq reduces envelope stress by controlling expression of envelope-localized proteins and protein complexes in enteropathogenic *Escherichia coli*

A version of this chapter has been submitted for publication:
Vogt, S.L. and T.L. Raivio. (2013) *Molecular Microbiology* MMI-2013-13324.

3.1 Introduction

The envelope is a Gram negative bacterium's interface with the outside world, performing critical roles in nutrient acquisition, waste removal, surface attachment, and motility, among other functions. Malfunctions in the envelope compartment must therefore be rapidly detected and ameliorated. Numerous envelope stress responses, including the σ^E and Cpx responses, perform these tasks in *Escherichia coli*. The specific stress detected by the σ^E response is the misfolding of outer membrane proteins (OMPs). When β-barrel porins become misfolded, they expose a C-terminal motif that is detected by the inner membrane (IM) protease DegS, which initiates a proteolytic cascade ultimately leading to the degradation of the anti-sigma factor RseA and the release of active σ^E into the cytoplasm (2, 33, 96). RNA polymerase containing σ^E then initiates the transcription of a suite of genes whose products promote OMP folding and biogenesis, including proteases (*degP*), chaperones (*surA*, *fkpA*, *skp*), and the β-barrel insertion machinery (*bamA*, *bamB*, *bamC*, *bamD*, *bamE*), as well as the *rpoErseABC* operon encoding σ^E itself (12, 16, 22, 59, 71, 74-76).

A complementary envelope stress response is mediated by the Cpx two-component system, which consists of the IM-localized histidine kinase CpxA and the cytoplasmic response regulator CpxR. Although the molecular nature of the Cpx inducing cue has not yet been identified, several alterations to the envelope are known to activate the Cpx response, including alkaline pH (13, 61), alterations to the composition of the IM (14, 58), and overexpression of pilin proteins including PapE and PapG from uropathogenic *E. coli* (UPEC) and BfpA from enteropathogenic *E. coli* (EPEC) (42, 62). Since all of these cues have the potential to cause envelope protein misfolding, CpxA may sense some feature of misfolded proteins; however, the Cpx response appears to monitor the status of periplasmic and IM proteins, while OMPs are monitored by the σ^E response (93). In the presence of an inducing signal, CpxA autophosphorylates and then phosphorylates CpxR, which subsequently activates or represses the transcription of dozens of target genes (9, 73). CpxR positively regulates a collection of genes

encoding envelope-localized protein folding and degrading factors, including *degP*, *dsbA*, and *ppiA* (12, 15, 67). Another gene positively regulated by the Cpx response is *cpxP*, encoding a periplasmic inhibitor of the Cpx pathway that also possesses some chaperone activity. The Cpx response also represses the expression of a variety of envelope proteins that may exacerbate protein misfolding; these include numerous envelope-localized macromolecular complexes, such as flagella, type III secretion systems (T3SSs), and several types of pili (19, 35, 51, 54, 92, 97).

Both σ^E and CpxR help to maintain homeostasis in the envelope by regulating the transcription of genes with envelope-related functions. In addition, many envelope proteins are also regulated post-transcriptionally. This regulation is frequently mediated by small regulatory RNAs (sRNAs) and the RNA chaperone protein Hfq. sRNAs are non-coding RNA molecules approximately 50 to 300 nucleotides in length that alter gene expression in a variety of ways (83). Most of the well-studied sRNAs in *E. coli* act by base-pairing at or near the ribosome binding site of their target mRNA(s), thereby interfering with ribosome binding and preventing translation. However, sRNAs may also alter the secondary structure of their target mRNAs in order to increase translation or alter their rate of processing or degradation by RNases (25). Since the sRNAs are usually encoded at a different chromosomal locus than their target mRNAs, the region of complementarity between the two molecules is typically short and imperfectly matched. The conserved RNA binding protein Hfq is therefore usually required to facilitate formation of the sRNA-mRNA duplexes (90). Hfq has several additional effects on the cellular RNA pool, including protecting sRNAs from cleavage by RNases and altering the rate at which mRNAs or mRNA-sRNA duplexes are degraded (47, 90). *hfq* mutants have pleiotropic phenotypes as a result of the diversity of Hfq-RNA interactions.

Recent studies indicate that Hfq and sRNAs are intimately involved in the σ^E envelope stress response in several Gram negative organisms. Under conditions in which the σ^E envelope stress response is activated due to problems with OMP folding or insertion, σ^E promotes the transcription of two sRNA genes,

rybB and *micA* (41, 65, 85). These sRNAs bind to and repress translation of the transcripts for every major porin, many minor OMPs, and several other proteins (28, 41, 65). As a result, OMP synthesis is diminished, thereby reducing the burden on the periplasmic protein folding machinery and gradually reducing envelope stress. However, in *hfq* mutants, the stability and function of RybB, MicA, and numerous other sRNAs that repress translation of *omp* mRNAs is decreased (30, 91). Therefore, too many OMP precursors are translocated into the envelope, leading to OMP misfolding and chronic activation of the σ^E envelope stress response (7). Although σ^E activation has been described in *E. coli*, *Salmonella*, and *Vibrio cholerae hfq* mutants (17, 24, 31), the effect of the loss of Hfq upon other envelope stress responses has not been assessed.

Our goal in this study was to examine how deletion of *hfq* affects induction of the σ^E and Cpx envelope stress responses in both non-pathogenic (K-12) and enteropathogenic strains of *E. coli*. As expected, we found that the σ^E envelope stress response was activated in *Δhfq* mutants of both *E. coli* K-12 and EPEC. Here we show for the first time that deletion of *hfq* activates the Cpx envelope stress response; however, Cpx activation occurred only in the EPEC *Δhfq* mutant and not in *E. coli* K-12. Importantly, the alterations to the envelope that induce the two stress responses in EPEC *Δhfq* are distinct, with altered porin expression causing σ^E pathway activity but increased pilus expression contributing to Cpx pathway activation. We further show that Hfq regulates pilus expression by controlling the stability of the *perA* transcript, encoding an EPEC master regulator of virulence. Our results indicate that Hfq plays a larger role in regulating the contents of the envelope than previously appreciated, particularly in pathogenic *E. coli*.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3-1. Unless otherwise stated, strains were cultured in Luria-Bertani (LB) broth at 37ºC

with aeration at 225 rpm. When required, isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen) was added to a concentration of 0.1 mM. Antibiotics (Sigma) were added when necessary for plasmid maintenance at the following concentrations: chloramphenicol (Cam), 25 μg/ml; kanamycin (Kan), 50 μg/ml.

3.2.2 Strain and plasmid construction

All EPEC mutants were constructed by allelic exchange (21). The *Δhfq* mutation was constructed by a 3-fragment ligation: regions ~1 kb upstream and downstream of *hfq* were amplified by PCR using primer sets hfqupF-hfqupR and hfqdownF-hfqdownR, respectively (primer sequences, including restriction sites, are given in Table 3-2). The PCR products and suicide vector pRE112 were restriction digested with the appropriate enzymes, and then all three fragments were ligated together. The other deletion mutations (*ΔompA*, *ΔompC*, *ΔompF*, *Δbfp*, and *ΔgadY*) were constructed by overlap extension PCR (39) using the primer sets listed in Table 3-2. Overlap PCR products were restriction digested and ligated into pRE112. Deletion constructs were transferred onto the EPEC chromosome as previously described (18). All mutations were confirmed by PCR.

All *E. coli* K-12 mutants were constructed by P1 transduction (79) of the appropriate mutation from the Keio collection (4). The kanamycin resistance markers were removed from transductants by Flp/FRT-mediated recombination (36) to produce unmarked deletions.

3.2.3 Luminescence assay

The activity of *lux* reporters in different strain backgrounds was measured as previously described (51). Normalized luminescence was calculated by dividing the raw luminescence by the optical density at 600 nm (OD_{600}) of the same culture in order to account for differences in cell numbers between samples. Assays were performed at least two times in quintuplicate.

3.2.4 Western blot analysis

Whole-cell lysates for Western blot analysis were prepared from subcultures grown in LB to an OD_{600} of 0.5 to 0.6. Culture densities were adjusted to give an OD_{600} of 0.5 in a volume of 1 ml; samples were then pelleted and resuspended in 50 µl 2× sample buffer (125 mM Tris [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 6% sodium dodecyl sulfate, 0.2% bromophenol blue). Electrophoresis and Western blotting were performed as previously described (8, 92). Quantification of Western blot signals was performed using a Bio-Rad ChemiDoc MP system with Image Lab software.

3.2.5 OM preparations

OM preparations were made essentially as described by Lobos and Mora (1991). Strains were subcultured 1:100 in 6 ml LB and grown to an OD_{600} of 0.6 to 0.7. A whole cell lysate was prepared by pelleting 1 ml of the culture and resuspending in 50 µl 2× sample buffer. The remaining 5 ml of culture were pelleted and resuspended in 1 ml of 100 mM Tris-HCl, pH 8.0. Cells were lysed by sonication and cell debris removed by centrifugation at 10,600 × *g* for 5 min in a microfuge at 4ºC. The supernatant was then centrifuged at 20,800 × *g* at 4ºC for 45 min to pellet the membrane fraction. Pellets were resuspended in 500 µl of solubilization buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2% Triton X-100) and incubated at 37ºC with agitation for 30 min to solubilize the IM fraction. Insoluble material containing the OM was pelleted as above, washed once in solubilization buffer, then pelleted again. Pellets were resuspended in 15 µl of 100 mM Tris-HCl, pH 8.0 containing 2% SDS. After addition of an equal volume of 2× sample buffer, whole-cell lysates and OM fractions were subjected to SDS-PAGE and staining with Coomassie Blue.

3.2.6 Localized adherence assay

The ability of EPEC strains to adhere to HEp-2 cultured cells was assessed as previously described (89), except that bacterial subcultures were grown in LB rather than the usual DMEM. The *Δhfq* mutant was subcultured 1:40, while the

wild-type was subcultured 1:400, as CFU counts showed that these inocula gave rise to equivalent numbers of CFUs at the end of the 1 h subculture. Cells and bacteria were incubated together for 45 min. Experiments were performed two times in triplicate.

3.2.7 RNA stability assay

The RNA stability assay was performed essentially as described by Hansen and Kaper (2009). Three replicate cultures of wild-type and *Δhfq* EPEC were grown in LB overnight, then subcultured 1:100 into fresh LB and grown at 37ºC with aeration to an OD_{600} of approximately 0.6. Rifampicin was added to a final concentration of 250 μg/ml. 0.9 ml samples were withdrawn after 0, 1, 2, and 4 minutes, and cells were immediately lysed in 0.9 ml TRIzol Reagent (Ambion). RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described (48). DNase treatment was performed on 1 μg of RNA using the TURBO DNA-*free* kit (Ambion), followed by cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen). Duplicate control samples were prepared in the same way, but without the addition of reverse transcriptase, to ensure a lack of genomic DNA contamination in the cDNA samples. qRT-PCR was performed by the relative quantification (ddCt) method, using primers rrsBF and rrsBR (Table 3-2) to amplify the endogenous control *rrsB* (16S rRNA), and primers perAFqPCR and perARqPCR (Table 3-2) to amplify *perA*.

3.2.8 Statistical analysis

Data analysis was performed using Prism version 5.0f (GraphPad Software, Inc.). For comparison of luminescence activity between strains, we used one-way analysis of variance followed by either Dunnett's (for comparing all mutants to a wild-type control) or Bonferroni's (for comparing selected pairs of strains) multiple comparison test. Localized adherence proportions were compared using Fisher's exact test.

3.3 Results

3.3.1 Effect of *hfq* deletion on σ^E and Cpx envelope stress responses

Since previous studies have revealed a link between loss of *hfq* and envelope stress in several Gram-negative organisms (17, 24, 31), we chose to examine the effect of deleting *hfq* on the σ^E and Cpx envelope stress responses in several strains of *E. coli*. We selected two *E. coli* K-12 strains: MC4100, in which the Cpx envelope stress response has been thoroughly characterized (68, 73), and W3110, which has undergone fewer modifications since its initial isolation than MC4100 and is thought to be more representative of a wild-type *E. coli* strain (34, 66). We also deleted *hfq* in a pathogenic strain background, EPEC strain E2348/69. The phenotypes of the three *Δhfq* mutants were consistent with previous reports (32, 87): all three mutants grew more slowly than their respective wild-type strains, the W3110 and E2348/69 *Δhfq* mutants exhibited reduced motility (MC4100 is non-motile), and E2348/69 *Δhfq* had increased type III secretion (T3S) relative to wild-type EPEC (data not shown).

In order to examine activity of the σ^E and Cpx envelope stress responses, wild-type and *Δhfq* mutants were transformed with plasmids encoding *rpoE-lux*, *degP-lux* and *cpxP-lux* transcriptional reporters. The *rpoE-lux* reporter is positively regulated by σ^E, *cpxP-lux* is positively regulated by the Cpx response, and *degP-lux* is positively regulated by both σ^E and Cpx (68). We found that the *rpoE-lux* and *degP-lux* reporters were activated in the *Δhfq* mutants of all three *E. coli* strains tested, with reporter expression in the *Δhfq* mutants 10- to 50-fold higher than in the respective wild-type strains (Figures 3-1A and 3-1B). These results suggest that the σ^E envelope stress response was activated in *Δhfq* mutants of both pathogenic and non-pathogenic *E. coli*. In contrast, the *cpxP-lux* reporter was significantly activated only in the EPEC *Δhfq* mutant, with reporter expression more than 11-fold higher than in wild-type EPEC (Figure 3-1C). There was less than a twofold difference in *cpxP-lux* expression between wild-type and *Δhfq* mutants of *E. coli* K-12 strains MC4100 and W3110 (Figure 3-1C). To confirm these results, we performed Western blotting to detect CpxR, expression

of which is also induced by activation of the Cpx pathway (72). CpxR protein levels were markedly higher in the EPEC *Δhfq* mutant than in the wild-type strain, while there was a smaller difference in CpxR levels between wild-type and *Δhfq E. coli* K-12 strains (Figure 3-1D). We therefore concluded that loss of Hfq led to activation of the σ^E envelope stress response in multiple *E. coli* strains, but the Cpx response was activated by loss of Hfq only in the EPEC background.

3.3.2 OMP misregulation induces the σ^E response in *E. coli Δhfq* mutants

Since σ^E pathway activation in *Salmonella* and *Vibrio hfq* mutants has previously been linked to aberrant OMP expression (7, 17), we examined the OMP profiles of our wild-type and *Δhfq E. coli* strains by SDS-PAGE and Coomassie Blue staining. In all three *Δhfq* mutants, we observed an altered OMP profile, with the abundance of a variety of major and minor OMPs changed in the mutant compared with the wild-type strain (Figure 3-2). These data were consistent with the hypothesis that alterations in OMP abundance were responsible for σ^E response activation in the *Δhfq* mutants. To further test this idea, we constructed deletion mutations in the genes encoding the major OMPs (OmpA, OmpC, and OmpF) in wild-type and *Δhfq* MC4100 and EPEC and examined the effect of these mutations on σ^E pathway activity. Deletion of either *ompA* or *ompC* from wild-type MC4100 and EPEC, and additionally deletion of *ompF* from EPEC, significantly reduced σ^E pathway activity ($P<0.05$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 3-3). Even in the MC4100 and EPEC *Δhfq* backgrounds, in which the σ^E response is strongly activated, deletion of *ompC* significantly reduced σ^E pathway activity ($P<0.05$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 3-3). These results further support the hypothesis that misregulation of OMP expression activates the σ^E response in *E. coli Δhfq*.

3.3.3 Bundle-forming pilus overexpression contributes to Cpx pathway induction in EPEC *Δhfq*

We next sought to explain the observation that the Cpx envelope stress

response is activated in EPEC *Δhfq* but not the *E. coli* K-12 *Δhfq* mutants. The Cpx pathway has been shown to be activated by overexpression of pilins (42, 62, 69); in particular, *bfpA*, which encodes the major pilin of the EPEC type IV bundle-forming pilus (BFP), activates the Cpx pathway when exogenously expressed in *E. coli* MC4100 (62). Since the BFP is encoded in the genome of EPEC but not *E. coli* K-12 strains, we hypothesized that misregulation of *bfp* expression in the EPEC *Δhfq* mutant could account for strain-specific activation of the Cpx response. By Western blotting, we found that expression of the BFP components bundlin (BfpA) and BfpB (encoding the pore-forming outer membrane secretin) was indeed elevated in EPEC *Δhfq* compared to the wild-type strain (Figure 3-4). BFP expression endows EPEC with its localized adherence (LA) phenotype, in which bacteria form adherent microcolonies on the surface of human epithelial cells (27). In agreement with its increased BFP protein expression, we found that EPEC *Δhfq* also had an enhanced ability to undergo LA, with 79.8% of cultured cells infected with the *Δhfq* mutant having at least one bacterial microcolony, while the wild-type strain formed microcolonies on only 52.3% of infected cells under the conditions tested ($P<0.0001$, Fisher's exact test). Importantly, the enhanced LA phenotype of the *Δhfq* mutant demonstrates that this strain's abundant BFP proteins are properly localized to the envelope, where they could be a source of envelope stress.

To assess the contribution of BFP overexpression to Cpx pathway activation in EPEC *Δhfq*, we deleted the *bfpA-L* gene cluster from both wild-type and *Δhfq* mutant EPEC, then measured *cpxP-lux* activity in each strain (Figure 3-5). Deletion of the *bfp* genes from EPEC *Δhfq* resulted in an approximately 50% reduction in *cpxP-lux* activity, suggesting that BFP overexpression contributes to Cpx pathway activation in this strain. However, *cpxP-lux* expression was still more than fourfold higher in the *Δhfq Δbfp* double mutant than in wild-type EPEC, implying that there are additional sources of envelope stress aside from BFP overexpression that activate the Cpx pathway in EPEC *Δhfq*. Interestingly, *cpxP-lux* activity was reduced below the wild-type level in EPEC *Δbfp* (Figure 3-5), suggesting that even basal BFP expression leads to mild induction of the Cpx

pathway.

Our results thus far indicated that OMP misregulation activated the σ^E response, while BFP misregulation activated the Cpx response in *Δhfq* mutants. We next asked whether activation of these two envelope stress responses resulted from distinct cues; that is, whether OMP misregulation in *Δhfq* mutants led to Cpx pathway induction and whether BFP misregulation contributed to σ^E induction in EPEC *Δhfq*. We examined this question by measuring *cpxP-lux* activity in the *Δomp* mutant strains and *rpoE-lux* activity in the *Δbfp* mutant strains. In general, deletion of major OMP-encoding genes had different effects on Cpx pathway activity than on σ^E pathway activity (Figures 3-6 and 3-3, respectively). In MC4100, the only *omp* deletion that significantly affected *cpxP-lux* activity was deletion of *ompF* in the wild-type *hfq*$^+$ background ($P<0.05$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 3-6A). In the EPEC *hfq*$^+$ background, deletion of any of the major OMPs caused a small (less than twofold) but statistically significant decrease in *cpxP-lux* expression ($P<0.05$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 3-6B). The only *omp* mutation that decreased *cpxP-lux* activity by more than 50% was deletion of *ompA* in the EPEC *Δhfq* background (Figure 3-6B). Interestingly, deletion of *ompC*, which strongly reduced σ^E activity in both MC4100 and EPEC (Figure 3-3), significantly increased Cpx pathway activity in the EPEC *Δhfq* background ($P<0.05$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 3-6B). Whereas deletion of the *bfp* genes from the EPEC *Δhfq* mutant significantly decreased *cpxP-lux* activity (Figure 3-5), the *bfp* deletion significantly increased *rpoE-lux* activity in this strain ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test) (Figure 3-6C). We therefore concluded that the σ^E and Cpx envelope stress responses were induced by distinct alterations to the envelope in *E. coli Δhfq* mutants.

3.3.4 Mechanism of regulation of BFP expression by Hfq

To better understand the mechanism by which Hfq regulates BFP expression, we assessed the expression of a *bfpA-lux* transcriptional reporter in

wild-type and *Δhfq* EPEC. As shown in Figure 3-7A, expression of the reporter was elevated approximately sevenfold in the *Δhfq* mutant. Since Hfq is a post-transcriptional regulator, the finding that transcription of the *bfp* gene cluster was increased in EPEC *Δhfq* led us to hypothesize that Hfq's effects on BFP expression are indirect, via a transcriptional regulator of the *bfp* genes. The only transcription factor known to directly activate expression of the *bfp* genes is PerA (86; see Figure 3-8 for a summary of BFP regulation). We therefore examined expression of a *perA-lux* transcriptional reporter in wild-type and *Δhfq* EPEC strains. Expression of the *perA-lux* reporter was also increased in EPEC *Δhfq* (Figure 3-7A), suggesting that Hfq could be affecting BFP expression by repressing the expression of PerA.

It is important to note that PerA activates expression of its own operon (53; Figure 3-8). Therefore, there are two possible explanations for the increased transcription of *perA* in EPEC *Δhfq*. The first possibility is that Hfq regulates *perA* expression indirectly, by binding to the mRNA encoding one of the numerous regulators of *perA* transcription (23, 56, 92). The second possibility is that Hfq regulates *perA* expression directly, by binding to and repressing translation or reducing stability of the *perA* mRNA. Repression of *perA* translation or stability would cause a reduction in PerA protein levels, thereby reducing *perA* transcription due to reduced autoactivation. Loss of Hfq-mediated translational repression would therefore lead to increased *perA* transcription in the *Δhfq* mutant. To separate these two possibilities, we examined expression of the *bfpA-lux* and *perA-lux* reporters in *hfq*$^+$ and *Δhfq* derivatives of an EPEC *ΔperA::kan* mutant. In this strain, we would still expect deletion of *hfq* to increase expression of the *perA-lux* reporter in the case of indirect regulation; however, no autoregulation of *perA* transcription can occur in this strain, so we hypothesized that deletion of *hfq* would not affect *perA* expression in the case of direct regulation. We found that in the *ΔperA::kan* mutant background, deletion of *hfq* had only a mild effect on expression of the *bfpA-lux* reporter (a 1.7 fold increase in expression in the *Δhfq* mutant, compared to the sevenfold increase seen when *hfq* was deleted in the *perA*$^+$ background) and no effect on expression of the *perA-*

lux reporter (Figure 3-7B). The greatly reduced effect of deletion of *hfq* on *bfpA-lux* expression in the *ΔperA::kan* mutant suggests that Hfq regulates BFP expression mainly via PerA. Furthermore, the virtually identical expression of the *perA-lux* reporter in the *ΔperA::kan* mutant compared to the *ΔperA::kan Δhfq* double mutant supports the idea that Hfq regulates *perA* expression directly, as explained above.

An alternative hypothesis to explain how Hfq regulates *perA* expression was proposed by Hansen and Kaper (32). Hfq acts in conjunction with the sRNA GadY to stabilize the transcript of the transcription factor GadX (63). GadX, in turn, is a transcriptional repressor of the *perABC* operon (78). Hansen and Kaper therefore suggested that, at low pH, loss of Hfq would be expected to lead to a decrease in GadY and GadX levels, thereby leading to increased *perA* expression (as summarized in Figure 3-8). We examined whether altered activity of GadY could account for the increased BFP and *perA* expression in the *Δhfq* mutant by constructing EPEC *ΔgadY* and *ΔgadY Δhfq* mutants. Deletion of *hfq* in the *ΔgadY* mutant background resulted in an increase in *bfpA-lux* and *perA-lux* expression comparable to the effect of an *hfq* deletion in wild-type EPEC (compare Figure 3-7C with Figure 3-7A). We therefore concluded that altered abundance of GadY sRNA in the EPEC *Δhfq* mutant could not account for the increased BFP expression that we observed.

Many of the pleiotropic phenotypes of *hfq* mutants are secondary to altered levels of alternative sigma factors in this strain, specifically increased σ^E activity (31; Figure 3-1C) and decreased σ^S activity (60). We therefore assessed the effects of changed levels of the alternative sigma factors σ^E and σ^S upon BFP expression (Figure 3-9). Increasing the cellular abundance of σ^E or decreasing the abundance of σ^S, in order to replicate the changes occurring in the *Δhfq* mutant, decreased the expression of the BFP (Figure 3-9), in contrast to the increased BFP expression observed in the *Δhfq* mutant (Figure 3-4). Therefore, altered sigma factor activity cannot account for Hfq's effects on BFP expression.

Since these experiments suggested that Hfq represses BFP expression via direct effects on *perA*, we assessed the stability of the *perA* transcript in wild-type

and *Δhfq* strains of EPEC. Rifampicin was added to mid-log phase cultures of the two strains to halt transcription, then RNA was isolated from samples taken 0, 1, 2, and 4 minutes post-rifampicin treatment. *perA* transcript abundance was measured by quantitative reverse transcriptase PCR (qRT-PCR). In wild-type EPEC, *perA* transcript abundance declined to less than 50% of its original level within 1 minute of rifampicin addition, whereas nearly 100% of *perA* transcripts still remained in the *Δhfq* mutant 2 minutes after rifampicin was added (Figure 3-10). This increased stability of the *perA* transcript in EPEC *Δhfq* strongly suggests that *perA* is a direct target of Hfq regulation.

3.4 Discussion

Proteins in the Gram-negative envelope carry out many tasks essential to cell survival, including transport of nutrients and wastes, attachment to surfaces, and motility, among others. Numerous regulatory pathways control expression of envelope proteins to ensure that they are produced under appropriate environmental and physiological conditions and in the correct quantities. In this work, we show that the RNA chaperone protein Hfq is an important regulator of envelope protein expression in *E. coli*. Deletion of *hfq* activated the σ^E envelope stress response in both *E. coli* K-12 and EPEC, as a result of the misregulation of OMP expression (Figures 3-1 to 3-3). In addition, the Cpx envelope stress response was activated in the EPEC *Δhfq* mutant, which can be partially explained by the overexpression of BFP in this strain (Figures 3-1, 3-4, and 3-5). These results indicate that Hfq plays a broad role in envelope homeostasis.

3.4.1 Activation of the σ^E envelope stress response

The σ^E envelope stress response was activated in *Δhfq* mutants of all three *E. coli* strains examined (MC4100, W3110, and EPEC E2348/69), as evidenced by increased activity of *rpoE-lux* and *degP-lux* transcriptional reporters in the mutants (Figure 3-1). Since the well-characterized inducing cue of the σ^E response involves the misfolding of OMPs (1), we examined OMP profiles of the wild-type

and *Δhfq* strains. We observed alterations in the abundance of numerous major and minor OMPs in *Δhfq* mutants in all three genetic backgrounds (Figure 3-2). Importantly, deletion of the major porin *ompC* significantly reduced σ^E activity in the MC4100 and EPEC *Δhfq* mutants (Figure 3-3), suggesting that overexpression of OMPs underlies the increased σ^E activity in the *Δhfq* mutants.

Interestingly, deletion of *ompC* had a bigger effect upon σ^E activity than deletion of either of the other major porins, OmpA or OmpF (Figure 3-3). The C-terminus of both OmpC and OmpF ends with the amino acids YQF, which is an inducing motif for the DegS protease that initiates the proteolysis cascade that leads to σ^E activation (96). In addition, both proteins activate σ^E when overexpressed (55). However, OM preparations from MC4100 *ompC* and *ompF* mutants showed that, during growth at 37ºC in LB, expression of OmpC is substantially higher than that of OmpF (data not shown), which could account for the larger effect of deleting *ompC* upon σ^E activity under these conditions. OmpA, on the other hand, does not terminate in a YXF motif (www.ecogene.org), and therefore its misfolding likely is not sensed by DegS. The small reduction in σ^E activity when *ompA* was deleted from the MC4100 *Δhfq* mutant (Figure 3-3A) could result from a reduced burden on periplasmic folding factors, allowing other OMPs like OmpC to fold more efficiently.

σ^E activity was not reduced to wild-type levels in any of the *Δhfq Δomp* double mutants (Figure 3-3). This likely indicates that σ^E activation in the *Δhfq* mutants results from the misregulation of numerous different OMPs. We observed altered abundance of numerous minor OMPs in the *Δhfq* mutants in our OM profiles (Figure 3-2), which could potentially be sensed by DegS.

Combined with findings in *V. cholerae* and *Salmonella* (17, 24), these results suggest that a conserved role of Hfq in γ-proteobacteria is to modulate OMP expression, thereby preventing induction of the σ^E response.

3.4.2 Activation of the Cpx envelope stress response

In contrast to the σ^E response, the Cpx envelope stress response was activated by deletion of *hfq* in a strain-specific manner. Expression of a *cpxP-lux*

reporter and anti-CpxR Western blots (Figure 3-1) indicate that the Cpx response is strongly activated in EPEC *Δhfq*, but not substantially activated in *Δhfq* mutants of either of the K-12 strains tested. Although a small increase in CpxR protein was observed in MC4100 and W3110 *Δhfq*, *cpxP-lux* assays show that the Cpx response was not significantly activated in these mutants (Figure 3-1). The increased CpxR protein levels in these strains could result from the loss of negative regulation of *cpxR* by the sRNA MicF (37) rather than from genuine envelope stress. One reason why the activation of the Cpx response is strain-specific is that overexpression of the BFP—an envelope structure that is not encoded in the genome of *E. coli* K-12—contributes to Cpx activation. Western blotting (Figure 3-4) indicated that the BFP proteins are produced at higher levels in EPEC *Δhfq*, and increased ability of the *Δhfq* mutant to adhere to cultured cells indicates that the pilus proteins are being correctly localized to the envelope in this strain, where they may be a source of envelope stress. Indeed, we observed that deletion of the *bfp* gene cluster from the *Δhfq* mutant significantly reduced Cpx pathway activity (Figure 3-5). These results are consistent with the previous finding that ectopic expression of *bfpA* in *E. coli* MC4100 activates the Cpx response (62).

Although overexpression of BFP appears to be a major contributor to activation of the Cpx response in EPEC *Δhfq*, Cpx pathway activity in the *Δhfq Δbfp* double mutant is still significantly higher than wild-type activity (Figure 3-5), indicating that there are other factors involved in Cpx pathway activation in the *Δhfq* mutant. The identity of these factors is currently unknown. One possible contributor is overexpression of *ompA* in the *Δhfq* mutant. We observed that deleting *ompA* from EPEC *Δhfq* reduced Cpx pathway activity by approximately 50% (Figure 3-6B). OmpA-mediated activation of the Cpx pathway was previously reported by Ma and Wood (2009), although it is unclear whether OmpA activates the Cpx response directly or indirectly. Interestingly, some structural studies of OmpA indicate that OmpA has a globular, α-helix-rich C-terminal domain that resides in the periplasm (82). Since other porins like OmpC

do not seem to possess a large periplasmic domain, perhaps this α-helical domain constitutes a unique signal for Cpx activation.

Cpx pathway activity in EPEC *Δhfq* may also be affected by the altered expression of pilins other than those comprising the BFP. Overexpression of several classes of pilins, including type 1, type IV, and curli, is already known to activate the Cpx response (94). The EPEC genome encodes numerous pilus operons in addition to the *bfp* gene cluster, including the type 1 fimbrial genes, several other type IV pilus clusters, and the *E. coli* common pilus (ECP) (40). Studies in *E. coli* K-12 have demonstrated that expression of the curli fimbriae is regulated by Hfq, via sRNA base-pairing with the mRNA of the transcriptional regulator CsgD (38, 43, 57, 84). Therefore, it is reasonable to expect that loss of Hfq may affect the expression of additional pilus proteins.

With the exception of *ompA* in EPEC, deletion of genes encoding major OMPs from the *E. coli Δhfq* mutants did not affect Cpx pathway activity (Figure 3-6). Conversely, deleting the *bfp* genes from EPEC *Δhfq* did not reduce σ^E pathway activity (Figure 3-6C). These results support a model in which these two envelope stress responses are induced by distinct cues – the misfolding of β-barrel OMPs in the case of σ^E, and the misfolding of periplasmic and/or IM proteins in the case of Cpx. This model contrasts a recent study showing that mutations that interfere with the folding of β-barrel OMPs (in genes encoding the Bam OMP insertion complex or OmpF itself) activate the Cpx pathway (26). The authors argue that both the σ^E and Cpx responses are required to effectively cope with aberrant assembly of OMPs. In contrast, OMP overexpression in our *Δhfq* mutants does not appear to be a significant factor in Cpx activation in *E. coli* K-12, and only deletion of *ompA* reduced Cpx activity in EPEC *Δhfq* (Figure 3-6). These data signify that an overabundance of OMPs (as in the K-12 *Δhfq* mutants) does not necessarily constitute an inducing cue for the Cpx system. Rather, the Cpx response appears to be induced only by mutations that debilitate OMP assembly, which could have numerous indirect effects on the envelope, or by overexpression of particular OMPs such as OmpA in EPEC. As a whole, our results suggest that the Cpx response plays a different physiological role than the σ^E response.

3.4.3 Hfq represses BFP expression via the *perA* mRNA

Numerous regulatory proteins and environmental conditions are known to influence the expression of the BFP, with activators including PerA, the *pst* operon, temperatures between 35 and 37ºC, and calcium ions; repressors include ammonium ions and CpxR (23, 70, 86, 92; Figure 3-8). We found that Hfq also repressed expression of the BFP (Figure 3-4). However, expression of a *bfp-lux* transcriptional reporter was elevated in EPEC *Δhfq* (Figure 3-7A), indicating that Hfq, which is a post-transcriptional regulator, acts indirectly to control *bfp* expression. The majority of Hfq's effect on BFP expression is via the transcriptional regulator PerA, since induction of the *bfp-lux* reporter is less than twofold when an *Δhfq* mutation is introduced into a *perA* mutant, compared with an approximately sevenfold induction in a $perA^+$ background (compare Figures 3-7A and 3-7B). We believe that this regulation of *perA* by Hfq is direct for several reasons. First, we ruled out several other regulators through which Hfq could conceivably affect *perA* expression. Deletion of *gadY*, encoding a sRNA that stabilizes the transcript of a transcriptional repressor of *perA* called GadX (63, 78), did not prevent induction of the *bfp-lux* and *per-lux* reporters when *hfq* was deleted (Figure 3-7C), indicating that altered levels of GadY are not responsible for the increased expression of BFP in EPEC *Δhfq*. We also showed that increased levels of σ^E and decreased levels of σ^S, which have been previously observed in *E. coli hfq* mutants (60, 85), caused a decrease in BFP expression (Figure 3-9), rather than the increase observed in the *Δhfq* mutant. Second, genetic experiments suggest that Hfq acts directly on *perA*. In a *perA* mutant background, deletion of *hfq* does not increase *per-lux* activity as *hfq* deletion does in a $perA^+$ background (Figure 3-7B). Since we would expect transcriptional induction to occur in a *perA* mutant if Hfq were regulating one of the characterized transcriptional regulators of *perA*, the simplest explanation for this result is that Hfq acts directly on the *perA* transcript in the wild-type strain. In the *Δhfq* mutant, this repression is lost, allowing PerA protein to accumulate to higher levels, which causes increased *per-lux* expression due to autoregulation (53). In a *perA* mutant, this autoregulation of the *per-lux* reporter cannot occur, and therefore deletion of *hfq* does not increase

expression of the *per-lux* reporter. Third, we observed that the *perA* transcript is stabilized by deletion of *hfq* (Figure 3-10). This result suggests that Hfq normally increases the rate of degradation of the *perA* transcript, which could be achieved by the reported interaction between Hfq and RNase E, the major enzyme responsible for mRNA turnover in *E. coli* (90).

One unresolved question is whether an Hfq-dependent sRNA is involved in the repression of *perA*. Although Hfq is primarily known for its role in assisting sRNA-mRNA interactions, it can also regulate translation independently of sRNAs in certain cases. For example, Hfq binds the *ompA* transcript *in vitro*, even in the absence of sRNAs, and prevents interaction of the transcript with the 30S ribosomal subunit (95). This interaction both represses translation of *ompA* and increases degradation of the transcript by RNase E. However, it was subsequently shown that the Hfq-binding sRNA MicA is required for growth phase regulation of *ompA* expression *in vivo* (88), making the significance of sRNA-independent regulation by Hfq *in vitro* unclear. Unfortunately, there is no straightforward way to identify which sRNA regulates a target mRNA. A BLASTn search of the E2348/69 genome failed to return any sRNAs with significant complementarity to the 5' end of the *perA* transcript. The identity of the sRNA(s) affecting *perA* expression, if any, will require further studies.

3.4.4 Hfq regulates virulence in pathogenic *E. coli*

In addition to our work with EPEC, the role of Hfq in the virulence of several other pathogenic strains of *E. coli* has also been examined. Kulesus *et al*. (46) reported reduced colonization by a UPEC *Δhfq* mutant in a mouse model of cystitis and pyelonephritis. *C. elegans* was used as a model host for wild-type and *Δhfq* mutants of UPEC, adherent-invasive *E. coli* (AIEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC); in all cases, the *Δhfq* mutants were found to be attenuated for virulence (6, 80). The reduced ability of *Δhfq* mutants to colonize a host may be at least partially attributable to their reduced ability to withstand stresses including low pH in UPEC, AIEC, and EHEC; reactive oxygen species and reactive nitrogen species in UPEC and AIEC; and

cationic antimicrobial peptides in UPEC (32, 46, 80). The stress sensitivity of UPEC *Δhfq* is comparable to that of a UPEC *ΔrpoE* mutant, suggesting that the reduced ability of *Δhfq* mutants to colonize their hosts could be related to problems with the envelope (46). Given the strong activation of both envelope stress responses in EPEC *Δhfq* (Figure 3-1), the envelope and therefore the virulence of this mutant is likely compromised as well.

Altered expression of virulence factors may also play a role in the decreased virulence of EHEC *Δhfq*. In EHEC strain EDL933, the locus of enterocyte effacement (LEE) genes encoding the T3SS are expressed during an earlier growth phase and at higher levels in the *Δhfq* mutant than in the wild-type strain (32, 77). This increase in T3S could be the result of Hfq's post-transcriptional regulation of two LEE-encoded regulators: the *grlRA* transcript was found to be more stable in EHEC *Δhfq* (32), while translation of *ler* was increased in the *Δhfq* mutant (77). In contrast, decreased expression of the LEE was observed in an *Δhfq* mutant of EHEC strain 86-24 (44), demonstrating a strain-specific role for Hfq in virulence regulation. The expression of the Shiga toxin-encoding genes $stx2_{AB}$ was elevated in *Δhfq* mutants of both EDL933 and 86-24 (44).

Taken together with our finding that BFP expression is enhanced in EPEC *Δhfq* (Figure 3-4), these studies show that multiple horizontally-acquired virulence genes are repressed by Hfq in attaching and effacing strains of *E. coli*. Shakhanovich *et al*. (2009) demonstrated that the overexpression of LEE genes in EHEC *Δhfq* is detrimental to growth, while we showed that the overexpression of BFP in EPEC *Δhfq* generates envelope stress (Figure 3-5). Hfq may therefore play an important role in moderating the potentially deleterious expression of horizontally-acquired genes in *E. coli*. In this role, the function of Hfq could be analogous to that of the transcriptional regulator H-NS, which binds to and represses expression of genes with lower GC content than the ancestral genome (3, 20). In pathogenic *E. coli* and *Salmonella*, horizontally-acquired virulence genes are typically located in low-GC genomic islands or plasmids. By repressing the expression of such genes, H-NS prevents inappropriate expression that could

pose a selective disadvantage, thereby allowing the genes to be maintained in the genome while they incur regulatory mutations that better integrate them into the cell's regulatory circuits. Interestingly, H-NS preferentially binds AT-rich DNA, while Hfq binds AU-rich RNA (29); therefore, both regulators could control the expression of the same target sequences at different stages of gene expression. A similar role for Hfq and sRNAs in binding the transcripts of horizontally-acquired genes has also been proposed for *Salmonella* (64, 81).

By regulating the expression of OMPs, the T3SS and the BFP, Hfq plays an important role in maintaining the function of the envelope compartment in EPEC and other strains of *E. coli*. Hfq's ability to tame the expression of horizontally-acquired genes may additionally be a critical factor in *E. coli*'s ability to successfully integrate new virulence genes into its regulatory programme.

3.5 Tables and Figures

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

Strain or plasmid	Description	Source or reference
Bacterial strains		
E2348/69	Prototypical EPEC O127:H6 laboratory strain	49
MC4100	F$^-$ *araD139 Δ(argF-lac)* U169 *rpsL150* (Str^R) *relA1 flhD5301 deoC1 ptsF25 rbsR*	11
W3110	F$^-$ λ$^-$ *rpoS(Am) rph-1 Inv(rrnD-rrnE)*	5
SV52	E2348/69 *Δhfq*	This study
RM5	MC4100 *Δhfq*	This study
SV97	W3110 *Δhfq*	This study
SV195	MC4100 *ΔompA*	This study
SV196	MC4100 *ΔompC*	This study
SV372	MC4100 *ΔompF*	This study
SV189	RM5 *ΔompA*	This study
SV190	RM5 *ΔompC*	This study
SV259	RM5 *ΔompF*	This study
SV383	E2348/69 *ΔompA*	This study
SV385	E2348/69 *ΔompC*	This study
SV387	E2348/69 *ΔompF*	This study
SV384	SV52 *ΔompA*	This study
SV386	SV52 *ΔompC*	This study
SV388	SV52 *ΔompF*	This study
SV235	E2348/69 *ΔbfpA-L*	This study
SV236	SV52 *ΔbfpA-L*	This study
JPEP20	E2348/69 *ΔperA::kan*	10
SV158	JPEP20 *Δhfq*	This study
SV233	E2348/69 *ΔgadY*	This study
SV234	SV52 *ΔgadY*	This study
Plasmids		
pRE112	Suicide vector for allelic exchange; Cam^R	21
pNLP19*	pNLP10 luminescence reporter plasmid containing *rpoE-rseABC* promoter; Kan^R	68
pNLP65*	pJW15 luminescence reporter plasmid containing *rpoE-rseABC* promoter; Kan^R	This study
pNLP11*	pNLP10 luminescence reporter plasmid containing *degP* promoter; Kan^R	68

pNLP27*	pJW15 luminescence reporter plasmid containing *degP* promoter; Kan^R	This study
pJW1*	pNLP10 luminescence reporter plasmid containing *cpxP* promoter; Kan^R	68
pJW25*	pJW15 luminescence reporter plasmid containing *cpxP* promoter; Kan^R	51
pCA24N	Vector control from ASKA library; Cam^R	45
pCA-*hfq*	IPTG-inducible *hfq* overexpression vector from ASKA library; Cam^R	45
pJW22	pJW15 luminescence reporter plasmid containing *perA* promoter; Kan^R	92
pJW23	pJW15 luminescence reporter plasmid containing *bfpA* promoter; Kan^R	92
pCA-*rpoE*	IPTG-inducible *rpoE* overexpression vector from ASKA library; Cam^R	45
pCA-*rssB*	IPTG-inducible *rssB* overexpression vector from ASKA library; Cam^R	45

* Note that pNLP10-based reporters (containing pSC101 origin) were used in *E. coli* K-12 strains, while pJW15-based reporters (p15A origin) were used in EPEC strains due to difficulties with transformation and maintenance of pNLP10 in this background. Aside from the origin of replication, pNLP10 and pJW15 are identical plasmids.

Table 3-2. Oligonucleotide primers used in this study.

Primer name	Sequence
hfqupF	5'-GAGAGGTACCGTACTGACTATCGTCCATTCC-3'
hfqupR	5'-GAGACTGCAGCCTTAGCCATTCTCTCTTTTCC-3'
hfqdownF	5'-GAGACTGCAGAACAGGACAGCGAAGAAACC-3'
hfqdownR	5'-GAGAGAGCTCGAATTTACCAAAGCGTGTTACC-3'
ompAUpF	5'-TTTTTCTAGAAATAGGGTTGATCTTTGTCG-3'
ompAUpR	5'-GACGAGAACTTAAGCTTTCATTTTTTGCGCCTCG-3'
ompADnF	5'-CGCAAAAAATGAAAGCTTAAGTTCTCGTCTGG-3'
ompADnR	5'-TTTTGAGCTCGAAACCTCATAGTGACCG-3'
ompCUpF	5'-TTTTTCTAGACTTTTGGATTGCGTGGG-3'
ompCUpR	5'-CAATCGAGATTAGAATTTCATGTTATTAACCCTCTG-3'
ompCDnF	5'-GTTAATAACATGAAATTCTAATCTCGATTGATATCG-3'
ompCDnR	5'-TTTTGAGCTCCGATAACATGAGAAAGACG-3'
ompFUpF	5'-TTTTTCTAGAGCATGAGCGTTATCTGG-3'
ompFUpR	5'-GGTATGCTATTAGAACTTCATCATTATTTATTACCCTC-3'
ompFDnF	5'-TAAATAATGATGAAGTTCTAATAGCATACCCCTTTG-3'
ompFDnR	5'-TTTTGAGCTCCACTGTCAGCAGCAACC-3'
bfpUpF	5'-TTTTTCTAGACGCCTGAATAACCTTCCG-3'
bfpUpR	5'-GCTATCCCGTAGAAACCATAAAAACTGTTTTCC-3'
bfpDnF	5'-TATGGTTTCTACGGGATAGCACCACCAG-3'
bfpDnR	5'-TTTTGAGCTCTGCTTTGTCTGTCTTTTGGG-3'
gadYUpF	5'-TTTTTCTAGAAATGCGAAATATGTCAGG-3'
gadYUpR	5'-CTCAGACATAAAGTTAAATATAACTTTTACTGG-3'
gadYDnF	5'-TATTTAACTTTATGTCTGAGTAAAACTCTATAA-3'
gadYDnR	5'-TTTTGAGCTCTAAATCCAGTCATCCAGC-3'
rrsBF	5'-TAATACCGCATAACGTCGCA-3'
rrsBR	5'-GCTAATCCCATCTGGGCAC-3'
perAFqPCR	5'-GTGCTTCATCTAAGAGCATCGT-3'
perARqPCR	5'-ATCGCCTAGTTTCCAGTTTTTG-3'

*underlining denotes a restriction enzyme sequence (*Kpn*I: GGTACC; *Pst*I: CTGCAG; *Sst*I: GAGCTC; *Xba*I: TCTAGA)

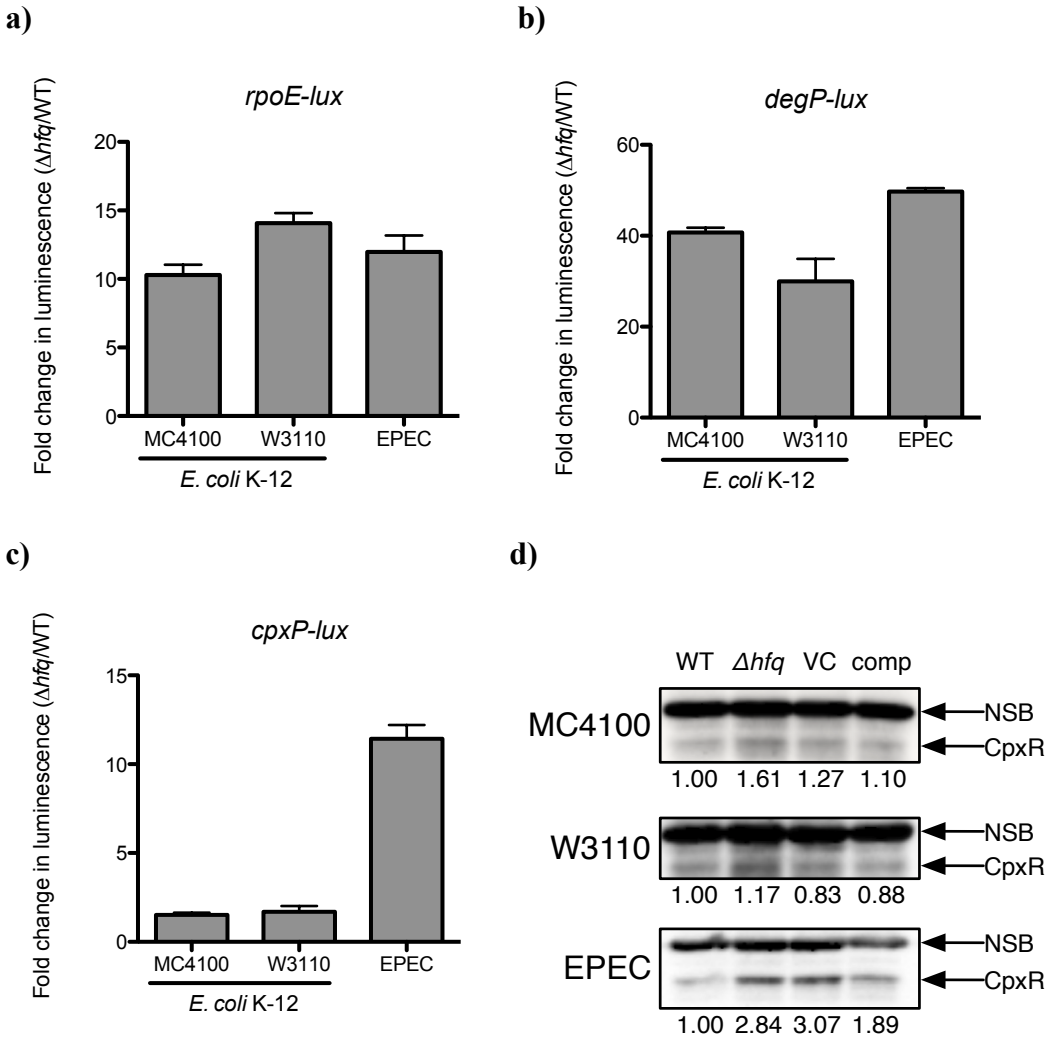

Figure 3-1. Activity of the σ^E and Cpx envelope stress responses in wild-type and *Δhfq* strains of *E. coli*. a-c) Luminescence assay results. To calculate fold changes, the normalized luminescence value for each replicate culture of the *Δhfq* mutant was divided by the average normalized luminescence value for the appropriate wild-type control at the same timepoint. Data shown were measured 6 h post-subculture and represent the mean and standard deviation of five biological replicate cultures. **d)** CpxR Western blots. The strains shown are wild-type (WT), *Δhfq* mutant, *Δhfq* mutant with vector control pCA24N (VC), and *Δhfq* mutant with complementation plasmid pCA-*hfq* (comp). The numbers below each lane indicate the intensity of the CpxR band in each sample, relative to the wild-type strain. IPTG was added to the vector control and complemented mutant cultures to induce expression of *hfq*. NSB, non-specific band that serves as a loading control.

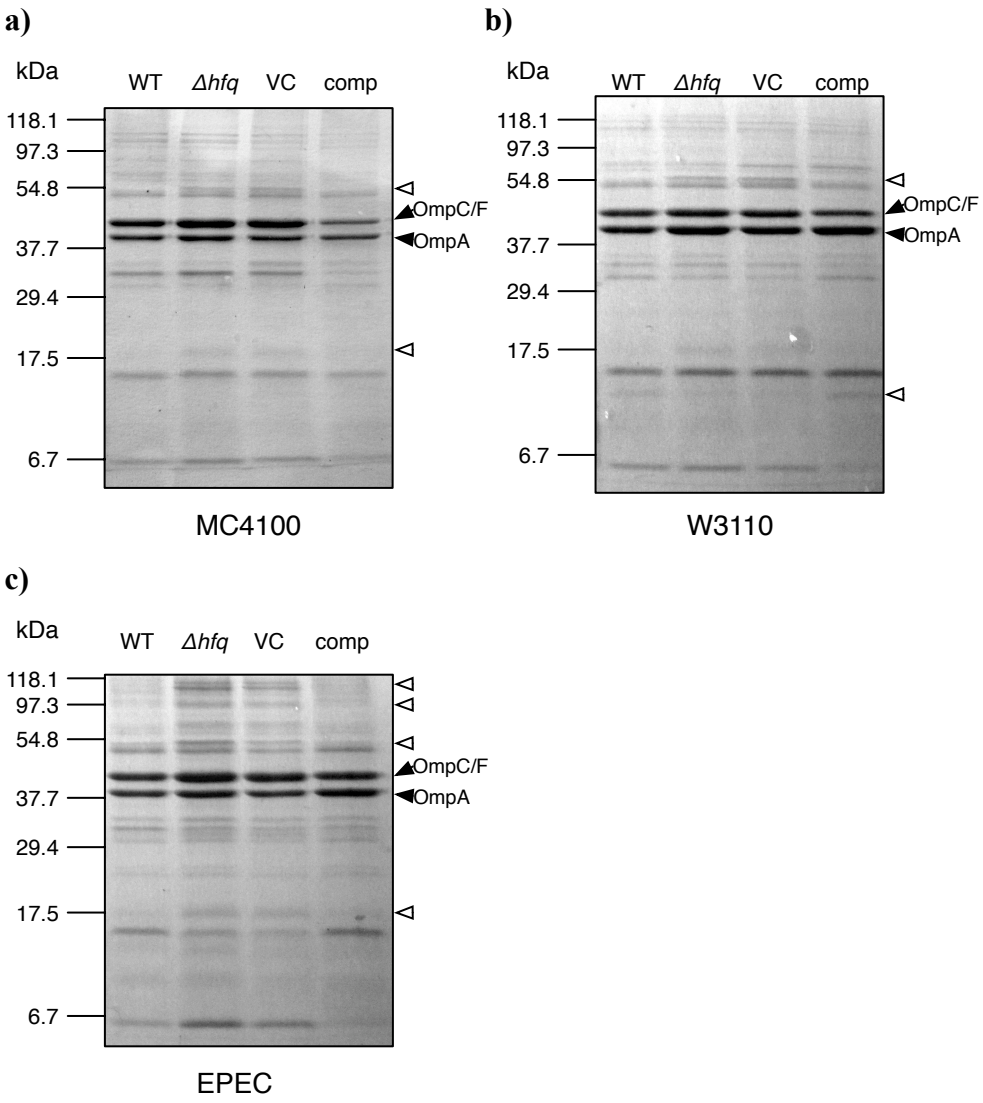

Figure 3-2. *E. coli Δhfq* mutants have altered OMP profiles. OM samples were extracted from MC4100 **(a)**, W3110 **(b)** and EPEC **(c)** strains as described in Experimental Procedures, then analysed by SDS-PAGE and Coomassie Blue staining. The strains shown are wild-type (WT), *Δhfq* mutant, *Δhfq* mutant with vector control pCA24N (VC), and *Δhfq* mutant with complementation plasmid pCA-*hfq* (comp). IPTG was added to the vector control and complemented mutant cultures to induce expression of *hfq*. Closed arrowheads indicate bands corresponding to major porins, while open arrowheads indicate bands corresponding to minor OMPs whose abundance was changed in the *Δhfq* mutant relative to wild-type.

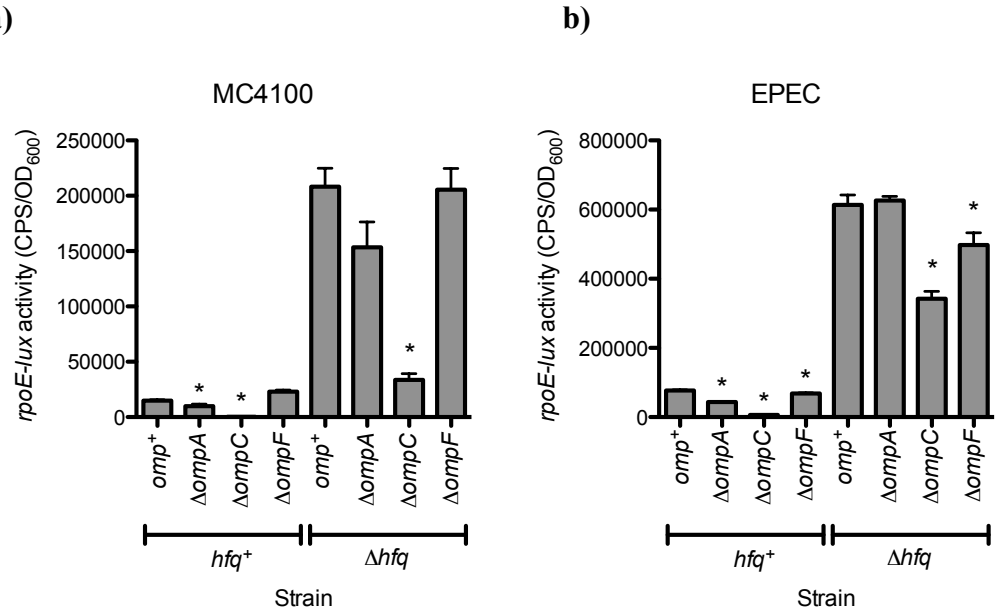

Figure 3-3. Deletion of major OMPs reduces activation of the σ^E envelope stress response. Luminescence assay results using the *rpoE-lux* reporter in MC4100 **(a)** and EPEC **(b)**. Data shown were measured 6 h post-subculture and represent the mean and standard deviation of five biological replicate cultures. The strains shown are wild-type (*omp*$^+$); *Δhfq*, *ΔompA*, *ΔompC*, and *ΔompF* single mutants; and *Δhfq ΔompA*, *Δhfq ΔompC*, and *Δhfq ΔompF* double mutants. Normalized luminescence was calculated by dividing raw luminescence (in cps, counts per second) by the OD_{600} of the culture. * indicates a statistically significant difference from the relevant *omp*$^+$ control strain ($P<0.05$, one-way ANOVA with Dunnett's multiple comparison test).

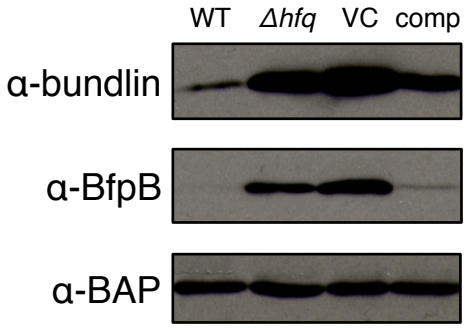

Figure 3-4. BFP expression is increased in EPEC *Δhfq*. Western blots showing levels of bundlin, BfpB, and bacterial alkaline phosphatase (BAP – loading control). The strains shown are EPEC wild-type (WT), *Δhfq* mutant, *Δhfq* mutant with vector control pCA24N (VC), and *Δhfq* mutant with complementation plasmid pCA-*hfq* (comp). IPTG was added to the vector control and complemented mutant cultures to induce expression of *hfq*.

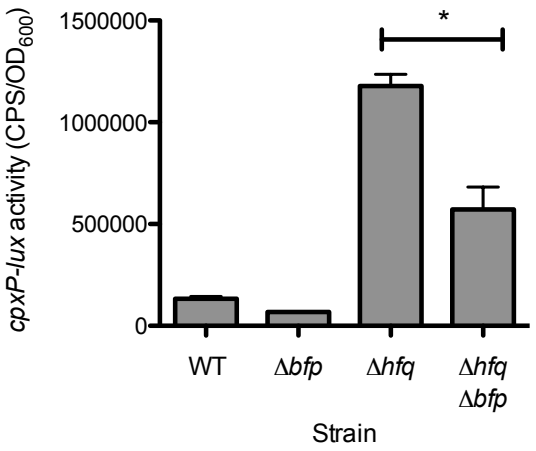

Figure 3-5. BFP expression contributes to activation of the Cpx response. Luminescence assay results using the *cpxP-lux* reporter. Data shown were measured 6 h post-subculture and represent the mean and standard deviation of five biological replicate cultures. The strains shown are wild-type (WT), *Δhfq* mutant, *ΔbfpA-L* mutant, and *Δhfq ΔbfpA-L* double mutant. Normalized luminescence was calculated by dividing raw luminescence (in cps, counts per second) by the OD_{600} of the culture. * indicates a statistically significant difference between strains ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test).

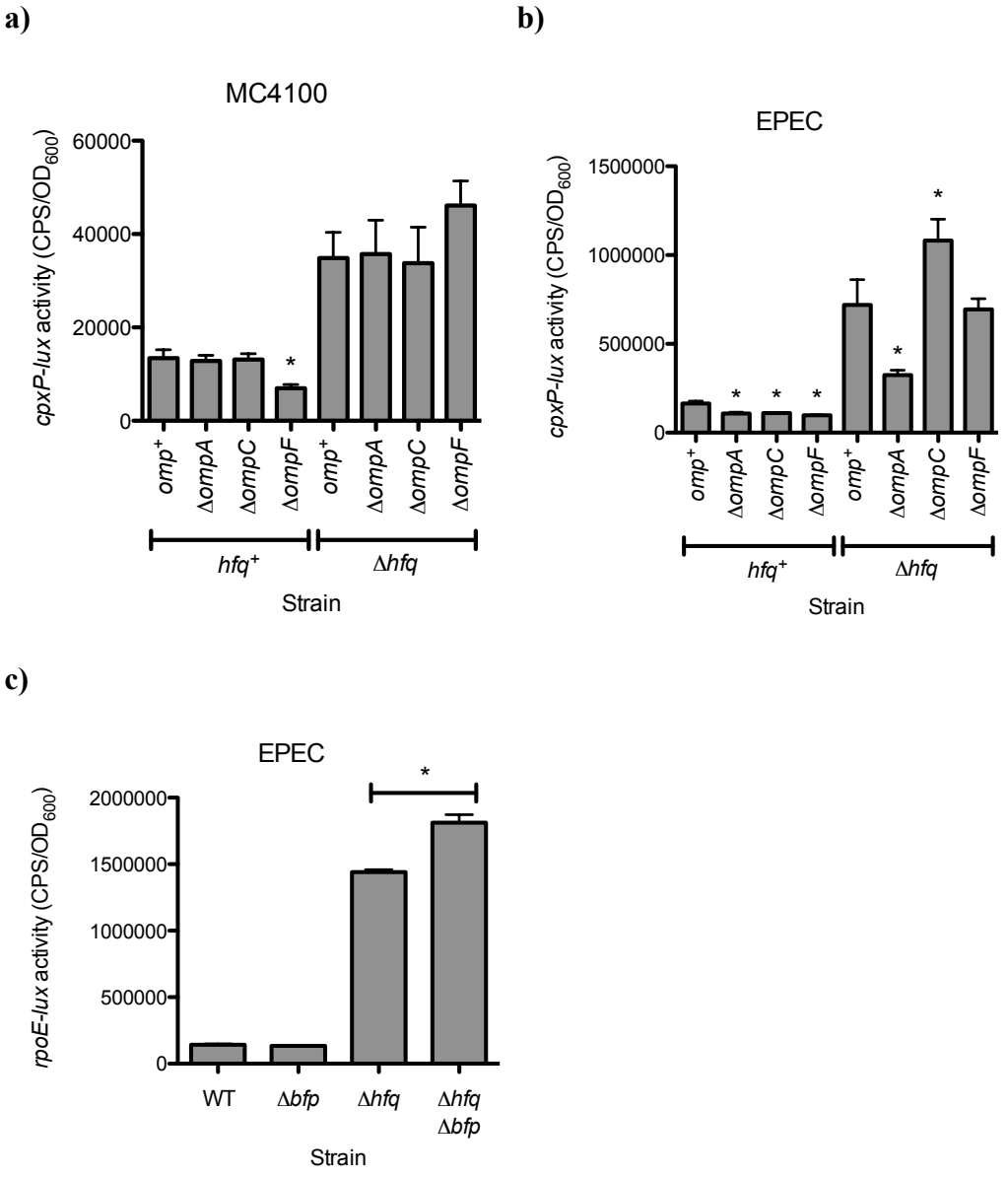

Figure 3-6. The σ^E and Cpx envelope stress responses are induced by distinct cues in *E. coli Δhfq*. Luminescence assay results for *cpxP-lux* **(a and b)** and *rpoE-lux* **(c)** reporters. Data shown were measured 6 h post-subculture and represent the mean and standard deviation of five biological replicate cultures. The strains shown are wild-type (WT), *Δhfq*, *ΔompA*, *ΔompC*, *ΔompF*, and *ΔbfpA-L* single mutants, and *Δhfq ΔompA*, *Δhfq ΔompC*, *Δhfq ΔompF*, and *Δhfq ΔbfpA-L* double mutants of MC4100 **(a)** and EPEC **(b, c)**. Normalized luminescence was calculated by dividing raw luminescence (in cps, counts per second) by the OD_{600} of the culture. In panels **(a)** and **(b)**, * indicates a statistically significant difference from the relevant *omp*$^+$ control (P<0.05, one-way ANOVA with Dunnett's multiple comparison test). In panel **(c)**, * indicates a statistically significant difference between strains (P<0.05, one-way ANOVA with Bonferroni's multiple comparison test).

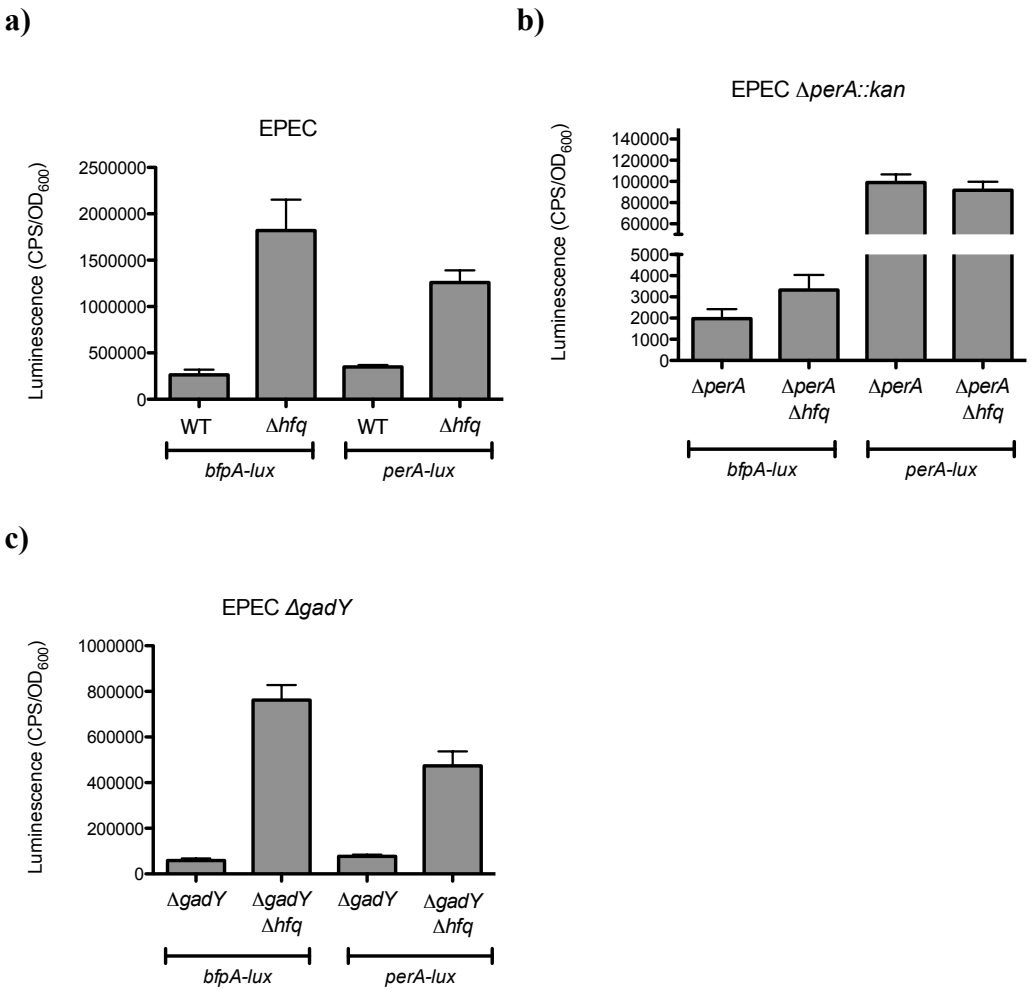

Figure 3-7. Increased BFP expression in EPEC *Δhfq* requires the regulator PerA but not the sRNA GadY. Luminescence assay results for *bfpA-lux* and *perA-lux* reporters. Data shown were measured 6 h post-subculture and represent the mean and standard deviation of five biological replicate cultures. The strains shown are wild-type (WT) and *Δhfq* mutants of EPEC E2348/69 wild-type **(a)**, EPEC *ΔperA::kan* **(b)**, and EPEC *ΔgadY* **(c)**. Normalized luminescence was calculated by dividing raw luminescence (in cps, counts per second) by the OD_{600} of the culture.

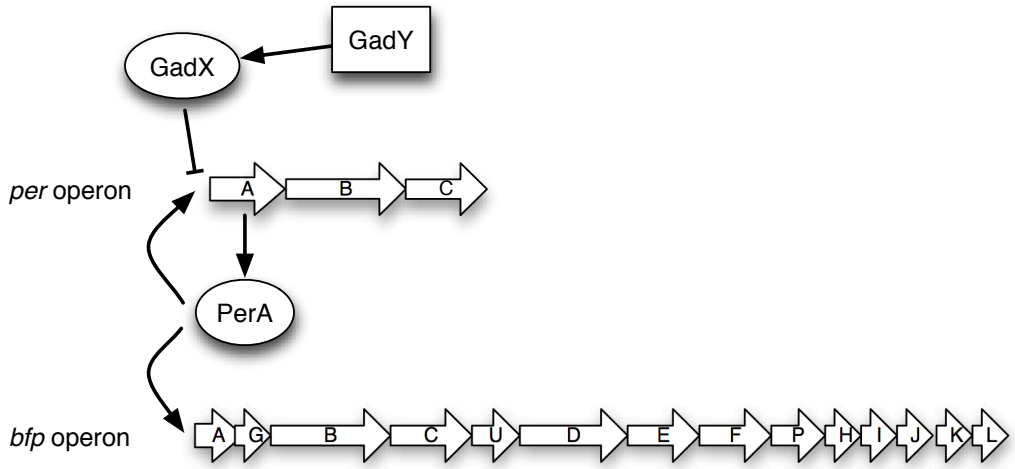

Figure 3-8. Summary of regulation of BFP expression. PerA directly activates *bfp* transcription, as well as autoactivating transcription of the *perABC* operon. *perA* transcription is repressed by GadX, whose production is increased by the GadY sRNA. Arrows indicate positive regulation, while lines with bars indicate negative regulation.

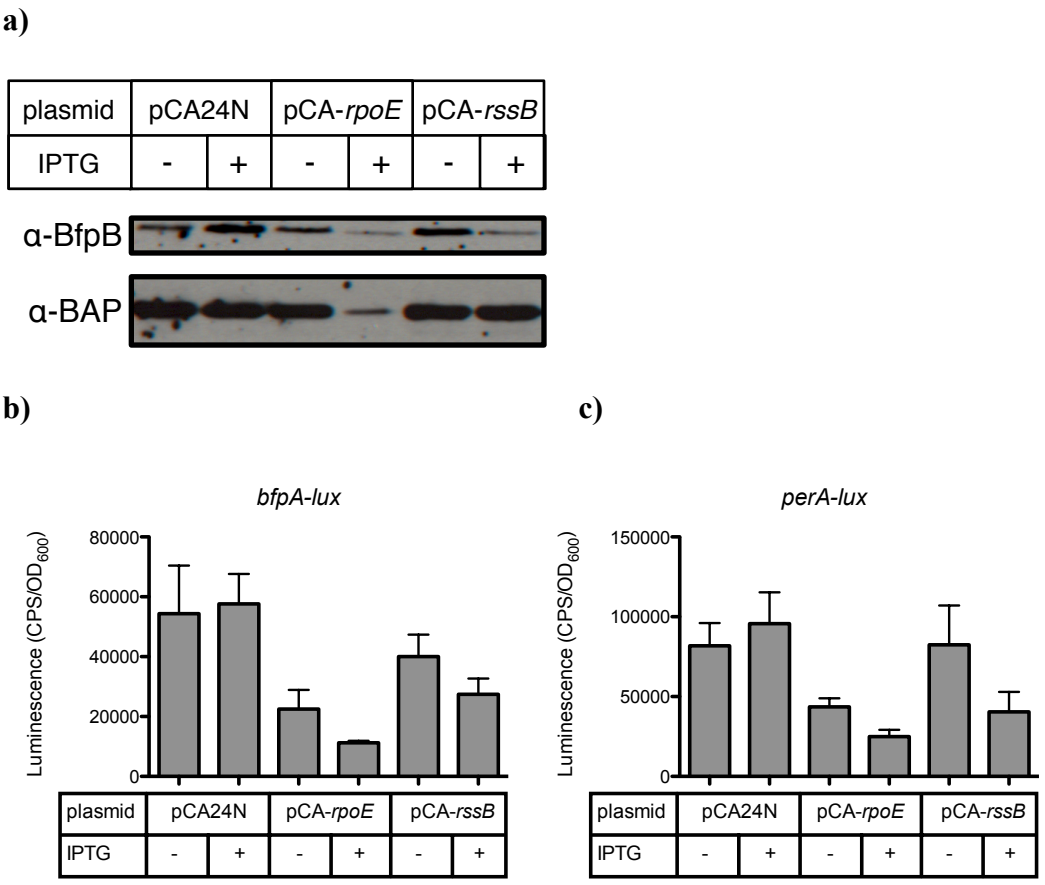

Figure 3-9. Increased σ^E or decreased σ^S levels do not account for increased BFP expression in EPEC *Δhfq*. σ^E expression was increased by transforming EPEC with pCA-*rpoE* (encoding σ^E), while σ^S expression was decreased by transforming EPEC with pCA-*rssB* (encoding RssB, a proteolytic adaptor for σ^S); the vector control for both plasmids is pCA24N. **a)** Western blots to detect BFP component protein BfpB, as well as the unrelated periplasmic protein bacterial alkaline phosphatase (BAP), which acts as a control for general disruption of the envelope. **b)** and **c)** Lux assays measuring expression of the *bfpA-lux* **(b)** and *perA-lux* **(c)** reporters. Luminescence was measured 4 h post-subculture; data represent the mean and standard deviation of five biological replicate cultures. Normalized luminescence was calculated by dividing raw luminescence (in cps, counts per second) by the OD_{600} of the culture. Where indicated, expression from plasmids was induced with 0.1 mM IPTG. Note that there is some leaky expression from the PT5-*lac* promoter in the pCA24N-based plasmids even in the absence of IPTG (our unpublished results).

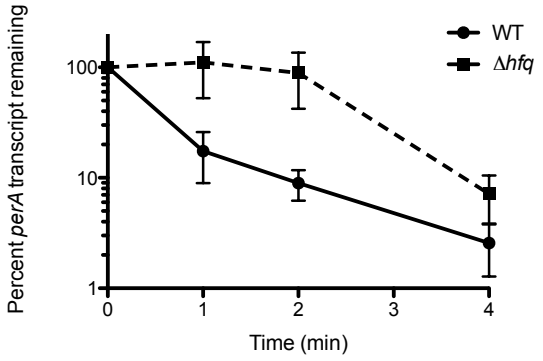

Figure 3-10. The *perA* transcript is more stable in EPEC *Δhfq*. Stability of the *perA* transcript was measured by treating mid-log phase cultures of E2348/69 wild-type (WT) and *Δhfq* grown in LB with 250 µg/ml rifampicin to stop RNA synthesis. Samples were withdrawn 0, 1, 2, and 4 minutes post-addition of rifampicin, followed by RNA extraction and cDNA synthesis. Levels of *perA* transcript were assessed by quantitative RT-PCR, using *rrsB* as an endogenous control. Each point represents the mean and standard deviation of three biological replicate cultures.

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CHAPTER 4

The Cpx envelope stress response regulates and is regulated by small non-coding RNAs

A version of this chapter has been submitted for publication:
Vogt, S.L., A.D. Evans, R.L. Guest, and T.L. Raivio. (2013)
Journal of Bacteriology JB-00905-13.

Note: The EMSA experiments in this chapter were performed by Randi Guest. The experiments in Figures 4-9 to 4-12 were performed by Alex Evans. Thanks also to Alex Evans for assistance with writing sections 4.3.5 and 4.3.6.

4.1 Introduction

Two-component systems (2CSs) are the primary means by which bacteria sense and respond to changes in their surroundings (70). Bacterial genomes frequently encode dozens of 2CSs, each of which detects a unique stimulus and performs a unique physiological role. Of the approximately 30 2CSs encoded in the *Escherichia coli* genome (52), the CpxAR 2CS is among the best characterized (reviewed in 69). The Cpx 2CS consists of the inner membrane (IM)-localized histidine kinase (HK) CpxA and the cytoplasmic response regulator (RR) CpxR. CpxA possesses two opposing enzymatic activities (60). In the presence of an inducing signal, CpxA acts as a kinase to phosphorylate CpxR at a conserved aspartate residue, thereby permitting CpxR to bind to DNA and modulate transcription. In the absence of an appropriate signal, CpxA acts as a CpxR~P phosphatase, keeping CpxR dephosphorylated and therefore inactive.

The molecular nature of the signal sensed by CpxA remains unknown; however, several cues that induce the Cpx pathway have been identified. These include alkaline pH (15), alterations to the composition of the IM (16, 47), and ectopic expression of pilins such as PapE, PapG, and BfpA in the absence of their cognate chaperones (31, 50). All of these cues are expected to generate misfolded IM and/or periplasmic proteins; the Cpx system is therefore considered an envelope stress response (69). The Cpx pathway is also induced by overexpression of the OM lipoprotein NlpE (17), which is believed to be an auxiliary regulator capable of sensing adhesion to hydrophobic surfaces (53). In accordance with the view of Cpx as an envelope stress response, many of the genes whose expression is most strongly increased by CpxR encode periplasmic protein folding and degrading factors, such as the protease/chaperone DegP (17), the disulphide bond oxidoreductase DsbA (14, 55), and CpxP, which functions as both a chaperone and a repressor of the Cpx response (59, 61, 75). CpxR also regulates a variety of other genes with envelope-related functions (62, 69); for example, expression of macromolecular complexes such as flagella and pili is

repressed during the Cpx response, thereby reducing protein traffic to an already troubled periplasm.

2CSs can participate in regulatory networks by interacting with other types of regulators. Many such regulatory networks also include small non-coding RNAs (sRNAs). sRNAs are regulatory molecules approximately 50 to 300 nucleotides in length (reviewed in 37, 65). The best-characterized type of sRNAs, *trans*-encoded sRNAs, act by base-pairing with target mRNAs, using short regions of imperfect complementarity. This base-pairing can have several different outcomes. sRNAs can negatively regulate expression of their target mRNAs by blocking ribosomal access to the mRNA's ribosome-binding site, thereby reducing translation, and/or by increasing degradation by RNases such as RNase E. Conversely, sRNAs can positively regulate mRNA expression by removing secondary structures in the mRNA that normally inhibit ribosome binding, thereby increasing translation, or by protecting the mRNA from degradation by RNases. Key to many of these activities is the RNA chaperone protein Hfq (reviewed in 68), which both stabilizes sRNAs and promotes annealing to their target mRNAs.

Interactions between 2CSs and sRNAs are numerous and can occur in both directions—2CSs can regulate the transcription of genes encoding sRNAs, while sRNAs can also regulate the translation and/or stability of mRNAs encoding 2CS components (reviewed in 22, 43). A prime example of a 2CS controlling the expression of sRNAs is EnvZ/OmpR, which activates the expression of three sRNA genes (*micF*, *omrA*, and *omrB*) and represses the expression of one sRNA (*micC*) (9, 13, 24). OmrA and OmrB, in turn, repress expression of the *ompR* mRNA, creating a negative feedback loop (25). Expression of mRNAs encoding 2CS proteins can also be regulated by sRNAs that are members of different regulons. Such is the case for the *phoP* mRNA, which encodes the RR of the PhoPQ 2CS. Expression of *phoP* is repressed by two sRNAs (MicA and GcvB), each of which is controlled by a different regulator (the alternative sigma factor σ^E and the transcription factors GcvA and GcvR, respectively), thereby allowing communication between these regulatory pathways (11, 12).

In this study, our aim was to determine whether the Cpx regulon also contains sRNAs. Preliminary evidence that CpxAR regulates the expression of sRNAs was obtained in a recent microarray examining changes in gene expression upon overexpression of NlpE (62). In this microarray, expression of several sRNA genes (*micF*, *omrA*, *omrB*, and *rprA*) was increased by NlpE overexpression, while expression of *cyaR* was repressed. Additional regulators and mRNA targets of these genes are already known and are summarized in Table 4-1. Interestingly, the majority of these sRNAs regulate the expression of mRNAs encoding envelope-localized proteins (indicated in bold in Table 4-1), which is in keeping with the role of the Cpx system as an envelope stress response. In the present work, we confirmed Cpx regulation of four of these sRNAs. We found that the Cpx response regulates the expression of *cyaR* and *rprA* through direct binding of CpxR to the promoters of these genes, while Cpx affects expression of *omrA* and *omrB* indirectly via its regulation of the EnvZ/OmpR pathway. We additionally found that these sRNAs endow the Cpx response with several regulatory network motifs, with CyaR participating in a feedforward loop to regulate the IM protein YqaE and RprA participating in a novel feedback loop with CpxR.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 4-2. Unless otherwise stated, strains were cultured in Luria-Bertani broth at 37ºC with aeration at 225 rpm. Where indicated, isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen) was added to a concentration of 0.1 mM. Antibiotics (Sigma) were added where appropriate at the following concentrations: amikacin (Amk), 3 μg/ml; ampicillin (Amp), 100 μg/ml; chloramphenicol (Cam), 25 μg/ml; kanamycin (Kan), 30 μg/ml (*E. coli* K-12 strains) or 50 μg/ml (EPEC strains); spectinomycin (Spc), 25 μg/ml; tetracycline (Tet), 10 μg/ml.

4.2.2 Strain and plasmid construction

E. coli K-12 mutants and overexpression strains were constructed by standard techniques for P1 transduction and transformation (63). Donor strains harbouring mutations in *mzrA*, *rpoS*, *csgD*, and *ydaM* were obtained from the Keio library (3). Where indicated, the kanamycin resistance cassette contained within these mutations was removed by Flp/FRT-mediated recombination (26) to produce markerless deletions. EPEC strains were transformed by electroporation as previously described (27).

sRNA-lux transcriptional reporters were constructed as previously described (72). Briefly, promoters of sRNA genes were amplified by PCR, using the primer sequences listed in Table 4-3. Purified PCR products and the pJW15 *lux* reporter vector (40) were digested with *Bam*HI and *Eco*RI, gel-purified, and ligated together. Correct insertion of promoter sequences was verified by PCR and sequencing. In addition, *sRNA-lux* reporters were transformed into strains harboring mutations in known regulators of each gene (*cyaA* for *cyaR-lux*; *ompR* for *micF-lux*, *omrA-lux*, and *omrB-lux*; and *rcsB* for *rprA-lux*); regulator mutations affected expression of all *lux* reporters as expected based on published results (13, 24, 30, 35, 41; data not shown).

The *cyaR*::kan mutation in strain SV514 was constructed by λ Red recombination (67). The FRT-flanked kanamycin resistance cassette was amplified from the Keio library using primers cyaRKOFor and cyaRKORev (Table 4-3). The purified PCR product was electroporated into strain DY378, which encodes λ Red recombinase functions. *cyaR*::kan mutations in kanamycin-resistant transformants were verified by PCR. The *cyaR*::kan cassette was then transduced into *yqaE'-lacZ* reporter strain NRD397.

4.2.3 Luminescence assays

Activity of *lux* reporters was measured as previously described (57). Strains were cultured overnight in LB at 30ºC with aeration, then subcultured 1:100 into fresh LB and grown at 37ºC with aeration for 4 h (with IPTG induction after 2 h if necessary). Normalized luminescence was determined by dividing raw

luminescence (in counts per second, cps) by the optical density (OD_{600}) of the same culture. Luminescence assays were performed at least twice with five replicate cultures each time.

4.2.4 Electrophoretic mobility shift assays (EMSAs)

Maltose binding protein (MBP)-CpxR was purified from JM109 (pMCR) as described (60) with a few exceptions. First, cells were disrupted by passage though a French pressure cell once at 20,000 psi. Second, the crude extract was incubated with the amylose resin overnight with gentle agitation for batch protein purification. This mixture was then poured into a column for subsequent washing and elution steps. MBP-CpxR at the indicated concentration was incubated in the presence of acetyl phosphate (20 mM) at 37ºC for 30 minutes in 15 µl of binding buffer [10 mM Tris (pH 7.4), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 µg/ml BSA, 1 mM DTT, 20 mM potassium glutamate, 10 mM $MgSO_4$]. Next, 1.5 pmol of purified, PCR-amplified promoter DNA was added and the mixture was incubated for another 30 minutes at 37ºC. Reactions were stopped by the addition of 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water). Reactions were electrophoresed on a 5% non-denaturing TBE polyacrylamide gel (Bio-Rad) in 1X TBE running buffer [89 mM Tris, 89 mM boric acid, 1 mM EDTA (pH 8.0)]. DNA was visualized with an ethidium bromide stain.

4.2.5 β-galactosidase assays

Overnight cultures of strains to be assayed were prepared by inoculating 2 ml of LB broth containing appropriate concentrations of antibiotics with single colonies and grown at 37ºC overnight with shaking at 225 rpm. For the P_{BAD}::*yqaE'-lacZ* reporter experiments, strains were subcultured 1:200 in fresh LB with antibiotics and grown at 37ºC with shaking for 6 h; arabinose was added to a final concentration of 0.01% 4 h post-subculture to induce reporter expression. For all other experiments, strains were subcultured 1:50 into fresh LB with antibiotics and grown at 37ºC with shaking to early stationary phase (5 h).

Strains harbouring sRNA overexpression plasmids were induced with 0.1 mM IPTG after 3 h growth. β-galactosidase activity was measured as previously described (7), with 5 µl of cell culture being added to 195 µl of 1X Z-buffer for strains carrying the *cpxP-lacZ* reporter gene due to high reporter activity. Each strain was assayed in triplicate.

4.2.6 Western blot analysis

Subcultures for whole-cell lysates of bacterial strains used for Western blot analysis were prepared by diluting overnight cultures 1:50 into 5 ml fresh LB containing appropriate concentrations of antibiotics. Cultures were grown to early stationary phase (5 h) at 37ºC with shaking, with induction of expression plasmids after 3 h of growth by addition of 0.1 mM IPTG. One-ml samples, standardized to the same optical density at 600 nm, were pelleted, and cell pellets were lysed in 50 µl of 2X SDS-PAGE loading dye [125 mM Tris (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 6% sodium dodecyl sulphate, 0.2% bromophenol blue]. Electrophoresis and blotting were performed as previously described (61) with rabbit α-MBP-CpxR (1:10,000 dilution) or α-MBP-CpxA (1:50,000 dilution) primary antibodies and α-rabbit-alkaline phosphatase secondary antibodies (Sigma, 1:25,000 dilution). Proteins were detected by chemiluminescence using a Bio-Rad ChemiDoc MP™ imaging system and an Immun-Star alkaline phosphatase chemiluminescence kit (Bio-Rad).

4.2.7 RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR)

To prepare for RNA extraction, strains were cultured overnight in LB in triplicate, then subcultured 1:100 into fresh medium. Subcultures were grown in one of two ways: for "immediate induction", 0.1 mM IPTG was added to each subculture at the time of inoculation. Subcultures were then grown at 37ºC with aeration to an OD_{600} of 0.5. For "delayed induction", subcultures were grown at 37ºC with aeration to an OD_{600} of 0.3, then induced with 0.1 mM IPTG and incubated for an additional 2 h at 37ºC. At the end of the growth period, a 1-ml

sample of each culture was pelleted in a microfuge. RNA was isolated from cell pellets using TRIzol Reagent (Ambion) as per manufacturer's instructions. One µg of RNA from each culture was treated with DNase I (Invitrogen) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen). A control reaction was also performed for each sample, in which no reverse transcriptase was added. qPCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) as previously described (57). Relative abundance of *rprA* transcripts was determined using the $\Delta\Delta C_T$ program, with *rpoD* as an endogenous control. The sequences of all primers used for qPCR are given in Table 4-3.

4.3 Results

In order to confirm the preliminary microarray results indicating that the sRNAs *cyaR*, *micF*, *omrA*, *omrB*, and *rprA* are members of the Cpx regulon (62), we examined the effect of Cpx pathway activation and inactivation upon expression of these genes using *lux* transcriptional reporters. As was the case with the microarrays, these analyses were conducted in both *E. coli* K-12 strain MC4100 and enteropathogenic *E. coli* (EPEC) strain E2348/69. We were able to confirm Cpx regulation of four of the five genes; expression of *micF* was not consistently altered by activation or inactivation of the Cpx response, as determined using either a *micF-lux* reporter or quantitative reverse transcriptase PCR (qRT-PCR) (data not shown).

In order to assess whether the Cpx response affects expression of the *sRNA-lux* reporters, we compared expression of the reporters in wild-type MC4100 or EPEC to strains in which the Cpx response had been mutationally activated [*cpxA24* mutation, a constitutively activating mutation resulting from the deletion of 30 amino acids in the periplasmic domain of CpxA (60)] or mutationally inactivated (through insertional mutation of the RR gene *cpxR*). In addition, we also examined the effect of *nlpE* overexpression on reporter activity, since this method of activating the Cpx response was used in the microarray and has the advantage of acting through a wild-type Cpx 2CS (62).

4.3.1 CpxR directly represses expression of *cyaR*

In the microarray, CyaR was the only sRNA whose expression was downregulated upon *nlpE* overexpression (62). Using a *cyaR-lux* transcriptional reporter, we confirmed that Cpx activation via the *cpxA24* allele causes a twofold or greater decrease in *cyaR* expression in both MC4100 and EPEC (Figure 4-1A). Activation of the Cpx response through overexpression of *nlpE* also caused a significant decrease in *cyaR-lux* activity in EPEC but not in MC4100 (Figure 4-1B). These results match closely with the microarray data, in which *nlpE* overexpression in EPEC but not MC4100 caused a threefold decrease in *cyaR* expression (62). Interestingly, inactivation of the Cpx response through mutation of *cpxR* also decreased *cyaR-lux* expression (Figure 4-1A), although the effect of the *cpxR* mutation was consistently smaller than the effect of the *cpxA24* mutation.

In order to determine whether Cpx repression of *cyaR* expression is direct or indirect, we performed an electrophoretic mobility shift assay (EMSA) to assess the ability of a purified MBP-CpxR fusion protein, which has previously been shown to complement a *cpxR* mutation (60), to bind to the *cyaR* promoter *in vitro*. We found that addition of 50 pmol or more of MBP-CpxR to the *cyaR* promoter DNA caused the appearance of a distinct CpxR-DNA complex (Figure 4-1C). The *cyaR* promoter had lower affinity for MBP-CpxR than the positive control, *cpxP*, which gave a shifted band with 25 pmol of protein (Figure 4-2A), but higher affinity than the negative control, *rpoD*, which did not bind to MBP-CpxR unless at least 100 pmol of protein was added (Figure 4-2B). We therefore concluded that CpxR weakly bound the *cyaR* promoter region, which could explain the relatively small change in expression of *cyaR* (two- to fourfold repression) when the Cpx response was activated (Figures 4-1A and B).

4.3.2 CpxR directly regulates expression of *rprA* in EPEC

To examine Cpx regulation of *rprA*, we transformed an *rprA-lux* reporter into wild-type and mutant strains of both MC4100 and EPEC. However, we were unable to detect luminescence activity in any of the MC4100 strains tested (data

not shown), suggesting that *rprA* is not expressed or is expressed at extremely low levels in this strain of *E. coli*. Since the same reporter construct was used in both MC4100 and EPEC, we do not believe that the lack of expression reflects a mutation in the *rprA* promoter in MC4100. Rather, the lack of expression of the reporter in MC4100 could reflect a regulatory difference between the strains, such as altered expression or activity of the Rcs pathway, which controls *rprA* expression (41).

Expression of the *rprA-lux* reporter was strongly repressed by the *cpxA24* mutation in EPEC, while the *cpxR* null mutation had little effect on *rprA-lux* activity (Figure 4-3A). Unexpectedly, activation of the Cpx response by overexpression of *nlpE* had the opposite effect on the *rprA-lux* reporter, enhancing its expression approximately 25-fold (Figure 4-3B), which correlates well with the eightfold increase in *rprA* expression observed in the microarray (62). EMSA analysis showed that MBP-CpxR bound to the *rprA* promoter region (Figure 4-3C), with similar affinity as for the *cpxP* promoter positive control (Figure 4-2A). Three distinct shifted bands were observed when MBP-CpxR was added to the *rprA* promoter DNA (Figure 4-3C), suggesting the presence of multiple CpxR binding sites in the promoter region.

We considered several possible explanations for the opposite effects of the *cpxA24* mutation and overexpression of *nlpE* on *rprA-lux* activity. First, we speculated that the Cpx response could have dose-dependent effects on *rprA* expression, since the *cpxA24* mutation is an extremely strong activator of the Cpx pathway and likely leads to higher levels of phosphorylated CpxR than does overexpression of *nlpE* (57). To address this possibility, we repeated the luminescence assay in Figure 4-3B using a range of IPTG concentrations (from 1 μM to 10 mM) to induce varying levels of *nlpE* overexpression. At concentrations of 10 μM IPTG or below, *nlpE* overexpression had no effect on *rprA-lux* activity compared to the vector control, while all higher concentrations of IPTG activated *rprA-lux* expression (Figure 4-4). Therefore, our data do not provide support for dose-dependent effects on *rprA-lux* expression, although it is still possible that the *cpxA24* mutation activates the pathway more strongly than

the highest level of *nlpE* overexpression tested. Second, we hypothesized that *nlpE* overexpression could have Cpx-independent effects that could lead to increased *rprA* expression. However, using qRT-PCR, we found that *nlpE* overexpression had little effect on *rprA* expression in a *cpxR* mutant (Figure 4-5), ruling out Cpx-independent effects of *nlpE* overexpression. Third, we hypothesized that the timing of Cpx pathway induction could affect *rprA* expression. In the *cpxA24* mutant, the Cpx pathway is constitutively induced at all growth phases. In contrast, in our *nlpE* overexpression luminescence assays, we induced *nlpE* overexpression when the bacteria had already reached mid-log phase. Using qRT-PCR, we found that inducing *nlpE* overexpression at the time of inoculation of subcultures (i.e. lag phase) caused repression of *rprA* expression, whereas inducing *nlpE* overexpression in mid-log phase caused activation of *rprA* expression (Figure 4-5). We concluded that the timing of Cpx pathway activation determined whether expression of *rprA* was activated or repressed.

4.3.3 The Cpx response indirectly regulates expression of *omrA* and *omrB*

Although *omrA* and *omrB* are encoded by neighbouring genes, each sRNA gene is transcribed as a monocistronic transcript from its own promoter (24). We therefore created *lux* reporters for both promoters and examined the effects of activating or inactivating the Cpx pathway upon their expression. Both reporters were activated by the *cpxA24* mutation in both MC4100 and EPEC (Figure 4-6A), although *omrA-lux* was more strongly activated than *omrB-lux* (14- to 25-fold activation for *omrA-lux*, compared with three- to fourfold activation for *omrB-lux*). Mutation of *cpxR* did not significantly affect expression of the reporters (Figure 4-6A). Both *omrA-lux* and *omrB-lux* were strongly activated by *nlpE* overexpression; in fact, even leaky expression of *nlpE* from pCA-*nlpE* in the absence of IPTG was sufficient to increase *omrA-lux* and *omrB-lux* expression compared to the vector control (Figure 4-6B).

Previously, Gerken and colleagues reported that activation of the Cpx response increased expression of an *omr-lacZ* reporter in a manner dependent on the connector protein MzrA (21). Expression of *mzrA* is activated by CpxR; the

IM protein MzrA then physically interacts with the periplasmic domain of EnvZ, promoting phosphorylation of OmpR and activating expression of OmpR-regulated genes such as *omrA* and *omrB* (20, 21). However, the *omr-lacZ* reporter in Gerken *et al*.'s study was constructed in such a way that the *omrA* promoter was deleted; it was therefore unclear whether Cpx activation of both *omrA* and *omrB* was dependent on MzrA, or if this was the case only for *omrB*. To assess the contribution of MzrA to Cpx activation of the *omr* genes, we constructed *ΔmzrA* and *cpxA24* single and double mutants of MC4100 and measured *omrA-lux* and *omrB-lux* activity in each strain. As shown in Figure 4-7, introduction of the *cpxA24* allele into a *ΔmzrA* mutant did not significantly increase expression of either *omrA-lux* or *omrB-lux*. Therefore, we concluded that the Cpx pathway activated expression of both *omrA* and *omrB* indirectly through its effects on MzrA and the EnvZ/OmpR pathway.

4.3.4 Cpx regulation of both *cyaR* and *yqaE* creates a feedforward loop

We next turned to the question of what roles sRNAs could play in the Cpx response. Previous studies have shown that sRNAs frequently participate in regulatory network motifs such as feedforward loops, in which a regulator controls expression of a target gene both directly (by binding to its promoter) and indirectly, by regulating expression of a third gene, which is itself a regulator of the target gene (5). We identified a potential feedforward loop consisting of CpxR, CyaR, and the IM protein YqaE. Transcription of the *yqaE* gene was previously shown to be activated by the Cpx response (62), while translation of *yqaE* is known to be repressed by CyaR (35). Combined with our results from Figure 4-1, these data suggested that CpxR could increase expression of *yqaE* both directly, by binding to its promoter, and indirectly, by decreasing expression of *cyaR*.

In order to address whether CpxR directly regulates transcription of *yqaE*, we performed an EMSA. We found that MBP-CpxR binds to the *yqaE* promoter with similar affinity as to the *cpxP* positive control (Figures 4-8A and 4-2A), confirming that the previously reported transcriptional regulation of *yqaE* is

direct. To assess whether the Cpx response regulates translation of *yqaE*, we used a previously described P_{BAD}::*yqaE'-lacZ* translational reporter (35). Transcription of this construct is driven by the arabinose-inducible P_{BAD} promoter and is therefore not subject to regulation by CpxR. When the Cpx response was activated by the plasmid pCA-*nlpE*, expression of the *yqaE* translational reporter increased approximately twofold relative to the vector control (Figure 4-8B). The majority of the Cpx enhancement of *yqaE* translation was CyaR-dependent, since pCA-*nlpE* increased reporter expression only ~1.2fold in a *cyaR*::kan mutant strain (Figure 4-8B). Our data indicate that Cpx repression of *cyaR* expression gives rise to a coherent feedforward loop, in which CpxR both directly and indirectly activates *yqaE* expression.

4.3.5 RprA overexpression inhibits the Cpx response

In addition to feedforward loops, many sRNAs also regulate the expression of their own regulator, creating a feedback loop (5). To determine whether OmrA, OmrB, RprA or CyaR function in a positive or negative feedback loop with the Cpx response, changes in *cpxP-lacZ* activity were assayed by β-galactosidase assay following overexpression of these genes in *E. coli* MC4100. MicF, although not a member of the Cpx regulon (data not shown), was included in these analyses because it has previously been shown to repress *cpxR* translation (28). Activity of the *cpxP-lacZ* transcriptional reporter decreased by roughly twofold following overexpression of *rprA* (Figure 4-9A). Small but significant changes in reporter gene activity were also recorded following overexpression of *omrA* and *omrB* ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test). Activity of a *degP-lacZ* transcriptional reporter was also inhibited to a similar degree by RprA overexpression but not by MicF, OmrA, or OmrB (Figure 4-9B). Overexpression of CyaR had a small but significant effect on *degP-lacZ* expression (Figure 4-9B), but since CyaR did not affect the *cpxP-lacZ* reporter (Figure 4-9A), this effect is unlikely to be dependent on the Cpx pathway. Since *degP* is transcriptionally activated by both the Cpx 2CS as well as the σ^E stress response (17, 18), we also assayed expression of a uniquely σ^E-

controlled transcriptional reporter, *rpoHP3-lacZ*, and found that it was not inhibited by RprA overexpression (Figure 4-9C).

Western blots against CpxR and CpxA showed that overexpression of RprA did not result in a decrease in intracellular abundance of either of these proteins (Figure 4-10). This result implies that repression of the Cpx pathway by RprA does not result from direct translational inhibition of either *cpxA* or *cpxR*, but rather from indirect inhibition of the pathway. As previously demonstrated by Holmqvist *et al*. (28), a significant decrease in both CpxA and CpxR protein levels was observed upon overexpression of MicF (Figure 4-10).

4.3.6 Cpx pathway inhibition by RprA is independent of RprA's known targets and dependent on CpxR

In order to determine whether inhibition of the Cpx pathway by RprA occurred through one of RprA's known targets, *rpoS*, *ydaM* and *csgD* (33, 42, 46), we deleted each of these individually and assayed for a disappearance of Cpx repression upon *rprA* overexpression. Overexpression of RprA in *E. coli* W3110 strains harbouring deletions of *rpoS*, *ydaM*, or *csgD* still resulted in a decrease in *cpxP-lacZ* reporter activity (Figure 4-11), suggesting that inhibition of the Cpx pathway by RprA does not occur via its regulation of these target genes.

Since RprA does not appear to inhibit the Cpx response by acting directly on the transcripts of *cpxA* or *cpxR,* or through any of its published targets, we sought to determine whether inhibition of Cpx activity by RprA involves sensing of an envelope-localized inducing cue by CpxA by assessing whether mutations to *cpxA, cpxP* or *cpxR* in *E. coli* W3110 abolished inhibition of the Cpx pathway by RprA. Experiments with a *degP-lacZ* reporter revealed that RprA overexpression was still able to repress the Cpx pathway in *cpxA*::cam and *cpxP*::kan strains, but that inhibition disappeared almost entirely in a *cpxR*::spc strain (Figure 4-12A). These data suggested that inhibition by RprA occurs not through its regulation of some envelope-localized component sensed by CpxA but rather by signalling through CpxR.

It has previously been shown that phosphorylation of CpxR can occur in a CpxA-independent manner by the small molecular weight phosphodonor acetyl phosphate, a product of the Pta-AckA pathway (71). Thus, we investigated the possibility that Cpx inhibition by RprA may occur through the Pta-AckA pathway by deleting both *pta* and *ackA* and measuring inhibition of the *cpxP-lacZ* reporter by RprA. No difference between the wild-type strain and the *pta-ackA*::Tn*10* strain was observed upon RprA overexpression (Figure 4-12B). Therefore, RprA inhibits the Cpx pathway via CpxR in a Pta-AckA-independent manner.

4.4 Discussion

Two-component systems and sRNAs are both widely used by bacteria to regulate gene expression in response to environmental changes. In recent years, many connections between these two types of regulators have been revealed, with at least six of *E. coli*'s 30 2CSs shown to regulate the expression of one or more sRNAs, and numerous sRNAs demonstrated to directly or indirectly regulate 2CS activity (22). In this work, we showed for the first time that the Cpx 2CS regulates the expression of sRNAs. We found that CpxR regulates expression of *cyaR* and *rprA* by direct binding to their promoters, while indirectly regulating *omrA* and *omrB* expression through the connector protein MzrA and the EnvZ/OmpR 2CS. These Cpx-regulated sRNAs create new regulatory motifs not previously identified within the Cpx response, including both feedforward and negative feedback loops.

4.4.1 Cpx regulation of sRNA expression

Microarray analysis suggested that several sRNA genes – *cyaR*, *micF*, *omrA*, *omrB*, and *rprA* – could be members of the Cpx regulon (62). Here, we identified two of these sRNA genes, *cyaR* and *rprA*, as direct targets of Cpx regulation (Figures 4-1 and 4-3). Electrophoretic mobility shift assays demonstrated that CpxR bound to the promoters of these genes more strongly than to the negative control, *rpoD* (Figures 4-1C, 4-2B, and 4-3C). Furthermore, the

affinity with which CpxR bound to the sRNA promoters correlated with the strength of regulation. *cyaR*, whose promoter was bound weakly by CpxR, was weakly repressed by activation of the Cpx response, while *rprA*, with higher binding affinity to CpxR, was more strongly regulated by Cpx activation (Figures 4-1 and 4-3).

Cpx regulation of *rprA* expression followed an unusual pattern. When the Cpx response was activated constitutively (Figure 4-3A) or during lag phase (Figure 4-5A), expression of *rprA* was strongly repressed. However, when the Cpx response was activated in mid-log phase (Figures 4-3B and 4-5B), expression of *rprA* was activated. At this time, the explanation for this behaviour is unknown. Since CpxR binds directly to the *rprA* promoter (Figure 4-3C), the growth phase-dependent regulation could reflect physical interactions with other regulators that bind to the *rprA* promoter. Currently, only two other regulators of *rprA* expression are known – RcsB, which is believed to activate *rprA* expression by directly binding to its promoter (41), and LrhA, a repressor of *rprA* expression whose mechanism of regulation is unknown (54). CpxR could potentially facilitate or interfere with the binding of one of these regulators, or another currently unknown regulator, to the *rprA* promoter in a growth phase-dependent manner.

We noted that inactivation of the Cpx response by mutation of *cpxR* did not strongly affect the expression of any of the sRNA genes tested (Figures 4-1A, 4-3A, and 4-6A), although *cyaR* expression was slightly but significantly decreased in the *cpxR* mutant. In contrast, some 2CSs are required for expression of the sRNA genes in their regulons; for example, expression of *omrA* and *omrB* is undetectable in an *ompR* null mutant (24). These results raise the question of whether there are any sRNAs whose expression is completely dependent on the Cpx system. Previously, we found that expression of numerous intergenic regions was altered upon *nlpE* overexpression, using a high-density tiling microarray (62). Further studies will hopefully elucidate whether any of these intergenic regions encode novel, Cpx-dependent sRNAs.

4.4.2 Cpx-controlled feedforward loops

Interactions between regulators can produce regulatory network motifs that have unique properties. One of these motifs is the feedforward loop, in which a regulator controls the expression of a target gene both directly (by binding to its promoter) and indirectly (by regulating another regulator that also affects expression of the target) (1, 5). Feedforward loops can be classified as either coherent, if the direct and indirect routes of regulation are both positive or both negative, or incoherent, if one route is positive and the other negative (1). sRNAs have been shown to participate in both coherent and incoherent feedforward loops (45). We have identified a coherent feedforward loop, in which CpxR activates expression of *yqaE* both directly, by binding to its promoter and increasing *yqaE* transcription (Figure 4-8A; 62), and indirectly, by repressing expression of *cyaR* and thereby increasing translation of *yqaE* (Figure 4-8B; 35). This feedforward loop can be sub-categorized as a type 4 coherent feedforward loop (1), meaning that the transcriptional regulator (CpxR) positively regulates the target gene (*yqaE*), and negatively regulates the secondary regulator (CyaR), which represses expression of the target.

Beisel and Storz recently described a similar type 4 coherent feedforward loop containing the sRNA Spot42 (6). In this case, the catabolite repression protein CRP represses expression of Spot42 but activates the expression of a variety of genes involved in utilization of alternative carbon sources, such as the glucitol/sorbitol permease gene *srlA* and the L-fucose isomerase gene *fucI*. Spot42, in turn, represses translation of these metabolic genes. The authors found that inclusion of Spot42 in the feedforward loop reduced leaky expression of the alternative carbon source genes when glucose was present and CRP was inactive, when compared to transcriptional regulation by CRP alone. Additionally, Spot42 changed the dynamics of target gene regulation. When CRP was activated, Spot42 caused a delay in target gene activation, which could help to prevent activation of the target genes in response to transient signals. Conversely, when CRP was inactivated, Spot42 caused a faster decrease in target protein levels than could be

achieved by transcriptional regulation alone. This feature allows cells to adapt more rapidly to glucose-replete conditions.

Given the related architecture of the CpxR-CyaR-*yqaE* feedforward loop, CyaR could play a similar role to Spot42, reducing leaky expression of *yqaE* under Cpx-inactive conditions, delaying *yqaE* activation in response to Cpx pathway induction, and increasing the speed at which *yqaE* expression is repressed when the Cpx pathway is inactivated. Since the function of YqaE is currently unknown, the benefits of such regulation are difficult to predict. However, it was shown that a *yqaE* mutant was more resistant to several envelope-damaging compounds in a Biolog Phenotype Microarray (62). This observation suggests that increased expression of *yqaE* may make cells more sensitive to some toxic agents. Therefore, CyaR repression of *yqaE* could help to prevent leaky or premature expression of *yqaE* that could increase sensitivity to these chemicals, ensuring that *yqaE* is only expressed when the Cpx response is fully activated.

In addition to the feedforward loop involving CyaR and *yqaE*, sRNAs could create additional Cpx-controlled feedforward loops that were not investigated here. For example, CpxR is known to directly repress the transcription of *csgD*, encoding the master regulator of curli expression (32, 51, 58). Since translation of *csgD* is repressed by RprA (33, 46), it is possible that Cpx activation affects both the transcription and the translation of *csgD* through its regulation of *rprA* expression (Figure 4-3).

4.4.3 sRNAs affecting Cpx pathway activity

Recent studies have identified numerous sRNAs that affect the expression or activity of 2CSs. This control of 2CSs by sRNAs can result in either the formation of a feedback loop or communication between different regulatory systems, depending on whether the regulatory system that controls expression of the sRNA is the same or different from the regulator that is regulated by the sRNA (22). Our data, combined with that of Holmqvist *et al.* (28), indicate that both types of sRNA regulation affect the Cpx pathway, with RprA mediating

feedback regulation and MicF possibly permitting communication with other regulators.

sRNAs can mediate feedback regulation in two ways – a direct feedback loop occurs when the sRNA directly targets the transcript of its own regulator, while an indirect feedback loop occurs when the sRNA targets a different gene that affects the expression or activity of its own regulator (5). Our data demonstrate that RprA forms a negative, indirect feedback loop with CpxR. Overexpression of RprA decreased expression of two Cpx-regulated genes (Figure 4-9), but did not decrease the abundance of CpxR or CpxA proteins (Figure 4-10), suggesting that RprA modulates the activity of the Cpx pathway rather than its expression. Since signals can enter the Cpx pathway through numerous signalling components (69), we overexpressed RprA in mutants lacking the Cpx system components CpxP, CpxA, and CpxR in order to gain more information about the mechanism of RprA feedback regulation. This experiment showed that RprA's effects on the Cpx pathway were dependent on CpxR, but not on CpxA (Figure 4-12A). Surprisingly, RprA-mediated repression was not dependent on the Pta-AckA pathway (Figure 4-12B), which was previously shown to be the major CpxA-independent source of CpxR phosphorylation when cells are grown in the presence of excess glucose (15). Furthermore, RprA repression of Cpx activity was not dependent on any of RprA's known targets (Figure 4-11), suggesting that additional targets of RprA regulation remain to be identified.

There are several possible mechanisms by which RprA could conceivably influence the activity of CpxR without altering its expression. One possibility is that RprA regulates a non-cognate HK (i.e. not CpxA) that is capable of cross-phosphorylating CpxR. We do not favour this possibility, however, since crosstalk between 2CSs has been shown to be unlikely to occur *in vivo* (23, 64). Another exciting possibility is that RprA regulates the expression of a novel auxiliary regulator that influences CpxR activity. Such auxiliary regulators can affect the activity of an RR by modulating its rate of phosphorylation or dephosphorylation or its ability to bind to DNA (48). Identification of the mechanism by which RprA

influences Cpx pathway activity could therefore shed light on both the cellular role of this sRNA and on the regulation of 2CS activity.

We found that overexpression of MicF decreased the abundance of both CpxR and CpxA (Figure 4-10), as expected based on the previous report that MicF decreases expression of a *cpxR-gfp* translational fusion (28). Curiously, despite this decrease in Cpx protein levels, overexpression of *micF* did not significantly decrease expression of the Cpx-regulated genes *cpxP* and *degP* (Figure 4-9). We considered the possibility that MicF overexpression might affect Cpx activity only under pathway-activating conditions, since gene regulation by CpxR depends not only on its abundance but also on its phosphorylation status. However, when we repeated the β-galactosidase assay from Figure 4-9A in LB broth at pH 8, which is an inducing condition for the Cpx pathway (15), we still found no effect of MicF overexpression on *cpxP-lacZ* activity (data not shown). Thus, the significance of MicF regulation of *cpxRA* is currently unclear. One possibility that we are currently investigating is that MicF overexpression could have differential effects on Cpx regulon members – decreased levels of CpxR could have a larger effect on its ability to bind to low affinity promoters than its ability to bind to high affinity promoters like *cpxP*. If this hypothesis is correct, MicF could provide a mechanism for its regulators, including EnvZ/OmpR and Lrp, to restrict the size of the Cpx regulon to include only its most important targets.

In summary, we have demonstrated that sRNAs comprise a previously unrecognized part of the Cpx regulon, with roles in both feedforward and feedback regulation. In addition, multiple sRNAs affect the expression or activity of CpxR. These results demonstrate that sRNAs link the Cpx response to a variety of other cellular regulators. Such regulatory connections may play an important role in *E. coli*'s ability to withstand envelope stress.

4.5 Tables and Figures

Table 4-1. sRNAs identified in the NlpE overexpression microarray with their known regulators and mRNA targets.

sRNA	Regulator(s) of sRNA expression	mRNA target(s)*	References
CyaR	Crp/cAMP, σ^E	***ompX***, *luxS*, *nadE*, ***yqaE***	30, 35
MicF	EnvZ/OmpR, Lrp, MarA, SoxS, Rob, H-NS	***ompF***, *lrp*, ***phoE***, *cpxR*	2, 10, 13, 19, 28, 39, 49, 66
OmrA	EnvZ/OmpR	***ompT***, ***cirA***, ***fepA***, ***fecA***, *ompR*, *csgD*, *flhD*	24, 25, 29, 36
OmrB	EnvZ/OmpR	***ompT***, ***cirA***, ***fecA***, *ompR*, *csgD*, *flhD*	24, 25, 29, 36
RprA	RcsCB, LrhA	*rpoS*, *csgD*, *ydaM*	33, 41, 42, 46, 54

*targets listed in boldface encode envelope-localized proteins

Table 4-2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Bacterial strains		
JM109	F' *traD36 lacI^q Δ(lacZ)M15 proA$^+$B$^+$*/ e14 (McrA$^-$) *Δ(lac-proAB) thi gyrA96* (Nal^r) *endA1 hsdR17*(r_k^- m_k^+) *relA1 supE44 recA1*	73
MC4100	F$^-$ *araD139 Δ(argF-lac)* U169 *rpsL150* (Str^r) *relA1 flhD5301 deoC1 ptsF25 rbsR*	8
TR51	MC4100 *cpxR*::spc^r	61
TR10	MC4100 *cpxA24* (Amk^r)	61
E2348/69	Prototypical EPEC O127:H6 laboratory strain	38
ALN88	E2348/69 *cpxR*::kan^r	50
ALN195	E2348/69 *cpxA24* (Amk^r)	40
ALN234	E2348/69 *cpxR*::cam^r	40
SV415	MC4100 *ΔmzrA*	This study
SV416	TR10 *ΔmzrA* (Amk^r)	This study
DY378	W3110 λ*c*I*857* Δ(*cro-bioA*)	74
NRD397	MG1655 *mal::lacI^q ΔaraBAD lacI'*::P_{BAD}::*yqaE'-lacZ*	35
SV514	NRD397 *cyaR*::kan^r	This study
W3110	F$^-$ λ$^-$ *rpoS(Am) rph-1 Inv(rrnD-rrnE)*	4
2K1056	W3110 Δ(*argF-lac*) U169	56
TR50	MC4100 λRS88[*cpxP'-lacZ*$^+$]	60
AE613	2K1056 λRS88[*degP'-lacZ*$^+$]	This study
AE614	2K1056 λRS88[*cpxP'-lacZ*$^+$]	This study
AE663	AE614 *rpoS*::kan^r	This study
AE664	AE614 *ydaM*:: kan^r	This study
AE666	AE614 *ΔcsgD*	This study
AE667	AE613 *cpxA*:: cam^r	This study
AE668	AE613 *cpxP*:: kan^r	This study
AE669	AE613 *cpxR*:: spc^r	This study
AE670	AE614 *pta-ackA*::Tn*10* (tet^r)	This study
Plasmids		
pMCR	Overexpresses a functional MBP-CpxR fusion (Amp^R) from the pMal-C vector	60
pCA24N	Vector control from ASKA library; Cam^r	34
pCA-*nlpE*	IPTG-inducible *nlpE* overexpression vector from ASKA library; Cam^r	34
pJW15P*cyaR*	pJW15 luminescence reporter plasmid containing *cyaR* promoter; Kan^r	This study

pJW15P*omrA*	pJW15 luminescence reporter plasmid containing *omrA* promoter; Kan^r	This study
pJW15P*omrB*	pJW15 luminescence reporter plasmid containing *omrB* promoter; Kan^r	This study
pJW15P*rprA*	pJW15 luminescence reporter plasmid containing *rprA* promoter; Kan^r	This study
pBR-plac	pBR322 expression vector containing IPTG-inducible P_{lacO-1} promoter; Amp^r	24
pBR-Plac-cyaR	pBR-plac-based IPTG-inducible *cyaR* overexpression vector; Amp^r	35
pBR-Plac-micF	pBR-plac-based IPTG-inducible *micF* overexpression vector; Amp^r	44
pBR-Plac-omrA	pBR-plac-based IPTG-inducible *omrA* overexpression vector; Amp^r	24
pBR-Plac-omrB	pBR-plac-based IPTG-inducible *omrB* overexpression vector; Amp^r	24
pBR-Plac-rprA	pBR-plac-based IPTG-inducible *rprA* overexpression vector; Amp^r	44

Table 4-3. Oligonucleotide primers used in this study.

Primer name	Sequence*
PcyaRFEcoRI	5'-TTTTGAATTCTGGTTATACTGTGTGGCTCC-3'
PcyaRRBamHI	5'-TTTTGGATCCGAGGTGGTTCCTGGTACAGC-3'
PomrAForEcoRI	5'-TTTTGAATTCCATCAATCTGTAACAGTAACCG-3'
PomrARevBamHI	5'-TTTTGGATCCCACCAATCAATACCTCTGGG-3'
PomrBForEcoRI	5'-TTTTGAATTCTGGTCGCCATGAAAATACC-3'
PomrBRev2BamHI	5'-TTTTGGATCCGTAATTCATGTGCTCAACCC-3'
PrprAForEcoRI	5'-TTTTGAATTCAAATTCTCGAAGAACTTGGC-3'
PrprARevBamHI	5'-TTTTGGATCCTTCACTCAGGGGATTTCC-3'
cyaRKOFor	5'-TAGAAACCGATCACATACAGCTGCATTTATTAAGGTTATCATCCGTTTCATTCCGGGGATCCGTCGACC-3'
cyaRKORev	5'-TGTGTGGACGTGACCAGAAATAAATCCTTTTATTTCATTGTATTACGCGTTGTAGGCTGGAGCTGCTTCG-3'
rpoDF (qRT-PCR)	5'-TGCGTATGCGTTTCGGTATC-3'
rpoDR (qRT-PCR)	5'-GCGGGTAACGTCGAACTGTT-3'
rprAF (qRT-PCR)	5'-AGCATGGAAATCCCCTGAGTG-3'
rprAR (qRT-PCR)	5'-ATCGTGGGAGATGGGCAAAG-3'
cpxPFor (EMSA)	5'-CAGCTCTCGCTGATCATCAAC-3'
cpxPRev (EMSA)	5'-CTGACGCTGATGTTCGGTTA-3'
rpoDFor2 (EMSA)	5'-ATGAATAAGTGTGGATACCG-3'
rpoDRev (EMSA)	5'-TCACCTGAATGCCCATGTCG-3'
PcyaRFor (EMSA)	5'-TGGTTATACTGTGTGGCTCC-3'
PcyaRRev (EMSA)	5'-GAGGTGGTTCCTGGTACAGC-3'
PrprAFor (EMSA)	5'-AAATTCTCGAAGAACTTGGC-3'
PrprARev (EMSA)	5'-TTCACTCAGGGGATTTCC-3'
PomrAFor (EMSA)	5'-CATCAATCTGTAACAGTAACCG-3'
PomrARev (EMSA)	5'-CACCAATCAATACCTCTGGG-3'
PomrBFor (EMSA)	5'-TGGTCGCCATGAAAATACC-3'
PomrBRev (EMSA)	5'-GTAATTCATGTGCTCAACCC-3'
yqaE-fwd-EMSA	5'-GTGCCGGGGGAACCGCTAAG-3'
yqaE-rev-EMSA	5'-TTACCGAGCAGCACGCCGAG-3'

*Underlined sequences denote restriction sites (*Bam*HI: GGATCC; *Eco*RI: GAATTC).

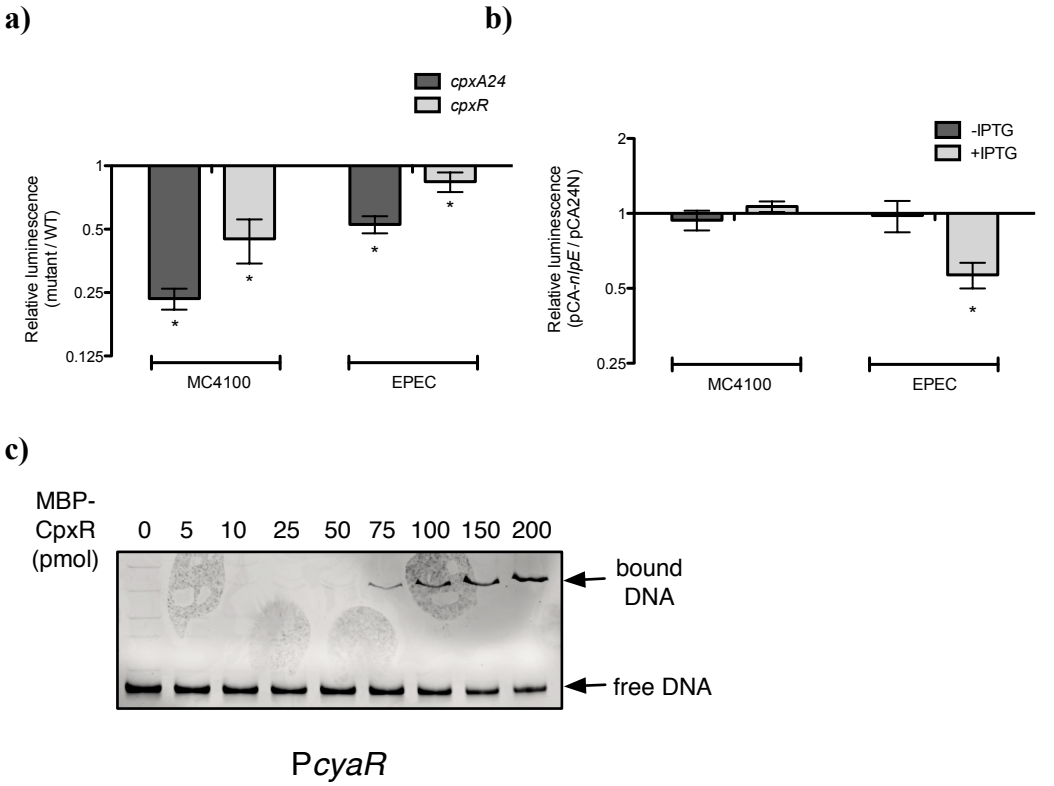
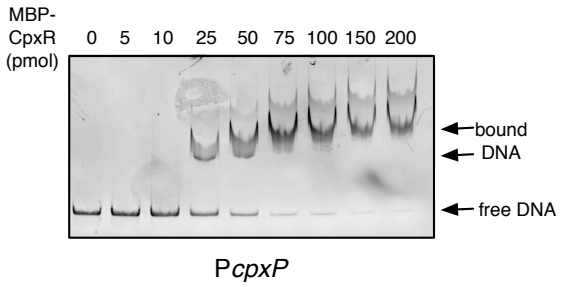


Figure 4-1. CpxR directly represses expression of *cyaR*. a) Luminescence assays comparing *cyaR-lux* reporter expression in wild-type (WT), *cpxA24* (Cpx-activating mutation) or *cpxR* (Cpx-inactivating mutation) strains of MC4100 and EPEC. **b)** Luminescence assays comparing *cyaR-lux* reporter expression in wild-type MC4100 and EPEC carrying vector control pCA24N or the overexpression plasmid pCA-*nlpE*. Luminescence was normalized to the OD_{600} of the culture. Data for luminescence assays represent the mean and standard deviation of five replicate cultures. **c)** EMSA. A PCR product containing the *cyaR* promoter region was incubated alone or with increasing concentrations of MBP-CpxR protein, then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining. * denotes a statistically significant difference from the relevant wild-type or vector control ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test).
Panel **(c)** courtesy of Randi Guest.

a)



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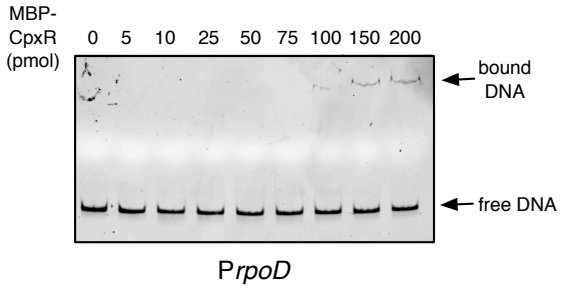


Figure 4-2. CpxR binding to positive and negative control promoters (EMSAs). PCR products containing the *cpxP* promoter (**a**, positive control for CpxR binding) or *rpoD* promoter (**b**, negative control for CpxR binding) were incubated alone or with increasing concentrations of MBP-CpxR protein, then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining.
Figure courtesy of Randi Guest.

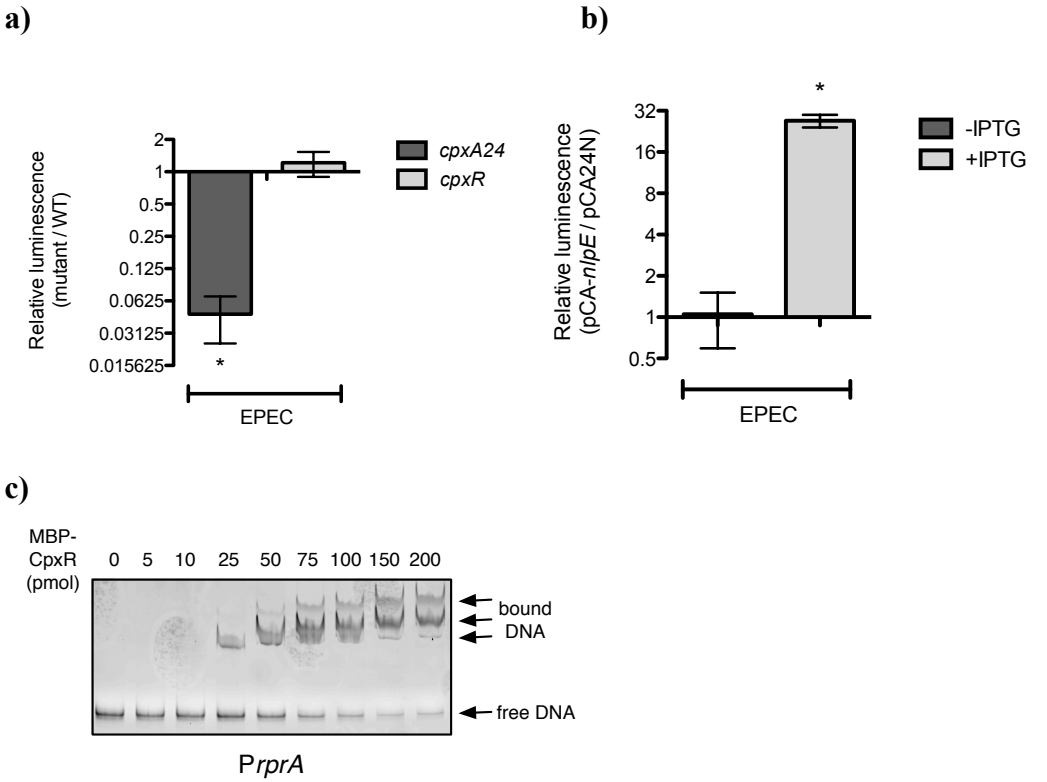


Figure 4-3. CpxR directly regulates expression of *rprA* in EPEC. a) Luminescence assays comparing *rprA-lux* reporter expression in wild-type (WT), *cpxA24* (Cpx-activating mutation) or *cpxR* (Cpx-inactivating mutation) strains of EPEC. **b)** Luminescence assays comparing *rprA-lux* reporter expression in wild-type EPEC carrying vector control pCA24N or the overexpression plasmid pCA-*nlpE*. Luminescence was normalized to the OD_{600} of the culture. Data for luminescence assays represent the mean and standard deviation of five replicate cultures. **c)** EMSA. A PCR product containing the *rprA* promoter region was incubated alone or with increasing concentrations of MBP-CpxR protein, then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining. * denotes a statistically significant difference from the relevant wild-type or vector control ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test).
Panel **(c)** courtesy of Randi Guest.

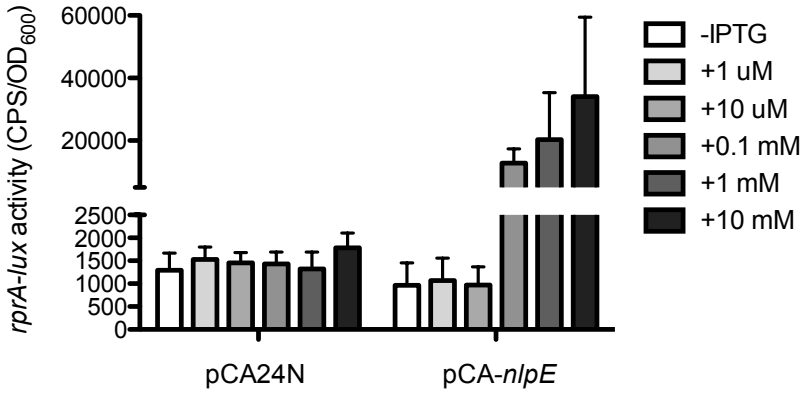


Figure 4-4. Increased induction of *nlpE* overexpression does not result in repression of *rprA* expression. Luminescence assay comparing *rprA-lux* activity in wild-type EPEC carrying either vector control pCA24N or *nlpE* overexpression plasmid pCA-*nlpE* with varying concentrations of inducer (IPTG). Luminescence assays were performed as described in Materials and Methods. Data represent the mean and standard deviation of five biological replicate cultures.

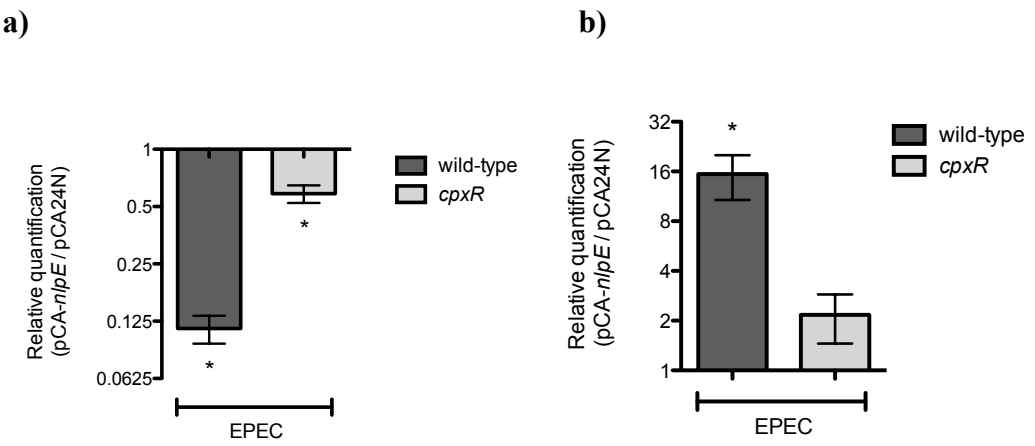
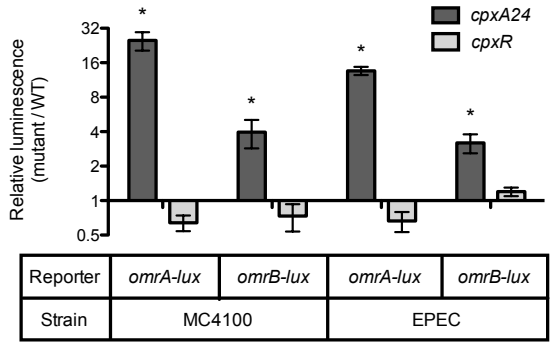


Figure 4-5. Direction of Cpx regulation of *rprA* is determined by the timing of Cpx pathway induction. qRT-PCR analysis comparing *rprA* expression in wild-type or *cpxR*::kan strains of EPEC carrying either the vector control pCA24N or the overexpression plasmid pCA-*nlpE*. **a)** Expression of *nlpE* was induced immediately upon subculture ("immediate induction" conditions described in Materials and Methods). **b)** Expression of *nlpE* was induced in mid-log phase ("delayed induction" conditions described in Materials and Methods). Data represent the mean and standard deviation of three biological replicate cultures. * denotes a statistically significant difference from the relevant vector control ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test).

a)



b)

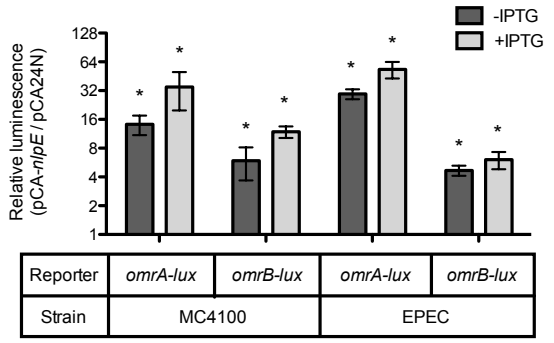


Figure 4-6. The Cpx response activates expression of *omrA* and *omrB*.
a) Luminescence assays comparing *omrA-lux* and *omrB-lux* reporter expression in wild-type (WT), *cpxA24* (Cpx-activating mutation) or *cpxR* (Cpx-inactivating mutation) strains of MC4100 and EPEC. **b)** Luminescence assays comparing *omrA-lux* and *omrB-lux* reporter expression in wild-type MC4100 and EPEC carrying vector control pCA24N or the overexpression plasmid pCA-*nlpE*. Luminescence was normalized to the OD_{600} of the culture. Data for luminescence assays represent the mean and standard deviation of five replicate cultures.
* denotes a statistically significant difference from the relevant wild-type or vector control ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test).

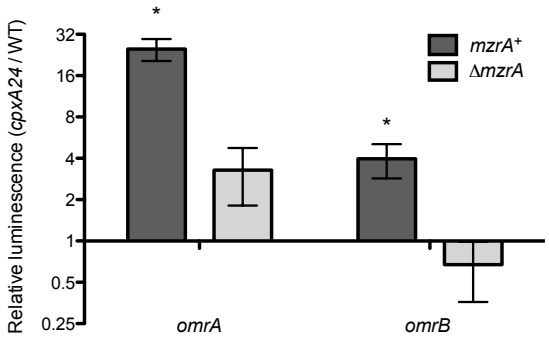
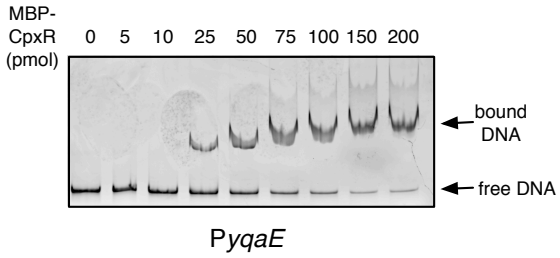


Figure 4-7. The Cpx pathway regulates expression of *omrA* and *omrB* indirectly via MzrA. Luminescence assays comparing *sRNA-lux* reporter expression in wild-type and *cpxA24* (Cpx-activating mutation) strains of MC4100, in *mzrA*$^+$ and *ΔmzrA* strain backgrounds. Luminescence was measured 4 h post-subculture and normalized to the OD_{600} of the culture. Luminescence assays were performed at least twice; one representative experiment is shown. * denotes a statistically significant difference from the relevant *cpxA*$^+$ control ($P<0.05$, one-way ANOVA with Bonferroni's multiple comparison test).

a)



b)

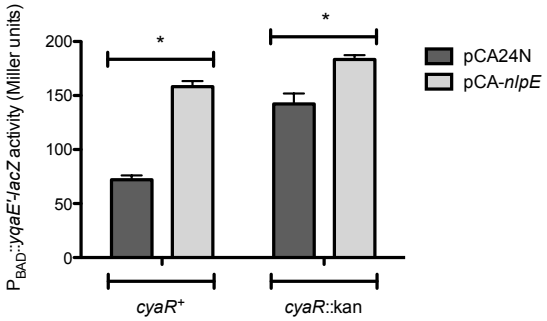


Figure 4-8. The Cpx response regulates *yqaE* expression at both the transcriptional and translational levels. a) EMSA. A PCR product containing the *yqaE* promoter region was incubated alone or with increasing concentrations of MBP-CpxR protein, then subjected to 5% native polyacrylamide electrophoresis. DNA was detected with ethidium bromide staining. **b)** β-galactosidase assay comparing expression of a P_{BAD}::*yqaE'-lacZ* translational reporter in the presence of the vector control pCA24N or the *nlpE* overexpression plasmid pCA-*nlpE*, in strains harbouring a wild-type copy of *cyaR* or a *cyaR*::kan mutation. Data represent the mean and standard deviation of three replicate cultures. * denotes a statistically significant difference from the relevant vector control (P<0.05, one-way ANOVA with Bonferroni's multiple comparison test). Panel **(a)** courtesy of Randi Guest.

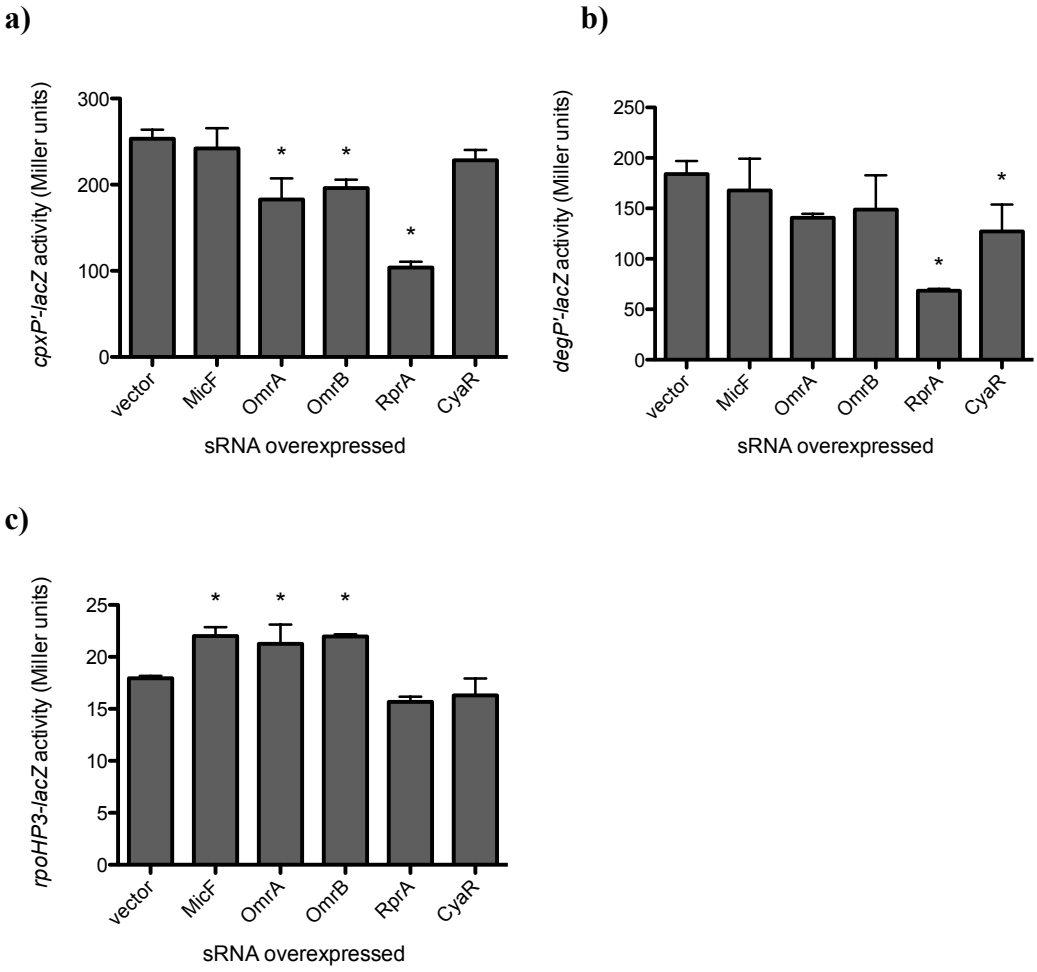
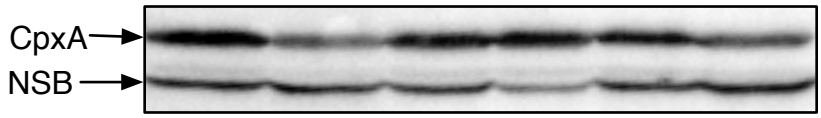


Figure 4-9. Overexpression of *rprA* inhibits Cpx pathway activity. MicF, OmrA, OmrB, RprA, and CyaR were overexpressed from the pBR-plac plasmid in *E. coli* MC4100 by inducing the cultures with 0.1M IPTG. Activity of the transcriptional reporter gene fusions *cpxP-lacZ* **(a)**, *degP-lacZ* **(b)**, and *rpoHP3-lacZ* **(c)** was measured by β-galactosidase assay. * denotes a statistically significant difference from the vector control (P<0.05, one-way ANOVA with Bonferroni's multiple comparison test).
Figure courtesy of Alex Evans.

a)



Plasmid	VC	MicF	OmrA	OmrB	RprA	CyaR
CpxA	1.00	0.53	0.90	0.95	0.86	0.65

b)

Plasmid	VC	MicF	OmrA	OmrB	RprA	CyaR
CpxR	1.00	0.64	0.95	1.09	1.22	1.08

Figure 4-10. Overexpression of *rprA* does not decrease CpxA or CpxR protein levels. The effect of overexpressing MicF, OmrA, OmrB, RprA, and CyaR on CpxA and CpxR protein levels was measured by Western blot with primary antibodies against either CpxA **(a)** or CpxR **(b)**, with a non-specific band (NSB) as a loading control. Quantification of CpxA and CpxR bands was performed using a ChemiDoc MP imager (Bio-Rad) with Image Lab software and is shown beneath the images.
Figure courtesy of Alex Evans.

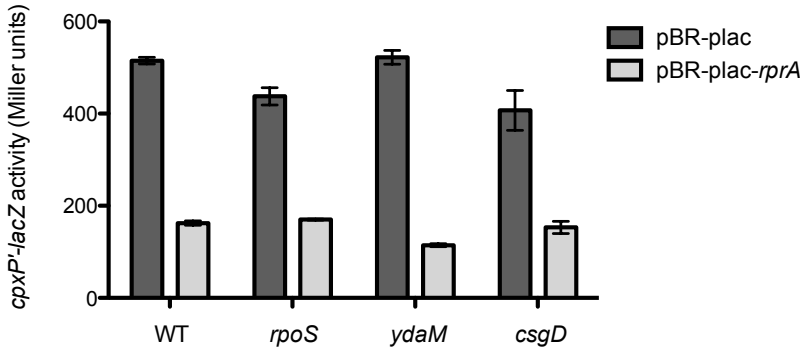
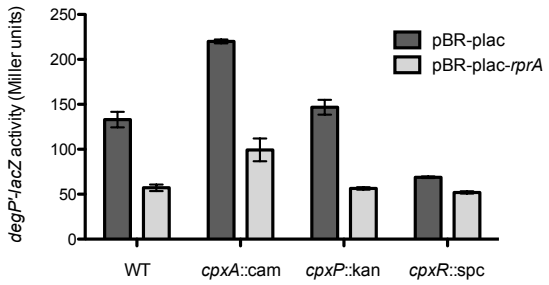


Figure 4-11. Inhibition of the Cpx pathway by RprA is not dependent on any of RprA's known target genes. β-galactosidase assay showing the effect of *rprA* overexpression on activity of a *cpxP-lacZ* reporter in wild-type (WT) and *rpoS, ydaM*, and *csgD* mutant strains. Data represent the mean and standard deviation of three replicate cultures.
Figure courtesy of Alex Evans.

a)



b)

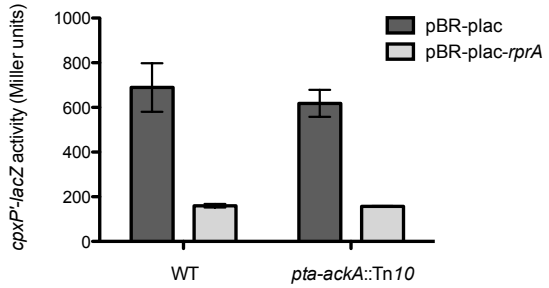


Figure 4-12. Inhibition of the Cpx pathway by RprA is CpxR-dependent but CpxA-independent. a) β-galactosidase assay showing the effect of *rprA* overexpression on activity of a *degP-lacZ* reporter in wild-type (WT), *cpxA*::cam, *cpxP*::kan, and *cpxR*::spc mutant strains. **b)** β-galactosidase assay showing the effect of *rprA* overexpression on activity of a *cpxP-lacZ* reporter in wild-type and *pta-ackA*::Tn*10* mutant strains. Data represent the mean and standard deviation of three replicate cultures.
Figure courtesy of Alex Evans.

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CHAPTER 5

General Discussion

5.1 Overview

The purpose of this thesis was to better understand regulatory interactions between the Cpx envelope stress response, Hfq and small RNAs, and EPEC's major adhesin, the bundle-forming pilus. More specifically, this work examined two major questions: first, whether and how the Cpx envelope stress response and the RNA chaperone Hfq affect expression of the BFP. Given that the BFP plays a crucial role in the initial interaction of EPEC with its host, the factors governing BFP regulation have broad implications with respect to the pathogenesis of EPEC and other related pathogens. Second, this work examined the nature of regulatory interactions between the Cpx two-component system and Hfq/sRNAs. A full description of the connections between cellular regulators is necessary in order to understand the complex responses that bacteria like *E. coli* undertake during periods of stress.

5.2 Complex Regulation of BFP Expression

Typical EPEC strains harbour the EPEC adherence factor (EAF) plasmid, which encodes the type IV bundle-forming pilus (12, 27). Both *in vitro* and *in vivo* studies have demonstrated the importance of the BFP for EPEC pathogenesis. A study using Caco-2 brush border cells demonstrated that BFP are strictly required for early adherence to host cells (10 minutes after infection), although other adhesins contribute to adherence later in the infection process (4). In human volunteer studies, EPEC strains either lacking the entire EAF plasmid or containing a mutation in the *bfpA* gene, encoding the major BFP pilin, were significantly impaired in their ability to cause diarrhoea compared to the wild-type strain (3, 27).

Previous work has shown that expression of this important virulence determinant is regulated by many environmental and genetic factors. BFP expression *in vitro* is maximal at 37ºC, in media containing calcium but lacking ammonium ions (44). The only transcriptional regulator known to bind directly to

the *bfp* promoter is the EAF-encoded protein PerA (also called BfpT) (53). Transcription of *perA* itself is affected by temperature and ammonium ions in a similar manner as the *bfp* operon, suggesting that these signals affect *bfp* expression via PerA (36). In addition, a variety of regulatory proteins affect the expression of *perA*, including the acid resistance regulator GadX (50), the quorum sensing signal synthase LuxS and regulator QseA (51), and the Pst phosphate transport system (10). Another crucial regulator of *perA* expression is PerA itself; *perA* expression is greatly diminished in the absence of autoactivation (36). Signal integration through PerA allows BFP expression to be attuned to environmental and physiological conditions.

5.2.1 Transcriptional regulation of BFP

This work describes two additional regulators that control the expression of BFP at the transcriptional level (Figure 5-1). The first of these is the Cpx envelope stress response (see Chapter 2). Activation of the Cpx response, through either mutation of *cpxA* or overexpression of *nlpE*, reduces expression of the BFP. Cpx repression acts at the level of transcription of the *bfp* genes, since a *bfp-lux* transcriptional reporter is repressed when the Cpx response is activated. Furthermore, activation of the Cpx response does not affect BFP expression when transcription of the *bfp* gene cluster is driven by an inducible promoter. The Cpx response likely affects *bfp* transcription via PerA, since a *perA-lux* reporter is also repressed when the Cpx response is activated, and BFP expression can be restored in the *cpxA24* pathway-active mutant by overexpression of *perA* from a plasmid.

The mechanism by which the Cpx pathway affects PerA expression is unknown. However, there are several possibilities. First, CpxR could bind directly to the *perA* promoter to repress *perA* transcription. However, the observations that there is no consensus CpxR box in the *perA* promoter and that *nlpE* overexpression takes several hours to repress *perA* expression argue against this option. This possibility could be addressed by performing electrophoretic mobility shift assays to determine whether CpxR binds to the *perA* (or *bfpA*) promoter *in vitro*. A second possibility is that the Cpx response reduces translation or stability

of the *perA* transcript, perhaps by regulating one or more sRNAs. One piece of evidence against this mechanism is that *nlpE* overexpression still represses BFP expression in a *Δhfq* mutant (data not shown). Therefore, if the Cpx response is regulating *perA* via a sRNA, this sRNA must act in an Hfq-independent manner. Post-transcriptional effects of the Cpx pathway on *perA* could be investigated by performing an RNA stability assay or examining the expression of a *perA* translational reporter under Cpx inducing vs. uninducing conditions. A third option for Cpx regulation of PerA expression is that regulation is post-translational. This type of regulation by the Cpx response has been observed for the F-plasmid regulatory protein TraJ, which is degraded by the Cpx-regulated protease HslVU when the Cpx response is activated (24). This mechanism of regulation could account for the slow repression of *perA-lux* expression after *nlpE* overexpression, as *perA* transcription would not decrease until after the protease had accumulated to sufficient levels to significantly decrease the concentration of PerA protein in the cell, thereby reducing autoactivation of *perA* expression. An interesting experiment in this regard would be to examine the stability of the PerA protein under Cpx inducing and uninducing conditions.

The second regulator of BFP expression described in this work is Hfq (see Chapter 3). Hfq represses transcription of the *bfp* genes; this repression is lost in a *ΔperA* mutant, demonstrating that Hfq, like most other regulators of the BFP, acts through PerA. Hfq most likely controls PerA expression by direct binding to the *perA* transcript, since stability of the *perA* mRNA is significantly increased in an *Δhfq* mutant. In order to detect an *in vivo* interaction between the *perA* transcript and Hfq, we performed immunoprecipitation of a tagged Hfq protein, followed by extraction of the bound RNAs and detection by Northern blotting. However, the *perA* transcript could not be detected by this method, likely due to its extremely low stability (half-life of less than one minute in wild-type cells). Interaction between Hfq and the *perA* transcript could potentially be examined *in vitro*, using electrophoretic mobility shift assays with purified Hfq and *in vitro*-transcribed *perA*.

A major future direction for this project is to determine which sRNA(s), if any, affect *perA* expression. The Gottesman laboratory has constructed a library of overexpression plasmids for *E. coli* Hfq-binding sRNAs (35); this library could be transformed into EPEC to determine whether any of the sRNAs repress BFP expression. However, if it is an uncharacterized and/or EPEC-specific sRNA that regulates *perA* expression, this approach will not be successful. In that case, an EPEC genomic library could be prepared in an overexpression vector and screened for any constructs that affect expression of a *perA-lacZ* translational reporter. This approach successfully identified the sRNA RybC (ChiX) as a regulator of *dpiB* expression in *E. coli* (34). If either of these approaches is successful, it will be interesting to determine whether the sRNA(s) that affects *perA* and BFP expression also regulates other EPEC virulence genes. Hansen and Kaper have shown that Hfq also reduces stability of another regulator-encoding transcript, *grlRA*, in the related pathogen, enterohaemorrhagic *E. coli* (EHEC) (17). It is possible that one or more sRNAs help to coordinate the expression of plasmid-encoded virulence genes like the BFP and chromosomally-encoded virulence genes like the type III secretion system in EPEC.

Both Cpx and Hfq act as repressors of BFP expression. What advantage could repressing expression of a major virulence determinant like the BFP confer to EPEC? One clue comes from the observation that overexpression of *bfpA*, either ectopically in MC4100 (41) or in the EPEC *Δhfq* mutant (Chapter 3), causes activation of the Cpx pathway. Activation of the Cpx response could indicate that inappropriate expression of the BFP damages the envelope in some way, such as causing harmful aggregation of pilins or overwhelming the folding capacity of periplasmic chaperones. Cpx repression of *bfp* transcription could therefore help to maintain homeostasis in the envelope, by reducing expression of pilus proteins under conditions where the envelope protein folding machinery is already struggling. In addition to the BFP, Hfq represses several other horizontally-acquired genes in EPEC and EHEC, including those encoding the T3SS and the Shiga toxin 2 (stx_{2AB}) operon (17, 23, 49). Repression of these horizontally-

acquired genes could help to mitigate the fitness costs associated with their expression, such as envelope damage.

5.2.2 Post-translational effects on BFP stability

Another major finding of this work is that the Cpx response affects BFP expression on multiple levels – while *bfp* transcription is repressed by CpxR, the Cpx response also promotes BFP biogenesis at the post-translational level (Figure 5-1; see Chapter 2). These post-translational effects are mediated by the Cpx-regulated periplasmic protein folding factors DsbA, DegP, and CpxP. DsbA was previously shown to be responsible for catalysing the formation of a disulphide bond in the bundlin protein that is required for its stability (59). Humphries *et al.* also observed a BFP biogenesis defect in an EPEC *degP* mutant; this phenotype could be complemented with a DegP construct in which the protease activity had been removed by mutation of the active site serine, suggesting that it is DegP's chaperone activity rather than its protease activity that is required for BFP biogenesis (19). In addition, CpxP was recently shown to possess chaperone ability *in vitro* (45, 60); it is therefore possible that CpxP is an additional factor involved in the proper folding of BFP proteins.

This multilevel regulation of BFP expression by the Cpx response is in fact a common theme among protein misfolding responses. These stress responses often consist of both an "activation arm", which increases the expression of protein folding factors, and a "repression arm", which decreases the expression of proteins prone to misfolding. In the case of the σ^E envelope stress response, the activation arm involves upregulation of periplasmic chaperones and the Bam β-barrel assembly complex, while the repression arm is mediated by the sRNAs RybB and MicA, which repress translation of many *omp* mRNAs (13, 29, 48). Similarities can also be found in the unfolded protein response (UPR), which is a eukaryotic response to protein misfolding in the endoplasmic reticulum (ER) (reviewed in 57). During the UPR, transcription of ER-localized chaperones is enhanced, while translation of cellular mRNAs is decreased due to phosphorylation of the translation factor eIF2α, thereby reducing the protein

folding load in the ER. The fact that these signalling principles are shared between multiple stress responses suggests that they are likely an effective means to maintain homeostasis in extracytoplasmic compartments.

5.3 Role of the Cpx Response in EPEC Pathogenesis

Understanding how the Cpx response contributes to EPEC pathogenesis has been a major focus of research in our laboratory in recent years. We have investigated the effects of both inactivating and activating the Cpx response upon EPEC virulence determinant expression. Inactivation of the Cpx pathway through mutation of *cpxR* significantly decreases expression of the BFP, resulting in decreased autoaggregation and localized adherence (41); however, the *cpxR* mutation does not significantly affect type III secretion (30). Conversely, activation of the Cpx response decreases expression of numerous envelope-localized virulence determinants, including the BFP (Chapter 2), the T3SS (30), and flagella (31). The virulence of the *cpxR* (pathway inactive) and *cpxA24* (pathway constitutively active) mutants was examined in a *Galleria mellonella* infection model (26). While the *cpxR* mutant is slightly attenuated in its ability to kill the *G. mellonella* larvae, the *cpxA24* mutant is almost completely avirulent and is unable to multiply inside the larvae. The slight virulence defect in the *cpxR* mutant is unlikely to be related to its reduced BFP elaboration, since a *ΔbfpA-L* mutant is fully virulent in the *G. mellonella* model (26). Although the reason for the severe virulence defect of the *cpxA24* mutant is currently unknown, it could be related to increased susceptibility to the host immune response, since the *cpxA24* mutant was threefold more sensitive than wild-type EPEC to the antimicrobial peptide cecropin (26).

These results have implications for the role of the Cpx response in the life cycle of EPEC and other attaching and effacing (A/E) pathogens. The data suggest that a basal level of Cpx activity would be important when the bacteria are inside their host, as inactivation of the Cpx response impairs BFP elaboration (41). The data also suggest that activation of the Cpx response would be

detrimental inside a host, as Cpx activation leads to the removal of numerous important virulence structures from the envelope (Chapter 2; 30, 31).

One way to study interactions of A/E pathogens and their hosts is to use the *Citrobacter rodentium* infection model. Both EPEC and *C. rodentium* possess the locus of enterocyte effacement, a genomic island that encodes the T3SS and some of its effectors, and therefore both organisms use a similar virulence strategy to infect their hosts (40). However, whereas EPEC is a human pathogen that does not cause representative disease in a mouse model (39), *C. rodentium* is a natural mouse pathogen. Intriguingly, experiments with *C. rodentium* indicate that the Cpx response is strongly induced during the early parts of the infection process, as compared to during growth *in vitro* (S. Gruenheid, personal communication). How can these data be reconciled with the knowledge that Cpx activation causes downregulation of virulence structures? One possibility is that the Cpx response provides locational information during infection. After oral administration, *C. rodentium* colonizes the mouse caecum for approximately three days prior to colonizing the colon, which is considered the main site of infection (58). Activation of the Cpx response could potentially prevent initiation of type III secretion and A/E lesion formation until the bacteria reach a suitable site for infection. Another important locational consideration within the host is the proximity to the epithelial layer. As an A/E pathogen, *C. rodentium* infects the epithelial surface (40). Activation of the Cpx response could potentially occur in the lumen of the intestine, where structures like the T3SS are not needed and where envelope-damaging agents like bile salts are common (1). The *C. rodentium* model is a useful tool for studying interactions of A/E pathogens and their hosts. More detailed study of the timing and location of Cpx activation during the infection process, as well as infection studies with Cpx-activated and -inactivated mutants, will hopefully shed more light on the role of the Cpx response *in vivo*.

5.4 Regulatory Connections Between CpxAR and sRNAs

A second major goal of this work was to elucidate the regulatory connections between the Cpx response and Hfq and sRNAs (Figure 5-2). These experiments were inspired by recent studies demonstrating that sRNAs are intimately involved in both two-component system signalling and the σ^E envelope stress response. Six of *E. coli*'s 30 two-component systems are currently known to regulate the expression of at least one sRNA (14). Regulation can also proceed in the opposite direction; sRNAs have been shown to directly regulate the translation of mRNAs encoding components of four different two-component systems (14). Similar regulatory connections exist between sRNAs and the σ^E envelope stress response. σ^E activates the transcription of two sRNA-encoding genes, *rybB* and *micA*, which together repress the expression of over 30 genes, including many outer membrane proteins (13, 20, 52, 54). sRNAs also control activity of the σ^E response; however, this regulation occurs through an indirect mechanism, in contrast to the direction regulation of many two-component system transcripts by sRNAs (2). Numerous sRNAs – including RybB and MicA but also other sRNAs that are not part of the σ^E regulon, such as MicF and MicC – repress the expression of outer membrane protein mRNA translation (15, 56). By repressing expression of outer membrane proteins, these sRNAs decrease σ^E pathway activation. When activity of these sRNAs is lost, such as in an *hfq* mutant strain, outer membrane proteins accumulate and the σ^E response becomes constitutively activated (16).

Because the Cpx system is both a two-component system and an envelope stress response, it seemed likely that sRNAs could also be involved in this regulatory system. This work examined both aspects of the connection between the Cpx response and sRNAs: regulation of the expression of sRNA genes by the Cpx response, and effects of Hfq and sRNAs on expression and activity of the Cpx pathway.

5.4.1 Regulation of sRNA expression by CpxAR

Preliminary evidence that the Cpx two-component system regulates the expression of sRNAs was obtained from a microarray examining the effects of *nlpE* overexpression, in which it was found that activation of the Cpx response increased the expression of four sRNAs (MicF, OmrA, OmrB, and RprA) and decreased the expression of one (CyaR) (46). *lux* transcriptional reporters were used to confirm that expression of *omrA* and *omrB* is activated by the Cpx response, while *cyaR* expression is repressed and *rprA* expression is either activated or repressed, depending on the growth phase in which the Cpx response is activated (see Chapter 4). Furthermore, gel-shift assays showed that CpxR binds to the promoters of *cyaR* and *rprA*, making them direct targets of Cpx regulation.

Several benefits have been proposed for the inclusion of sRNAs in two-component system and other transcription factor regulons (reviewed in 2, 14, 33). By regulating additional targets that are not controlled by the two-component regulator at the transcriptional level, sRNAs can increase the size of the regulon and broaden the response to stress. Conversely, sRNAs can also regulate the translation of genes that are regulated by the two-component system at the transcriptional level. This multi-level regulation sets up a network motif called a feedforward loop, which can have different regulatory properties than transcriptional regulation alone. For example, combined repression of a target at both the transcriptional and translational levels can provide tighter control, as the sRNA can repress translation of any transcripts that are produced when the transcriptional repressor briefly dissociates from the target gene's promoter. Feedforward loops can also change the dynamics of gene expression, with the sRNA introducing delays or speeding up target regulation in response to changes in the biological stimulus. The separate layer of regulation provided by sRNAs also allows evolution of regulatory control over target genes whose promoters are crowded with transcription factor binding sites, as the sRNA can bind to the transcript without necessitating a change in the promoter sequence that might disrupt regulation by other transcription factors. Finally, sRNAs can also reverse

the direction of regulation by some regulators that are, by nature, restricted to gene activation or gene repression. For example, inclusion of sRNAs in the σ^E regulon permits σ^E to repress the expression of outer membrane proteins, even though a sigma factor is inherently limited to gene activation (13).

Several of these benefits of sRNA regulation may apply to the Cpx-regulated sRNAs CyaR and RprA. For example, regulation of RprA could broaden the scope of the Cpx response because RprA positively regulates translation of the *rpoS* transcript, encoding the stationary phase sigma factor (32). Thus, RprA could tie the Cpx envelope stress response to induction of genes that enhance stress survival during stationary phase. We also identified several genes that are co-regulated by Cpx at the transcriptional level and either CyaR or RprA at the translational level. These include the inner membrane protein gene *yqaE*, which is activated by Cpx at the transcriptional level (Chapter 4; 46) and repressed by CyaR at the translational level (25), and the regulator of curli expression *csgD*, which is repressed by Cpx at the transcriptional level (21, 42, 43) and repressed by RprA at the translational level (22, 38). These feedforward loops have the potential to change both steady-state and dynamic expression of the target genes; it would be interesting to experimentally investigate the contribution of these sRNAs to target gene regulation during the Cpx response.

Cpx regulation of sRNAs may not be limited to its effects on *cyaR* and *rprA* expression. An important future direction for this project will be to determine whether the Cpx response regulates the expression of any novel sRNAs. The expression of 35 intergenic regions was altered by twofold or more in the *nlpE* overexpression microarray (46). Although some of these may represent unannotated open reading frames or 5' or 3' untranslated regions of adjacent genes, the presence of even a few novel Cpx-regulated sRNAs in these intergenic regions could significantly broaden the scope of post-transcriptional regulation by the Cpx response.

5.4.2 Effects of Hfq and sRNAs on Cpx pathway activity

In addition to Cpx control of sRNA expression, this work also examined the opposite direction of regulation – control of Cpx pathway activity by Hfq and sRNAs (Figure 5-2). Since the σ^E envelope stress response is activated in *Δhfq* mutants of *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Vibrio cholerae* (8, 11, 16), we postulated that the absence of Hfq could generate envelope stress that would also activate the Cpx pathway. Results showed that mutation of *hfq* had strain-specific effects on Cpx pathway activity, with the Cpx response being strongly activated in an EPEC *Δhfq* mutant but not activated or mildly activated in *E. coli* K-12 *Δhfq* mutants (see Chapter 3). The activation of the Cpx response in the EPEC *Δhfq* mutant is probably multifactorial, since deletion of either *ompA* or the *bfp* gene cluster reduced Cpx activity in an *Δhfq* background. However, Cpx activity remained above wild-type levels in both the *Δhfq ΔompA* and the *Δhfq Δbfp* double mutants, suggesting that neither OmpA nor BFP overexpression alone can account for the Cpx activation in the *Δhfq* mutant. It remains to be determined whether Cpx activity would be restored to wild-type levels in an *Δhfq ΔompA Δbfp* triple mutant, or whether there are additional factors contributing to Cpx activation. In addition, the sRNA(s) responsible for keeping Cpx activity at basal levels in a wild-type strain have yet to be identified. If the sRNA regulating expression of *perA* and therefore the BFP is identified as suggested in Section 5.2.1, it would be of interest to determine whether mutation of that sRNA alone would have a significant effect on Cpx activity. Other possible contributors to Cpx activation in the *Δhfq* mutant are RybB and MicA, which negatively regulate OmpA expression (13, 47, 55). Since overexpression of OmpA contributes to Cpx activation in the EPEC *Δhfq* mutant, it is possible that deletion of one or both of *rybB* or *micA* could have a similar effect.

This work also uncovered the effects of several specific sRNAs on Cpx activity, aside from the effects of the loss of Hfq. We confirmed Holmqvist *et al.*'s report that MicF directly represses *cpxRA* translation (18), as we observed decreased levels of both CpxR and CpxA protein in a strain overexpressing MicF (see Chapter 4). However, the effect of MicF on the Cpx regulon remains

uncertain. We were unable to observe any effect of MicF overexpression on expression of *cpxP-lacZ* or *degP-lacZ* reporters when strains were grown in LB. Even when cultures were grown at pH 8, which induces activity of the Cpx response (7), we still failed to observe any effect of MicF overexpression on a *cpxP-lacZ* reporter. Therefore, the physiological relevance of MicF regulation of *cpxRA* expression is still unknown. One future direction in this regard will be to examine expression of more Cpx regulon members, particularly ones that are weakly Cpx-regulated, in response to MicF overexpression. We predict that the decrease in CpxR levels mediated by MicF would likely have a stronger effect on the promoters for which CpxR has a lower affinity, as compared to high affinity promoters like *cpxP*.

We also uncovered an indirect effect of an sRNA on Cpx activity. Overexpression of RprA caused an approximately 50% decrease in Cpx pathway activity, without affecting levels of CpxR and CpxA proteins (see Chapter 4). This effect was independent of all of RprA's known regulatory targets and also independent of CpxA. RprA repression of Cpx activity was dependent on CpxR, but not on the Pta-AckA pathway, which has previously been shown to contribute to CpxA-independent phosphorylation of CpxR (7). Additional studies will be required to elucidate how RprA affects Cpx pathway activity. One possibility is that RprA regulates the expression of an undescribed auxiliary regulator of CpxR, which could change its phosphorylation status or ability to bind to DNA without altering protein levels.

There are several benefits to regulation of two-component system expression or activity by sRNAs. First, sRNAs can act as connectors between regulatory systems. One case where this has been experimentally demonstrated is for repression of the *phoP* response regulator transcript by the sRNA MicA (5). By repressing expression of *phoP*, MicA reduces expression of genes in the PhoPQ regulon. Since *micA* itself is regulated by σ^E (20, 54), this sRNA provides a link between the σ^E envelope stress response and the PhoPQ system. Since *micF* expression is controlled by a variety of regulators, including the EnvZ/OmpR two-component system, the global regulator Lrp, and the oxidative stress

regulator SoxS (6, 9, 28), MicF could be a mechanism for these regulators to influence activity of the Cpx pathway.

A second benefit of sRNA regulation of two-component systems is that they can exert negative or positive feedback on their own regulator. Feedback regulation by sRNAs can serve several purposes (2). Negative feedback loops can reduce response time, decrease variability in pathway activity between cells in a population, and adjust the relationship between signal intensity and strength of pathway activation. The benefit of RprA-mediated negative feedback on the Cpx response remains to be investigated. The regulatory loop formed by RprA and Cpx is particularly complex because of the growth phase-dependent effects of Cpx activation on *rprA* expression (Chapter 4). The role of RprA's negative feedback on the Cpx response could therefore be growth phase-dependent as well.

Finally, recent studies have found that several regulators, such as the curli regulator *csgD* and the flagellar regulator *flhDC*, are hubs for sRNA regulation (37). It is therefore possible that additional sRNAs also regulate expression of the Cpx response, either directly by binding to the *cpxRA* transcript or indirectly by affecting Cpx pathway activation. This possibility could be addressed by screening the Gottesman laboratory's sRNA overexpression library (35) for sRNAs that affect levels of the CpxR or CpxA proteins or expression of Cpx-regulated genes such as *cpxP*.

5.5 Concluding Remarks

The Gram-negative envelope is a structurally and functionally complex cellular compartment. It participates in every interaction of a bacterial cell with its environment, with roles in obtaining nutrients, bacterium-host interactions, and signal transduction, among many others. Regulators that determine which envelope constituents are produced in response to environmental cues are therefore a major determinant of bacterial adaptation and survival, including the ability of pathogens to successfully infect their host. This thesis provided further insight into how the Cpx envelope stress response and small regulatory RNAs

control expression of envelope components, particularly the bundle-forming pilus, as well as how these regulators interact with each other. A full understanding of these regulatory systems may have practical applications in the design of antimicrobial compounds with novel cellular targets, in addition to enhancing our understanding of how bacterial cells adapt to their environment.

5.6 Figures

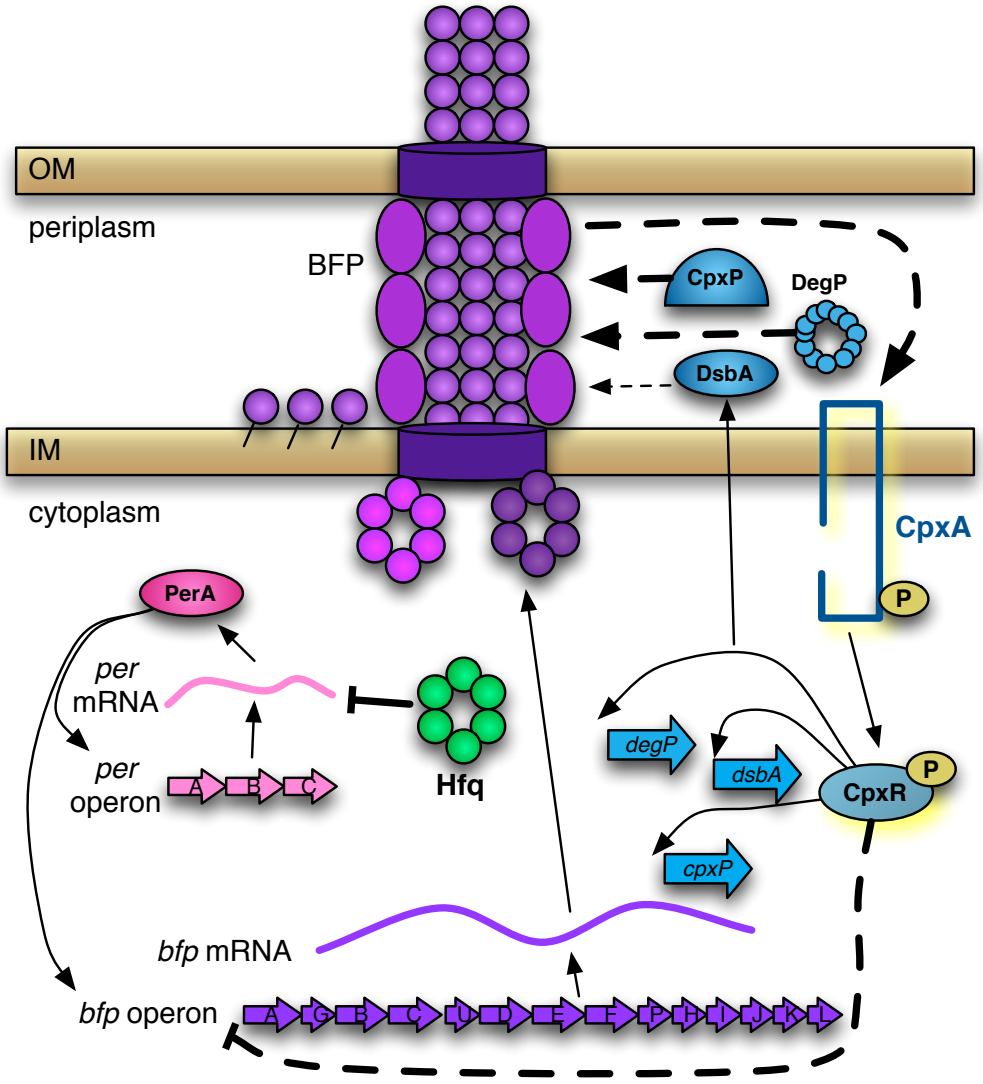

Figure 5-1. Summary of regulation of bundle-forming pilus expression. Transcription of the *bfp* operon, encoding all of the BFP components, is activated by the transcriptional regulator PerA. PerA also autoactivates expression of the *perABC* operon. In the presence of envelope stress, the CpxA histidine kinase autophosphorylates then phosphorylates the response regulator CpxR. Through a currently unknown mechanism (indicated by the dashed line), CpxR represses transcription of the *bfp* operon. CpxR also activates transcription of *degP*, *dsbA*, and *cpxP*, genes encoding periplasmic protein folding factors that promote biogenesis of the BFP through an unknown mechanism, likely by assisting folding of periplasmic BFP components. The sRNA chaperone protein Hfq represses BFP expression by decreasing stability of the *perA* mRNA. When BFP components are overexpressed, such as in an *Δhfq* mutant, the Cpx pathway is activated. Bolded arrows and lines indicate regulatory connections described for the first time in this thesis. OM, outer membrane; IM, inner membrane; BFP, bundle-forming pilus; P, phosphate.

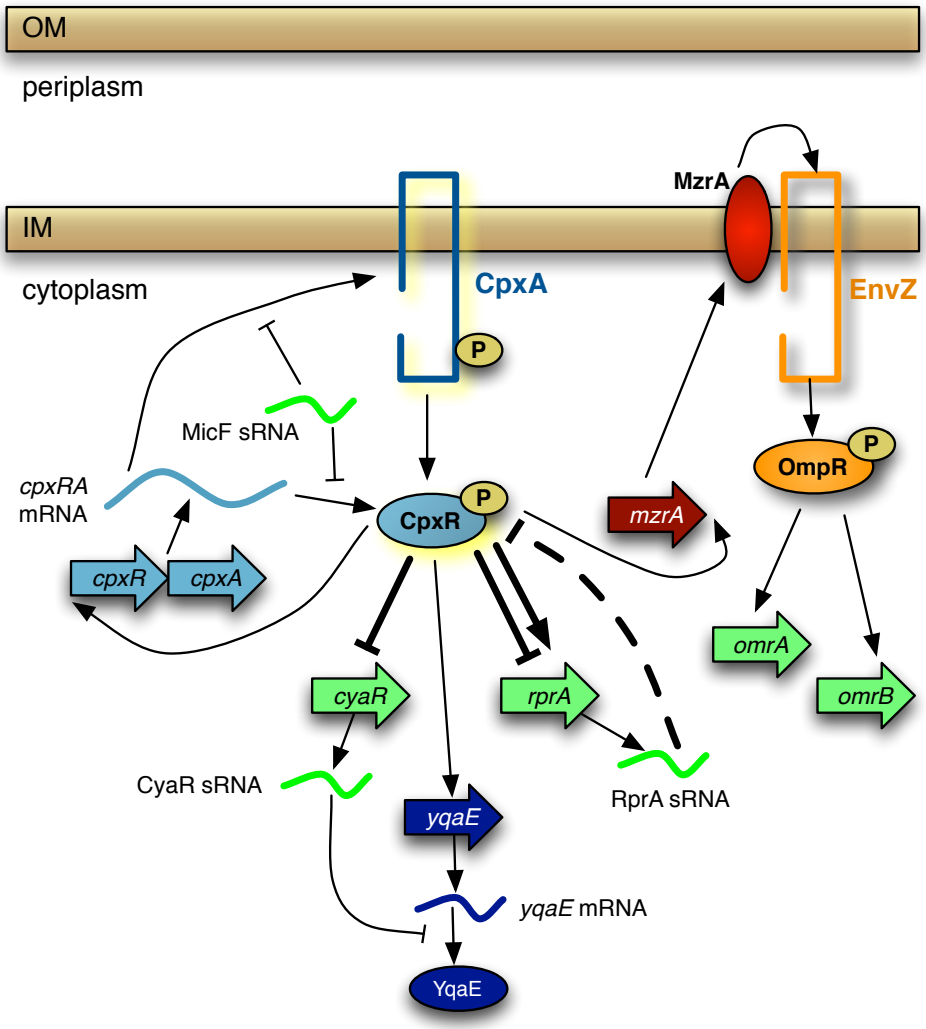

Figure 5-2. Regulatory connections between the Cpx two-component system and small RNAs. In the presence of envelope stress, the CpxA histidine kinase autophosphorylates then phosphorylates the response regulator CpxR. CpxR directly regulates the transcription of two sRNA genes: *cyaR* transcription is repressed, while *rprA* transcription is either activated or repressed, depending on the growth phase. CpxR indirectly regulates the transcription of the sRNAs OmrA and OmrB by activating transcription of the inner membrane protein MzrA; MzrA physically interacts with EnvZ, increasing the activity of the EnvZ/OmpR two-component system, which activates *omrA* and *omrB* transcription. CpxR regulates the expression of the inner membrane protein YqaE in two ways: directly at the transcriptional level, and indirectly at the translational level through repression of CyaR, creating a feedforward loop. Two sRNAs affect expression or activity of the Cpx response: RprA indirectly represses activity of CpxR by an unknown mechanism (indicated by dashed line), while MicF represses translation of the *cpxRA* mRNA. Bolded arrows and lines indicate regulatory connections described for the first time in this thesis. OM, outer membrane; IM, inner membrane; P, phosphate.

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