CHAPTER 6

Two-Component Signaling and Gram Negative Envelope Stress Response Systems

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Introduction

Bacteria have evolved complex stress responses that allow them to respond to their surroundings. In Gram negative bacteria, these stress responses can be compartmentalized into the cytoplasmic and extracytoplasmic (envelope) stress responses. The extracytoplasmic stress response monitors the integrity of the envelope, which consists of the outer membrane (OM), inner membrane (IM) and the periplasm. The OM is an atypical membrane in that it is asymmetrical with lipopolysaccharides found only on the outer facet. Functions of the OM include acting as a permeability barrier, allowing for transport via porins and avoiding phagocytosis.[1] The IM is composed of phospholipids and proteins. The IM is involved in many activities including energy generation and conservation, biosynthetic and catabolic reactions, signal transduction and acting as a hydrophobic barrier to control and maintain the intracellular concentrations of cytoplasmic ions/molecules.[2] The periplasm, which lies between the IM and OM, contains the peptidogylcan layer that is involved in maintaining cell shape. Although it has been thought that the periplasm is extremely viscous due to its high protein content, it was recently shown that the viscosity of the periplasm is not that much different than the cytoplasm, with an average diffusion rate of $9.0 \pm 2.1\ \mu m^2 s^{-1}$ in the cytoplasm and $2.6 \pm 1.2\ \mu m^2 s^{-1}$ in the periplasm.[3] The periplasm is involved in processing essential nutrients for transport, biogenesis of major envelope components, detoxification and buffering the cytoplasmic environment from external stresses to maintain growth and viability.[4]

Escherichia coli has at least five known stress responses that allow it to monitor envelope homeostasis, the σ^E, Cpx (Conjugative Pilus Expression), Bae (Bacterial Adaptive Response), Psp (Phage-Shock-Protein) and Rcs (Regulator of Capsular Synthesis) responses. Of these five stress responses three of them are controlled by two-component systems and thus, two-component signal transduction is centrally involved in the bacterial response to envelope stress. Although there is some overlap, each of these pathways has been shown to respond to different stresses and regulate different processes. The σ^E pathway senses and responds mainly to stresses that involve OM protein (OMP) maintenance and folding.[5] The Cpx pathway is induced and mediates adaptation to, misfolded proteins in the periplasm, specifically those associated with the periplasmic face of the inner membrane.[6] The Cpx response has also been shown to sense and facilitate adhesion to abiotic surfaces.[7] The Bae pathway is involved in detoxification by ridding the cell of toxic

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Bacterial Signal Transduction: Networks and Drug Targets, edited by Ryutaro Utsumi.

compounds.[8,9] The Phage-shock-protein response monitors both the proton motive force and the integrity of the inner membrane.[10] The Rcs pathway seems to be involved in the formation of surface structures.[11,12]

The σ^E Envelope Stress Response

Discovery of σ^E

The use of ECF (ExtraCytoplasmic Function) sigma factors, which are divergent from σ^{70}, is one way in which bacteria sense and respond to their surroundings. Signal transduction events in response to alterations in the environment lead to an increased cellular concentration of a given ECF sigma factor, which can then associate with the core RNA polymerase (RNAP) to change gene expression.[13] To date, only two ECFs have been identified in *E. coli*, FecI (as reviewed by 14) and σ^E, which is essential.[15,16] The σ^E (σ^{24}) subunit was first identified for its ability to transcribe the *rpoH* (encodes an alternative sigma factor that regulates heat shock response) and *degP* (*htrA*) (encodes a periplasmic serine endoprotease/chaperone) genes, under heat shock conditions[17,18] (Table 1). When they initially identified σ^E, Erickson and Gross[17] purified the sigma factor responsible for transcribing *rpoH* from the P3 promoter, however they did not identify the gene that encoded the sigma factor. Taking different approaches Rouviere et al[19] and Raina et al[20] both determined that an ORF at minute 55.5 on the *E. coli* genetic map encoded σ^E, *rpoE*. It was later determined that *rpoE* was the first gene in a four gene operon[21,22] and the *rpoE* promoter is driven by σ^E.[19,20]

Activating Cues

From the discovery of σ^E, it was determined that one of the activating cues that stimulated σ^E-mediated transcription was heat shock conditions, an up shift in temperature above 42°C.[17]

Table 1. Members of the σ^E regulon that are involved in envelope homeostasis

Gene Name	Classification; Role	Essential
rpoE	ECF σ factor; role in envelope and oxidative stress responses	Yes
rseA	Regulatory protein; antisigma factor of σ^E	No
rseB	Regulatory protein; modulates RseA activity	No
rseC	Regulatory protein; possible positive regulator of σ^E	No
rseP (*yaeL*)	Zinc metalloprotease; degrades RseA	Yes
rpoD (σ^{70})	Housekeeping σ	Yes
rpoH (σ^{H})	Heat shock σ	Yes
degP (*htrA*)	Serine endoprotease with chaperone activity; Conditional; degradation of damaged periplasmic proteins	Temp[a] above 43°C
skp	Chaperone; OMP chaperone	No[b]
fkpA	PPI[c] and chaperone activity	No[b]
surA	PPI[c] and OM porin chaperone	No[b]
dsbC	disulfide bond isomerase with chaperone activity	No[b]
yifO	OM lipoprotein; OM biogenesis	Yes
yraP	OM lipoprotein; OMP assembly	No
yaeT	OMP assembly factor	Yes
micA	sRNA; reduces *ompA* RNA levels	No
rybB	sRNA; reduces *ompC* and *ompW* RNA levels	No

a. Temp, temperature
b. *skp, fkpA,surA, dsbC* single or double mutants had no deleterious affects on bacterial growth, however triple mutants displayed growth defects.
c. PPI, peptidylprolyl *cis, trans* isomerase

However heat shock conditions encompass many different signals that could elicit many different responses. To better understand the role of σ^E in the cell it was crucial that specific signals that activate σ^E be identified. From the periplasmic location of DegP, it was suggested that σ^E may be involved in monitoring the envelope.[23] To test this hypothesis, Mecsas et al[23] looked to see what affect overproduction and underproduction of OMPs would have on σ^E activity. Overexpression of OMPs such as, OmpX, OmpT, OmpF and OmpC resulted in an increase in σ^E activity, while limiting the amount of OMPs present reduced the activity of σ^E. To determine if the signal was generated in the cytoplasm or the envelope Mecsas et al[23] looked at what happened when OMP precursors were overexpressed and trapped in the cytoplasm in a *secB* mutant. They observed that the activity of σ^E resembled that seen when OMPs were limited, suggesting that σ^E is activated by a signal that originates in the envelope when OMPs are overproduced.[23] A few years later it was shown that ethanol, DTT (dithiothreitol) and puromycin, all compounds known to cause misfolded proteins, specifically increased σ^E activity.[20] It was also shown that σ^E senses and responds to the production of the misfolded P-pilus subunit PapG, when it is driven off-pathway because of the absence of its cognate chaperone, PapD.[24] All these observations suggested that σ^E senses and responds to misfolded proteins in the envelope. If this is true one would think that defects in envelope protein folding factors would also affect pathway activity. Mutations to protein folding genes, such as the *dsb* (encoding disulfide bond oxidoreductases) genes, *degP*, *surA* (a peptidylproyl *cis, trans* isomerase (PPI)/chaperone) and *fkpA* (PPI/chaperone), all caused an increase in pathway activity,[20,25] which reaffirmed that σ^E senses and responds to misfolded proteins, specifically to misfolded OMPs, in the envelope.

Regulation of σ^E

When Raina et al[20] and Rouviere et al[19] were trying to clone and analyze the gene encoding σ^E, they noticed that in the absence of induction the activity of σ^E was low, suggesting that it was negatively regulated by a possible antisigma factor. To try and identify a possible antisigma factor two approaches were taken. De Las Penas et al[21] took into account that the genes encoding sigma and antisigma factors are normally found in an operon. They looked at the sequence downstream from *rpoE* and identified three ORFs that were termed *rseABC* for regulator of sigma E. Missiakas et al[22] used transposon mutagenesis to identify genes that, when inactivated, led to an increase in σ^E activity. Their approach identified two such insertional mutations that seemed to be involved in σ^E regulation. These mutations localized to the second and third genes, *rseAB* respectively, in the *rpoErseABC* four-gene operon. Upon characterization of *rseABC*, it was determined that RseA is an inner membrane protein that transmits the signal generated in the envelope to σ^E.[21,22] RseA spans the membrane once with C-terminal periplasmic and N-terminal cytoplasmic domains. It is the cytoplasmic domain of RseA that interacts with σ^E to inhibit its activity and this is all that is needed for inhibition[22,26-27] (Fig. 1). Specifically, the −35 binding region 4.2 of σ^E has been shown to interact with RseA.[27] RseB, a periplasmic protein, is also a negative regulator of σ^E activity; however its role is not pivotal in transmitting the signal to σ^E.[21,22] RseB has a role in stabilizing RseA and the RseA~σ^E interaction[21,22,28,29] (Fig. 1). When RseB is absent there is a two-fold increase in activity compared to when it is present and RseA half-life is changed from 18.6 ± 3.1 mins to 44.3 ± 6.1 mins.[21,22,29] RseC, a cytoplasmic protein, does not seem to have a major role in the signaling cascade, although it has been suggested that it may be a positive regulator of σ^E.[22]

The effects of RseB on RseA and the observation that the relative RseA synthesis rate was the same under inducing and non-inducing conditions suggested that RseA is posttranslationally modified in response to inducing cues.[29] RseA is cleaved in a two step process by two inner membrane proteases, first by DegS, a serine protease and then RseP (YaeL), a zinc metalloprotease[29-30] (Fig. 1). Under inducing conditions, it is not RseA that senses the misfolded proteins, but rather the PDZ domain of DegS.[31] PDZ domains are found in diverse signaling proteins and are involved in protein:protein interactions.[32] Under non-inducing conditions the PDZ domain of DegS inhibits it proteolytic activity, preventing it from degrading RseA (Fig. 1). However, under inducing conditions, the PDZ domain binds the C-terminus of misfolded OMPs, which contain

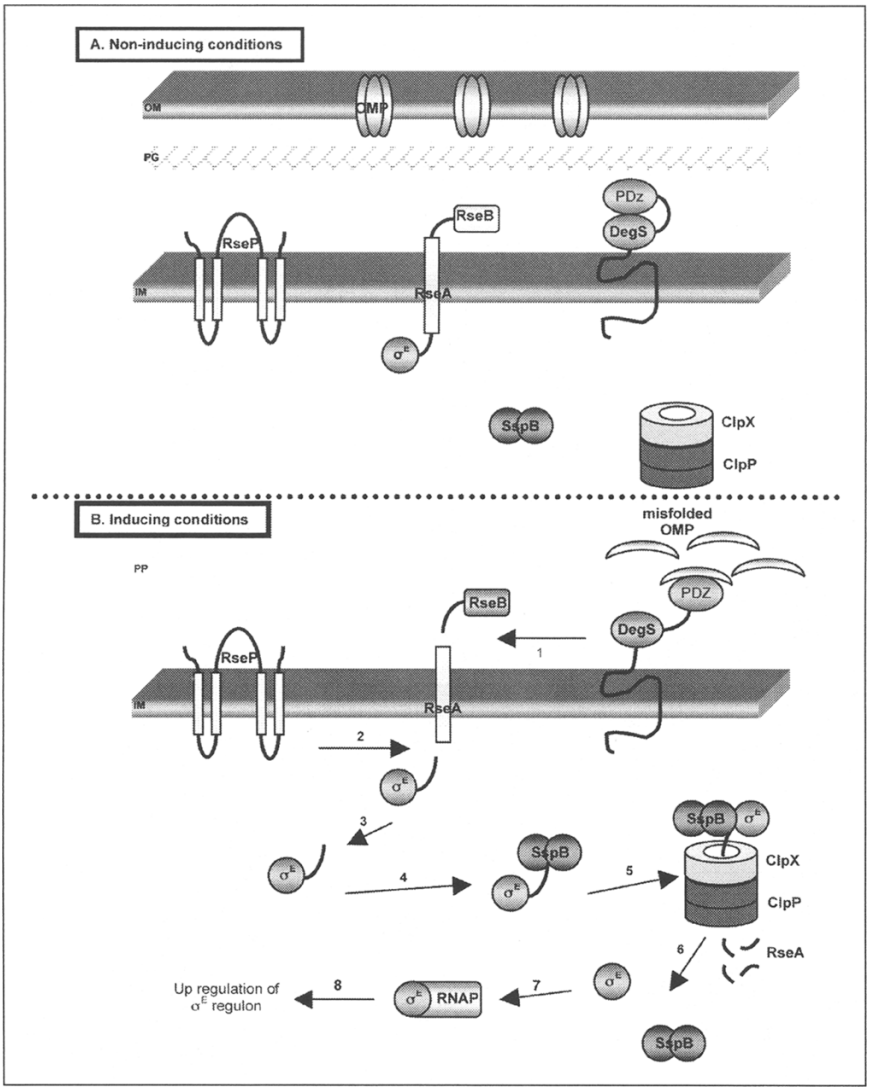

Figure 1. Model of the σ^E pathway under induced and non-induced conditions. A. Under non-induced conditions, the PDZ domain of DegS inhibits degradation of RseA. RseB is bound to the periplasmic domain of RseA, stabilizing the RseA: σ^E interaction. The presence of RseB and the periplasmic domain of RseA inhibit RseP from degrading RseA. B. Under inducing conditions, the PDZ domain of DegS interacts with the C-terminal domains of misfolded OMPs. The activated DegS then cleaves RseA near the transmembrane domain on the periplasmic side (1). This releases the periplasmic domain of RseA and RseB, which relieves the inhibition on RseP. RseP is then able to cleave RseA near the transmembrane domain in the cytoplasm (2). This releases the cytoplasmic domain of RseA with σ^E still bound (3). SspB then delivers the cytoplasmic domain of RseA with σ^E to the ClpXP complex (4, 5). ClpXP degrades the cytoplasmic domain of RseA freeing σ^E (6). σ^E is now free to bind with RNAP and up regulate the regulon (7, 8). The numbered arrows indicate the sequential order in which σ^E is activated. OM, outer membrane, IM, inner membrane, PP, periplasm, PG, peptidoglycan, OMP, outer membrane protein, RNAP, RNA polymerase.

a YFF recognition peptide. This interaction activates DegS to cleave RseA adjacent to the transmembrane domain on the periplasmic side.[31] It has been determined that the cleavage by DegS is the rate determining step and that the DegS-cleaved RseA serves as a substrate for RseP proteolysis.[29,30,33] Once the periplasmic domain of RseA is removed then RseP is free to cleave RseA at the cytoplasmic cleavage site (HExxH), which is close to the transmembrane domain.[30] It is believed that the glutamine rich regions of RseA's periplasmic domain interact with the periplasmic PDZ domain of RseP, thus inhibiting it from degrading RseA until the proper stress is sensed.[30]

Grigorova et al[34] showed that RseB diminished RseP degradation of RseA. They speculated that the affect RseB had on RseP activity may have a role in sensing signals other than OMPs. It has been shown that RseB can interact with the misfolded periplasmic protein MalE31.[28] The hypothesis is that under inducing conditions that do not involve OMPs, RseB would be titrated away, where it may or may not interact with the misfolded proteins present. This removal of RseB would then allow RseP to cleave RseA independently of DegS.[34] If this does occur, one would speculate that the proteolysis is inefficient since the glutamine rich region in the periplasmic region of RseA that is important for RseP inhibition is still present. It has also been shown that the substrate for RseP is a DegS-cleaved RseA.[29,33] Thus the role of RseB in signal transduction remains mysterious. A better explanation for the inhibitory effect of RseB on RseP proteolysis of RseA may be that the glutamine rich region of RseA and RseB both contribute to the inhibition of RseP and that RseB may function to block any inappropriate degradation of RseA by DegS. In a similar fashion, RseB may also have a role in blocking the σ^E pathway from normal OMP misfolding "noise". Another possibility is that RseB is important in shutting off the σ^E pathway after OMP folding stress has been relieved.

After both cleavage events by DegS and RseP, the cytoplasmic domain of RseA is left intact which is still able to inhibit σ^E. This cytoplasmic domain of RseA must be degraded in order to free σ^E. After the cleavage by RseP, the cytoplasmic domain of RseA is targeted for degradation by ClpXP.[35] SspB, a ClpXP adaptor protein, recognizes the C-terminal VAA signal on RseA that is generated after RseP cleavage and delivers it to ClpXP, where it is degraded, releasing σ^E[35] (Fig. 1).

It was thought that RseA was the pivotal signal transduction protein of the σ^E pathway. However, recently it has been shown that σ^E activity increases upon entry into stationary phase independently of RseA.[36] This increased activity is due to an internal (cytoplasmic) signal related to starvation. Upon further analysis it was determined that the increased activity of the σ^E pathway is correlated with increased levels of ppGpp (3',5'-bispyrophosphate).[36] ppGpp is a global regulator that is the general signal of starvation.[37] Along with activating σ^E, ppGpp also activates σ^S and σ^N,[37] suggesting that it is used to coordinate a broad response to nutritional stress. The exact nature of how the signaling cascade between ppGpp and σ^E works is still not understood. However this is the first time that a cytoplasmic regulator has been shown to influence σ^E regulation. Understanding exactly how ppGpp regulates σ^E will open the door to understanding how envelope stress responses are regulated by both external and internal signals.

σ^E *Regulon*

The σ^E regulon has been extensively studied and it has been speculated that it consists of over 100 members. Originally, it was shown that the regulon consisted mainly of periplasmic protein folding and degrading factors, in keeping with a primary role in sensing and responding to misfolded proteins in the periplasm, especially OMPs. It is now known that the regulon contains members that are involved in multiple aspects of cell function including protein folding and degradation in the periplasm, assembly of outer membrane proteins, primary metabolism, transcription and translation, lipoproteins and lipid detoxification, DNA/RNA modification and repair, cell structure and division, regulation of small noncoding RNAs (sRNAs) and genes of unknown functions [17,25,38-43] (Table 1). For a comprehensive list of the members of the σ^E regulon please refer to the microarray papers by Rhodius et al[43] and Kabir et al.[40] This section will only deal with the members of the regulon whose main purpose is in envelope protein biogenesis and degradation.

Initially, the main members of the σ^E regulon identified were *rpoE* itself along with its regulators *rseABC*.[21,22] The first major characterization of the σ^E regulon utilized promoterless *lacZ* elements to identify constructs that showed increased β-galactosidase activity when σ^E was overexpressed.[38] *rseP*, another regulator of σ^E was added to the regulon after this screen was carried out.[38] It was found that σ^E not only regulates itself but also two other σ factors, *rpoD* (σ^{70}), the housekeeping sigma factor and *rpoH* (σ^H), the heat shock sigma factor.[17,18,38] Because of the regulation of *degP*, a periplasmic chaperone and protease and activation by OMPs, it was determined that σ^E monitors the envelope, so it was not surprising when periplasmic chaperones and folding catalysts were added to the σ^E regulon. These chaperones and folding catalysts include *skp*, a chaperone for OMPs, *fkpA*, a periplasmic peptidyl-prolyl-isomerase (PPI), *surA*, a periplasmic OMP chaperone, which also has PPI activity and *dsbC*, a disulfide bond isomerase which also has chaperone activity[38] (Table 1). Interestingly, prior to their identification as σ^E regulon members, DsbC, SurA, FkpA and Skp were identified for their ability to modulate σ^E activity when overexpressed or mutated.[25] Recent studies that characterized genes that were upregulated when σ^E was overexpressed have expanded the regulon to include proteins involved in OM assembly, such as *yifO*, an essential lipoprotein required for OMP biogenesis, *yraP*, a non-essential lipoprotein that is important for cell envelope maintenance and *yaeT*, an essential OMP assembly factor[41,44,45] (Table 1).

Interestingly, it was recently shown that σ^E alleviates OMP misfolding not only by inducing expression of OMP assembly and degradation factors but also by controlling expression of the OMPs themselves. When the σ^E pathway is induced many OMPs, such as OmpA, OmpC, OmpF, OmpW and OmpX are down-regulated.[40,43] However, none of the OMP promoters contain the recognition sequence for σ^E.[43] It was discovered that σ^E down-regulates OMPs upon pathway activation through the control of transcription of two sRNA, *micA* and *rybB*.[39] Upon induction of the σ^E pathway there is an increase in MicA and RybB levels. MicA and RybB go on to bind with *ompA* mRNA and *ompC/W* mRNA, respectively, decreasing the steady-state levels of these mRNAs.[39] Thus, σ^E mediated activation of these sRNA stops the accumulation of misfolded/mislocalized OMPs in the periplasm by controlling gene expression at the posttranscriptional level. This in turn, will allow the members of the σ^E regulon to more efficiently clear the misfolded OMPs present and return the cell to homeostasis.

σ^E *and Virulence*

The σ^E pathway is conserved throughout numerous Gram negative bacteria. While the σ^E pathway has been studied extensively in *E.coli* with relation to monitoring and maintaining OMPs, most work in other Gram negative bacteria has linked σ^E to virulence. σ^E is essential in *E.coli*, but its homologues are not essential in other bacteria. Probably the most widely studied σ^E homologues are in *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*. Studies of σ^E in various pathogens showed that *rpoE* mutations attenuate virulence (Table 2) (for a more comprehensive review on σ^E and pathogenesis please refer to the Raivio review ref. 46).

In *S. typhimurium*, *rpoE*/σ^E mutants exhibit decreased survival and proliferation in macrophages and epithelial cell lines.[47] This decreased virulence is attributed to an increased sensitivity to reactive oxygen species, like H_2O_2 and superoxide.[47-49] It was also shown when *S. typhimurium* enters into macrophages there is an increase in σ^E activity, allowing one to speculate that this increase in activity may help the bacteria survive against oxygen-dependent host defences.[49,50] Analysis of the σ^E regulon in *S. typhimurium* also showed that it contains genes important for pathogenesis, which may also be why *rpoE* mutants are attenuated with a 50% lethal dose (LD_{50}) five orders of magnitude higher than wild type.[47,51] The phenotypes seen with *rpoE* mutants in *S. typhimurium* are common among other *rpoE* mutants in different pathogens (Table 2). For example *rpoE* mutants in *Vibrio cholerae* have a decreased ability to survive within the intestines and have a LD_{50} three fold higher than wild type.[52] *Mycobacterium rpoE* mutants are unable to survive and grow within macrophages and when mice are infected with the mutants they have a delayed death.[53,54] σ^E/*rpoE* mutants in *Burkholderia pseudomallei* exhibit reduced survival in macrophages, which may be due to an increased sensitivity to H_2O_2.[55] As for the *P. aeruginosa rpoE* homologue, *algU*,

Table 2. Affects that rpoE mutants have on pathogenesis

Pathogen	Affects on Virulence
S. typhimurium	Decreased survival and proliferation in macrophages and epithelial cell lines increased sensitivity to reactive oxygen species, H_2O_2 and superoxide LD^{50a} is 5 fold higher than wild-type
V. cholerae	Decreased ability to survive in intestinal environments LD^{50} is 3 fold higher than wild-type intestinal colonization is 30 fold reduced
Mycobacterium	Unable to survive and proliferate in macrophages results in delayed death in infected mice
B. pseudomallei	Reduced survival in macrophages increased sensitivity to H_2O_2 reduced ability to form biofilms, which help in resistance to antibiotics and host immune systems
P. aeruginosa	Increased sensitivity to phagocytic killing reduced ability to form biofilms, which are important in establishing infection

a. LD^{50}, 50% lethal dose

mutants show increased sensitivity to phagocytic killing.[56] Thus, through the study of *rpoE* mutants in numerous pathogens it appears that σ^E mediates adaptation to oxidative stresses, however the mechanism by which this occurs is not known.

Essential Nature of σ^E

Not long after σ^E was discovered and cloned it was noticed that it was essential for bacterial growth at high temperatures.[16] Initially, it was thought that the reason for the essential nature of σ^E at high temperatures was due to the fact that it is needed to drive the temperature dependent promoters of *rpoH* and *degP*. However analysis of different *rpoE* mutants at 30° suggested that σ^E might be essential at all temperatures, since no two *rpoE* mutants had similar growth patterns/phenotypes, suggesting the accumulation of suppressor mutations.[19,20] Upon analysis of cells lacking σ^E it was determined they had acquired a suppressor in order to grow at low temperatures.[15] Understanding why and how σ^E is essential is key to fully understanding the role of σ^E within the bacterial cell. The σ^E regulon includes numerous genes that are essential for viability such as *rpoE* itself, *rpoD, rpoH* and *rseP*[38] (Table 1). It was also noted that single and double deletions of *dsbC, skp, surA or fkpA* did not have deleterious effects, however deletions of more then two of these genes conferred growth defects[38,57] (Table 1). Thus, one reason why σ^E is essential may be that it transcribes several genes that are essential for viability either alone or in combination. σ^E may also contribute to other undefined essential cellular functions, since recently it has been shown through microarray analysis that σ^E is involved in numerous cellular processes.[40,43]

As mentioned before the only way that σ^E mutants are able to grow is in the presence of suppressors.[15] To try and understand in further depth the essential nature of σ^E, Douchin et al[58] looked at how suppressors alleviated the need for RseP in the normal activation of σ^E. There could be two reasons why RseP is needed in the normal activation of σ^E. The first reason is that without RseP, RseA would not be fully degraded and ultimately there would be no release of σ^E and therefore no activation of the regulon. The second reason why RseP might be important is that *rseP* and *yaeT* are predicted to be in the same operon, so one could imagine that a mutation in *rseP* could affect *yaeT*, which is important in the insertion of OMPs into the OM.[44,45] It was found that overexpression of the sRNA, *rseX*, bypassed the RseP requirement for the activation of σ^E. RseX suppressed σ^E lethality by down regulating levels of OmpC and OmpA,[58] thus decreasing the potential stress caused by these proteins and ultimately altering the requirement for σ^E. Thus, this study suggests that *rseP* (and therefore σ^E) is essential because it permits sufficient activation of

the σ^E pathway to allow the cell to deal with basal levels of stress conferred by normal/unstressed OMP elaboration.

In conclusion there are three potential reasons why σ^E may be essential to the cell; the first being that the regulon encodes numerous genes with redundant functions. Thus if the bacteria loses an individual gene it still has other factors to overcome the deficit, however a loss of more than one would see that function eliminated resulting in reduced viability. The second reason that the σ^E pathway may be essential is that it is involved in many aspects of cell physiology. The third reason σ^E may be essential is that it controls the expression levels of OMPs through sRNAs, so when σ^E is absent OMPs are expressed at higher levels but there is no pathway to upregulate the assembly and folding factors to facilitate their biogenesis, thus leading to an enormous stress that cannot be overcome.

Future Research Questions

Although the σ^E pathway has been studied in depth and it is the most characterized envelope stress response in *E.coli*, there are still questions that remain to be answered. Fully understanding the essentiality of σ^E is probably the biggest question that still needs to be addressed. While initial studies of the σ^E response addressed how the pathway is controlled by signal transduction that occurs across the inner membrane, recent studies indicate cytoplasmic signal transduction events, such as those involving generation of ppGpp also affect σ^E regulated genes.[36] This begs the question of whether other cytoplasmic factors or signals exist that affect σ^E regulated gene expression. Another unanswered question concerns the role RseB plays in signal transduction. Is RseB involved in shutting off the σ^E pathway after the stress has been relieved, or is its role in preventing false signals from activating the pathway? Addressing these questions will shed light on how bacteria maintain homeostasis by integrating a variety of signal transduction mechanisms and pathways.

The Cpx Two-Component System

Cpx History and Components of the Signal Transduction Pathway

The discovery of the Cpx signal transduction pathway was originally not associated with studies of extracytoplasmic stress responses, but rather with a genetic screen for strains incapable of conjugation of the F plasmid.[59] Though non-essential for cell survival, gain-of-function mutations in the *cpx* locus yielded strains sensitive to elevated temperatures that possessed altered envelope protein composition, giving the first indication that the *cpx* genes may be involved with envelope stress.[60-63] Subsequent characterization of the *cpx* locus determined that it encoded two genes. Through analysis of fusion proteins and immunochemical experiments, the *cpxA* gene was determined to encode a transmembrane protein with sequence homology to the EnvZ two-component histidine kinase family.[64,65] Later analysis of the region upstream from *cpxA* uncovered its cognate response regulator, CpxR, which showed homology to the OmpR subfamily of DNA-binding proteins and together the CpxAR proteins were classified as a two-component signal transduction system.[66]

Subsequent studies uncovered a biological role for the Cpx two-component system. Using toxic, misfolded, mislocalized, secreted proteins, Silhavy and colleagues demonstrated that constitutively activated *cpx* mutants (*cpx**) endured high levels of misfolded and mislocalized envelope proteins.[67] These studies also demonstrated that the periplasmic protease, DegP, was an important component of the alleviation of toxicity.[67] Furthermore, the overexpression of a lipoprotein of unknown function, NlpE, was demonstrated to activate the Cpx pathway and also suppressed the toxicity of misfolded, mislocalized secreted proteins.[68] Since Cpx pathway activation rescued the cell from misfolded envelope proteins, the Cpx signal transduction system was proposed to control an envelope stress response in *E. coli*.[67,68]

Though CpxA and CpxR were initially classified as a traditional two-component signal transduction system, later work revealed a third signal transduction protein, CpxP.[69-71] CpxP was first identified as a small periplasmic protein that was induced in a Cpx-dependent manner in response to envelope stresses such as alkaline pH or overproduction of NlpE.[69] However in addition to being a downstream target of the Cpx system, the overproduction of CpxP resulted in a downregulation

of Cpx-mediated gene expression.[70] Moreover, it was shown that tethering of CpxP to the inner membrane prevented the activation of the Cpx system after harsh envelope stress due to spheroplasting (i.e., removal of the outer membrane).[71] Although no direct interaction between CpxP and CpxA has been shown, it has been demonstrated that CpxP-mediated inhibition requires the periplasmic sensing domain of CpxA, which suggests that CpxP interacts with the sensor domain of CpxA to inhibit the Cpx pathway.[70] In support of this hypothesis, CpxP could inhibit CpxA enzymatic activity in reconstructed proteoliposomes.[72] Overall, early studies showed that the Cpx signal transduction pathway regulates an envelope stress response of which DegP is a component and consists of the membrane bound sensor kinase CpxA, the cognate response regulator CpxR and the periplasmic inhibitory protein CpxP (Fig. 2).

Cpx Pathway Activation

The Cpx response is induced by envelope stressors that include alkaline pH, the overexpression of specific membrane-associated proteins and alterations in membrane structure.[6] In addition, adherence to abiotic surfaces has been shown to activate the Cpx response.[7] The first major

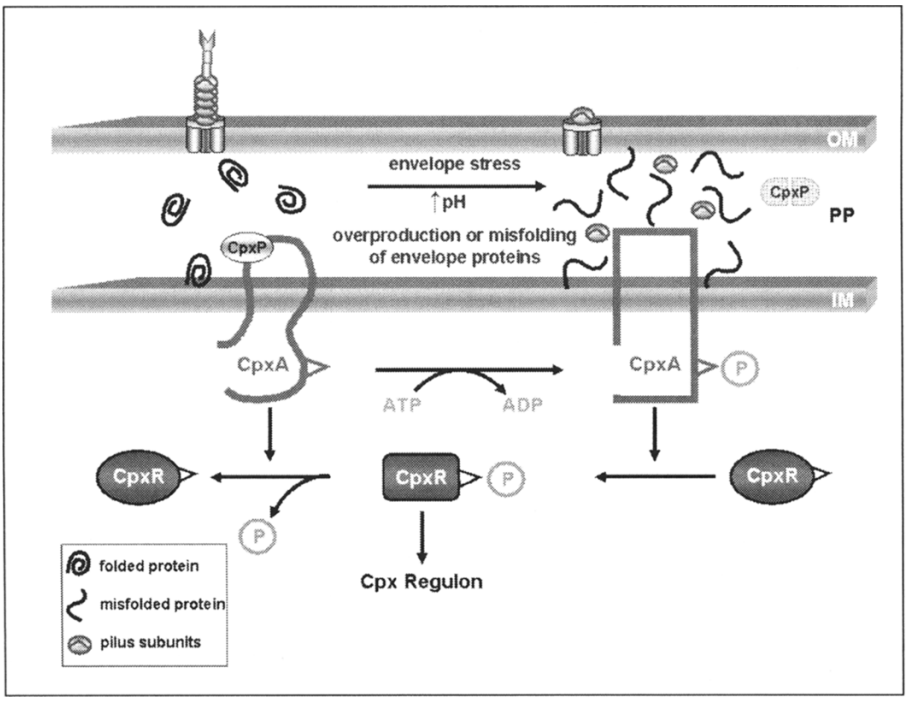

Figure 2. The Cpx envelope stress response. In non-inducing conditions, CpxA remains unphosphorylated, partly through interaction with the periplasmic protein, CpxP. Envelope stress brought about by either elevated pH or the presence of misfolded or overexpressed envelope proteins results in degradation of CpxP by DegP, thus relieving inhibition of CpxA. Inducing cues stimulate CpxA autokinase and kinase activities. The phosphorylated form of CpxA transfers the phosphate ion to CpxR which up-regulates transcription of several genes involved with protein folding, degradation and transcription regulation (e.g., *dsbA*, *degP*, *cpxRA*, *cpxP and* others). Once envelope stress is alleviated, CpxA acts as a phosphatase to remove the phosphate ion from CpxR thereby down-regulating the Cpx envelope stress response. Additional abbreviations: OM: outer membrane; PP: periplasmic space; IM: inner membrane. Figure adapted from Raivio et al, 1999, Raivio and Silhavy, 2001, Buelow and Raivio 2005.

inducing cue of the Cpx pathway was identified through a selection for multicopy suppressors of the toxicity exerted by secretion of a LamB-LacZ-PhoA fusion protein. This selection identified the overproduction of the outer membrane lipoprotein NlpE.[68] The mechanism of Cpx pathway activation by NlpE overexpression is not known, however NlpE overexpression results in protein mislocalization and likely misfolding, thus it is speculated that this may constitute part of the inducing cue.[6,68] In addition to NlpE, the overexpression of pilus subunits such as those of the uropathogenic *E. coli* (UPEC) P or Pap pilus (PapG and PapE) and the major subunit, BfpA, of the type IV bundle forming pilus of enteropathogenic *E. coli* (EPEC) has been shown to induce the Cpx system as well.[24,73] Since both Pap and Bfp subunits are misfolded and/or mislocalized when expressed in the absence of other pilus assembly components, these studies together with those on NlpE, provide strong evidence that misfolded proteins are a major Cpx pathway activating signal. It has been demonstrated that the Cpx system is induced during P pilus assembly and it is hypothesized that this is a result of occasional pilus subunits failing to follow the normal assembly pathway and becoming misfolded.[24,74] Thus, activation of the Cpx pathway by misfolded pilus subunits may have a physiological role during pilus assembly (discussed later); however, the molecular nature of the inducing cue that is generated by misfolded proteins is not known.

Interestingly, PapG was shown to activate the σ^E and Bae envelope stress responses as well; however, overproduction of PapE appears to specifically induce the Cpx pathway.[24,75,76] The specificity of PapE to induce only the Cpx system has been attributed to its N-terminal extension, as deletion of this motif abolished the ability of PapE overexpression to activate the Cpx response.[76] It is hypothesized that in the absence of the Pap chaperone, PapE adopts a specific conformation that induces CpxA and that this conformation requires the N-terminal extension. Thus, further analysis of Pap subunit folding intermediates is likely to lead to a better understanding of how misfolded proteins activate the Cpx response.

As both Pap and bundle forming pili play an essential role in adherence to host cells, it is interesting that NlpE has also been implicated in cell adherence. Otto and Silhavy demonstrated the link between NlpE and adherence,[7] as bacterial attachment to abiotic surfaces was shown to induce the Cpx response in an NlpE-dependent fashion. Furthermore, both *cpxR* and *nlpE* mutants exhibited defects in adhesion.[7] Together, the induction of the Cpx pathway in response to assembly intermediates of bacterial pili and adherence to abiotic surfaces highlights a possible role for the Cpx system in bacterial pathogenesis and biofilm formation (see below).

Elevated pH is another inducing cue of the Cpx stress response that has been demonstrated in both *Shigella* and *E. coli* species.[69,77] Strains carrying *cpx* null mutations are hypersensitive to alkaline pH and the expression of Cpx-regulated genes has been demonstrated to be induced by elevated pH.[69] In *Shigella*, mutations in *cpxA* altered gene expression of the virulence regulator, *virF*, in a pH-dependent manner.[77] Although the nature of the Cpx inducing cue generated at alkaline pH remains unknown, it seems likely that it involves denatured proteins, given that misfolded proteins are known to activate CpxA.[24,73]

Finally, two separate research groups identified alterations in the cell envelope that activated the Cpx system. Milkeykovskaya and Dowhan[78] first reported that the lack of phosphatidylethanolamine (PE) in the membrane resulted in the increased Cpx-dependent expression of the periplasmic protease *degP*. In another study by Silhavy and colleagues,[79] the accumulation of an intermediate of enterobacterial common antigen (ECA) synthesis resulted in the activation of the Cpx system.[79] In both cases, the alteration of the membrane composition stimulated the Cpx response. Again, it is not clear how changes in membrane composition activate CpxA. One possibility is that these changes alter protein folding in the envelope.

Cpx Signal Transduction

In the presence of the above mentioned envelope stresses, the activation of the Cpx response is thought to be mediated by classic two-component pathway signal transduction mechanisms. Typical two-component phosphotransfer reactions between the sensor kinase CpxA and the response regulator CpxR control the Cpx envelope stress response[72,80] (Fig. 2). Inducing cues

result in autophosphorylation of CpxA, likely at the conserved histidine residue.[72,80] Once phosphorylated, CpxA transfers the phosphate ion likely to the conserved aspartate residue on the cognate response regulator, CpxR.[72,80] CpxR is most closely related to the OmpR subfamily of response regulators that are classified as winged-helix-turn-helix DNA binding proteins.[81] Based on this homology, it is expected that CpxR~P stimulates transcription through direct contacts with the carboxy-terminal domain of the α subunit of RNA polymerase. In addition, experimental evidence indicates that CpxA also acts as a phosphatase of phosphorylated CpxR.[72,80] Furthermore, since constitutively activated *cpx* mutations diminished the phosphatase activity of CpxA, this indicates that the relative activity of the pathway is controlled by the ratio of CpxA kinase to phosphatase activities.[80]

Efforts to elucidate the inhibitory role of CpxP on the Cpx system have proved to be challenging and have consisted of numerous experimental approaches. Investigations of various gain-of-function CpxA* mutants revealed that deletion or point mutations in the periplasmic domain of CpxA resulted in the inability of CpxA to sense Cpx inducing cues.[80] The authors speculated that this domain of CpxA interacted with an inhibitory protein which would be titrated away in the presence of activating signals.[80] Follow up studies revealed that overproduction of CpxP inhibited the Cpx response and this inhibition was dependent on the periplasmic domain of CpxA, which suggested that CpxP interacted with CpxA.[70] In support of this hypothesis it was demonstrated that tethering CpxP to the inner membrane prevented full activation of the Cpx response by spheroplasting.[71] Further, Fleischer et al[72] recently showed that the addition of purified CpxP to reconstructed proteoliposomes containing CpxA lead to an inhibition of CpxA autokinase activity. Together, the cumulative data suggest that CpxP associates with CpxA in the periplasm and the CpxP:CpxA interaction has a negative regulatory effect on the Cpx envelope stress response.

To examine the mechanisms of CpxP inhibition, Buelow and Raivio performed a mutagenesis screen that identified several *cpxP* mutants that no longer could inhibit the Cpx pathway.[82] Examination of these *cpxP* mutants revealed mutations in a highly conserved N-terminal domain.[82] Some of these mutations resulted in a large decrease in CpxP stability that was restored by mutations of the periplasmic protease DegP.[82] This observation suggested that DegP might normally be involved with CpxP stability and Cpx signal transduction. In fact, both the Silhavy and Raivio research groups have now demonstrated the involvement of the periplasmic protease DegP with CpxP degradation.[82,83] Buelow and Raivio[82] demonstrated that the inducing cue of alkaline pH led to a DegP-dependent degradation of CpxP, while Isaac et al [83] showed that CpxP was required for the degradation of misfolded pilus proteins and also that CpxP degradation was enhanced under these conditions.[82,83] These experiments suggest that misfolded periplasmic proteins may associate with CpxP thus titrating CpxP away from CpxA and relieving CpxP-mediated inhibition of the Cpx response. CpxP could then bring the misfolded protein to DegP where both CpxP and the misfolded protein would be degraded. Another possibility is that conditions that lead to envelope protein misfolding also result in misfolding of CpxP, causing it to become a substrate for DegP.

It is interesting to note that high levels of CpxP do not completely shut off the Cpx pathway, nor does deletion of *cpxP* prevent increased activation of the Cpx system.[70,84] Thus, it is proposed that the role of CpxP is to help "fine tune" the Cpx pathway during envelope stress and/or to prevent inappropriate activation of the Cpx response.[82] The elucidation of the molecular mechanism of CpxP-mediated inhibition, its relief and its precise cellular role await further study.

The Cpx Regulon

Initial studies of genes regulated by CpxR identified three genes: *dsbA,* which encodes for the major periplasmic disulfide oxidoreductase, *degP,* coding for the periplasmic protease/chaperone and a gene encoding a peptidyl-prolyl isomerase, *ppiA.*[85,86] These findings were not surprising as each gene product is involved with proper envelope protein folding (*dsbA, degP* and *ppiA*) or degradation (*degP*). Through analysis of the upstream region of all three genes, Pogliano and colleagues[86] proposed a consensus binding site for CpxR (5′-GTAAN$_{(6-7)}$GTAA-3′) which has aided other research groups in identifying more putative Cpx-regulated genes.

There are currently 41 genes in 25 operons that are proposed to make up the Cpx regulon (Table 3). Examination of these genes reveals functional subcategories. One class consists of genes directly involved with envelope maintenance such as the aforementioned *dsbA*, *degP* and *ppiA* genes, as well as *psd*, *secA* and *spy*. Mutations in the *dsbA* locus resulted in misfolded envelope proteins due to the lack of disulfide bond formation.[87,88] Identification of DegP protease activity was shown through analysis of a *degP* mutant which was defective in breakdown of several misfolded periplasmic proteins, but not cytoplasmic proteins.[89] Though not essential for bacterial survival, proper protein folding in the periplasm is also mediated by peptidyl-prolyl isomerases such as *ppiA* and *ppiD*.[90,91] Identification of *ppiD* as a Cpx-regulated gene was based on the presence of putative CpxR binding sites in its upstream region,[91,92] the ability of a putative phosphatase that controls the Cpx response to down-regulate *ppiD* expression and its increased transcription in the presence of an uncharacterized *cpx** mutation.[91] However, *ppiD* has also been demonstrated to be under the regulatory control of the classical heat shock sigma factor, σ^H, which regulates the expression of

Table 3. List of genes and operons proposed to be under Cpx regulation

Gene(s)	Function	Proposed Cpx-Regulation[a]
Envelope Protein Maintenance		
degP	Periplasmic serine endoprotease	Positive
yihEdsbA	Disulfide oxidoreductase	Positive
ppiA	Periplasmic peptidyl-prolyl isomerase A	Positive
ppiD	Periplasmic peptidyl-prolyl isomerase D	Positive
secA	Secretion subunit A	Positive
psd	Phosphatidyl serine decarboxylase	Positive
spy	Spheroplast protein Y	Positive
Envelope Components		
ompC	Outer membrane protein C	Positive
ompF	Outer membrane protein F	Negative
nanC	N-acetylneuraminic acid (NAN) channel	Positive
acrD	Component of efflux pump	Positive
mdtABCD	Multi drug transporter	Positive
Signal Transduction		
cpxP	Periplasmic proteolytic adapter protein, modulator of CpxA	Positive
cpxRA	Cpx two-component regulators	Positive
rpoErseABC	Sigma E and regulators	Negative
Bacterial appendages (flagella, frimbriae, pilins) and chemotaxis		
motABcheAW	Motility and chemotaxis signal transduction proteins	Negative
tsr	Serine chemotaxis	Negative
aer	Aerotaxis receptor	Negative
csgDEFG, csgBAC	Curlin adhesin regulation and assembly	Negative
papBA	UPEC P pilus assembly and regulation	Negative
Unrelated to envelope components or stress		
ung	Uracil-DNA glycosylase	Positive or negative
aroK	Shikimate kinase I	Positive or negative
mviM	Putative virulence factor	Positive
htpX	Heat shock protease	Positive

a. For references of the putative Cpx regulation of these genes, see text.

cytoplasmic chaperones and proteases.[91] Though no experimental evidence exists, the upstream regulatory region of σ^H is speculated to contain a CpxR binding site, which suggests that σ^H may be under Cpx regulatory control.[86,92] Thus, further work needs to be done to determine whether the proposed Cpx-regulation of *ppiD* is direct or the result of the Cpx system influencing σ^H levels. The *psd* and *secA* genes encode for proteins that affect biogenesis of phospholipids and proteins destined for the bacterial envelope. These genes were proposed to be Cpx-regulated based on a bioinformatics screen for a CpxR consensus binding logo.[92] *psd* encodes a membrane-bound phosphatidylserine decarboxylase that is involved in phospholipid biosynthesis.[93] SecA is the ATPase subunit of the Sec bacterial protein translocation machinery which governs protein export across the inner membrane. The *spy* gene, of unknown function, may also be included in this category since its deletion causes induction of the σ^E response, suggesting a role in outer OMP biogenesis.[71] A *spy-lacZ* reporter was shown to be up-regulated by several Cpx-inducing conditions, including NlpE overexpression, spheroplasting and the presence of *cpxA** mutations.[71]

A second class of genes proposed to be Cpx-regulated includes genes encoding envelope proteins involved in transport, such as outer membrane proteins OmpC, OmpF and NanC and membrane channels involved with active efflux, including AcrD and MdtABCD.[94-96] CpxR has been shown to bind upstream of the *ompC*, *ompF*, *acrD* and *mdtA* promoters.[94,96] Additionally, mutations and conditions that activate the Cpx response lead to increased *ompC* and decreased *ompF* expression.[94] Deletion of *cpxR* diminished *nanC* expression, suggesting that this putative N-acetylneuraminic acid channel may be positively Cpx-regulated.[95] Interestingly, all genes listed in this class are strongly regulated by other regulatory pathways. The porin genes *ompC* and *ompF* have been shown to be under the control of the EnvZ-OmpR signal transduction system.[97] Various regulators including NanR, NagC and OmpR regulate the expression of *nanC*.[95,98] The genes encoding the drug transporters AcrD and MdtABCD are also regulated by another envelope stress response, the Bae system (see next section).[8,99,100] Further, the CpxR binding site has been shown to overlap those of OmpR upstream of *ompC* and *ompF* and those of BaeR upstream of the *acrD* and *mdtABCD* loci.[94,96] At the *acrD* and *mdtABCD* gene clusters, CpxR appears to function to enhance or facilitate BaeR-mediated gene expression, since inducible transcription is still seen in *cpx* mutants, although at a diminished level.[96] These data suggest CpxR may function as an accessory regulator at these loci.

Another class of Cpx-regulated genes consists of regulatory genes, encoding the Cpx and σ^E signal transduction proteins. Either constitutively activated *cpx** mutations or elevated pH levels resulted in increased levels of CpxA, CpxR and CpxP, indicating autoregulation of the Cpx system.[69,70,101] Autoregulation is noted in other stress response systems such as in σ^W and σ^X in *B. subtilus* and the *E. coli* σ^E stress response. This probably reflects a need for tight control of these stress responses when responding to potentially harmful environments (reviewed in ref. 5, 13). In addition to regulating the Cpx system, the Cpx response also negatively regulates the σ^E response.[92,102] CpxR~P binds upstream of the *rpoErseABC* locus which encodes σ^E and its regulators and mutations that inactivate or induce the Cpx response cause increased or decreased expression of the *rpoErseABC* gene cluster, respectively.[92,102] Presumably, this cross regulation between envelope stress signal transduction pathways reflects the need for the cell to coordinate its response to different envelope stresses.

A growing number of proposed Cpx-regulated genes can be grouped together as genes involved with the production or function of extracellular bacterial structures such as flagella or pili. These include the motility and chemotaxis gene clusters *motABcheAW*, *tsr* and *aer*, the *csg* locus encoding the curli adhesin, its assembly machinery and regulators and the *pap* gene cluster encoding the UPEC P pilus. Experimental evidence for the link between the Cpx response and motility is limited as the motility and chemotaxis genes were reported to be downregulated only in a mutant *cpx** background, while the deletion of *cpxR* had minimal effect on the transcription of *motABcheAW* and *tsr*.[101] Nevertheless, the deletion of *cpxR* results in increased motility,[101] thus suggesting an inhibitory role of the Cpx response system on motility. As the regulation of motility, chemotaxis and flagellar components is governed by the alternative sigma factor, σ^F, it remains unclear whether

Cpx exerts its regulatory effect directly on these motility and chemotaxis genes, or acts on genes higher up in the flagellar regulation hierarchy. Alternatively, the effects of the Cpx response on motility may also be partly due to posttranscriptional effects. Curli are extracellular proteinaceous fibers important for biofilm formation and have also been proposed to be negatively regulated by the Cpx response, however only under certain growth conditions.[103] Using electrophoretic mobility shift assays, it was shown that CpxR, in concert with OmpR, binds to the upstream region of the *csgDEFG* operon which encodes the curli biosynthesis regulator and transport genes.[104] In a *cpxR* mutant grown in high salt, the expression of the *csgD* reporter is increased, suggesting that CpxR may be involved in repression of the *csgDEFG* operon under certain conditions.[104] However the Cpx regulatory effect was mild (~2 fold difference) and only noted in specific media conditions, thus it may be that the Cpx response regulates curli production only under specifically defined experimental conditions. A direct regulatory link between the Cpx response and P-pili has also been demonstrated.[74,105] Analysis of the upstream regions of the *pap* operons revealed a CpxR binding site which overlaps with that of another regulatory protein, Lrp. Phosphorylated CpxR competes with Lrp for binding to certain sites within this *pap* switch region that directs phase-variable expression of the *pap* operon, resulting in a decrease in in vivo production of P pili.[105] Interestingly, all of the genes encoding these extracellular appendages appear to be negatively regulated by the Cpx response. It is tempting to speculate that these genes have evolved to be under Cpx control because it is desirable for the cell to shut down production of extracellular envelope-localized structures when envelope stresses causing misfolding of proteins are present.

The remaining genes (*ung, aroK, htpX* and *mviM*) proposed to be part of the Cpx regulon do not involve envelope structure, maintenance, or regulation and thus the rationale for their regulation by the Cpx stress response is unclear. The majority of these genes (*ung, aroK* and *mviM*) were identified to be Cpx-regulated from a genome-wide bioinformatics screen for CpxR consensus binding sites.[92] The *ung* gene encodes uracil-DNA glycosylase and is involved in DNA repair. While DeWulf and colleagues[92] showed that *ung* transcript levels were reduced in a *cpxR* null background and elevated in the presence of a constitutively active *cpxA** mutation, Ogasawara et al[106] showed that CpxR~P bound upstream of the *ung* gene and apparently repressed transcription, leading to reduced Ung enzymatic activity. The experiments were performed in different strain backgrounds and so it may be that this is the explanation for the contradictory results. Differences in Cpx-regulation of genes have also been noted with the *aroK* gene that encodes a shikimate kinase that is involved in aromatic amino acid metabolism. DeWulf et al[92] have suggested *aroK* is positively regulated by the Cpx response, however using a different strain background, Price and Raivio[102] showed *aroK* to be negatively regulated by the Cpx response. *mviM* encodes a putative virulence factor and is proposed to be positively regulated by the Cpx response.[92] The cytosolic heat shock protease, HtpX, was suggested to be under Cpx-regulatory control by another research group which showed that a *htpX-lacZ* fusion was up-regulated in the presence of a *cpxA** mutation and down-regulated by ablation of *cpxR*.[107] Since these genes encode proteins with an array of functions including cytosolic proteolysis (*htpX*), DNA and amino acid metabolism (*ung, aroK*) and virulence (*mviM*), it is not clear what their cellular role is as part of the Cpx regulon. A detailed analysis of how and when the Cpx system exerts its regulatory effects on these genes is required to properly confirm the role of each gene in the Cpx regulon. It may be that the Cpx response plays cytoplasmic roles in metabolism and protein quality control that have yet to be fully described.

Cellular Role of the Cpx Response

The Cpx system is proposed to regulate various genes with different functions not exclusively limited to envelope stress. In efforts to clarify the cellular role of the Cpx response, Price and Raivio[102] assayed the expression of the majority of the proposed Cpx regulon members in several *cpx* genetic backgrounds and in response to overexpression of NlpE, an inducer of the wild-type Cpx signal transduction pathway. The authors demonstrated a core group of genes that were strongly regulated by the Cpx response. The majority of these genes (*degP, dsbA, cpxP, spy, ompF,*

rpoErseABC and *psd)* are directly involved with envelope protein folding and therefore suggest the primary role of the Cpx response is as an envelope stress response.[102]

Stemming from its role in envelope maintenance, the Cpx response is also likely to be involved in bacterial pathogenesis since it affects bacterial adherence via NlpE and also pilus assembly which is necessary for host-cell attachment.[7,24,73,74] In addition, the Cpx system itself has been shown to be important for bacterial pathogenesis in several pathogens. Mutations in *cpxA* homologues render pathogenic *Salmonellae* incapable of attaching to and invading host cells.[108,109] In *Shigella* spp., the Cpx signal transduction system plays an essential role in regulation of the *virF* and *invE* activators of virulence determinant expression.[110-112] Several studies have provided indirect evidence to support the theory that the Cpx pathway plays a crucial role in *E. coli* pathogenesis. Null mutations in *cpxR* resulted in a Pap pilus assembly defect in laboratory strains of *E. coli* carrying a plasmid encoding all of the P pilus assembly genes, resulting in shorter pili when compared to wild-type strains.[74] In addition, a *cpxR* null mutation in enteropathogenic *E. coli* (EPEC) resulted in a decrease in the production of bundle forming pili and localized adherence, the first step in EPEC pathogenesis.[73] Furthermore, both Cpx-regulated folding factor DsbA and protease DegP have been shown to be essential for bacterial virulence in several pathogenic species including *Pseudomonas*, EPEC, *Vibrio*, *Salmonella*, *Shigella*, *Yersinia*, and *Bordetella*.[113-121] DegP and DsbA are thought to help maintain proper function of various envelope-localized virulence determinants such as pili and type III secretion apparati. On whole, the Cpx system appears to play a role in bacterial pathogenesis, however, questions remain as to whether the Cpx response is directly involved with regulating virulence factor gene transcription, or rather indirectly involved through mediating the expression of folding factors and proteases that ensure proper function of these virulence determinants.

The Bae Two-Component System

The BaeSR two-component system was the third signal transduction pathway to be officially classified as an envelope stress response in *Escherichia coli*. Based on the limited information available on the Bae pathway, it appears that its main function is to upregulate efflux pumps in response to toxic compounds. Here we will discuss what is presently known about Bae while highlighting important areas requiring further research.

Discovery and Classification as an Envelope Stress Response

Characterization of Cpx regulon member *spy* revealed that induction of this gene during spheroplast formation was only partially dependent on a functional Cpx pathway.[71] In an effort to identify the coregulator of *spy*, Raffa and Raivio[75] performed transposon mutagenesis on a strain carrying a *spy::lacZ* fusion. This approach generated a single insertional mutant that dramatically increased *spy* expression. The mutant was determined to have a BaeS gain-of-function mutation and was capable of activating *spy* transcription independent of Cpx. The *baeS* gene had previously been identified for its ability to complement mutations in the histidine kinase genes *envZ*, *creC* and *phoR*.[122] Sequence analysis revealed that BaeS is itself a putative histidine kinase, encoded in an operon with a proposed response regulator, BaeR. Together they constitute the BaeSR (bacterial adaptive response) two-component system.[122] Further investigation into the link between the BaeSR two-component system and the Cpx envelope stress response revealed that both pathways were capable of activating *spy* expression in response to a variety of envelope stresses. In addition, a *baeR cpxR* double mutant was more sensitive to envelope perturbations than either single mutant.[75] While these findings suggest significant overlap between the pathways, it should be noted that BaeSR was shown to have no effect on any other Cpx regulated gene and that not every Cpx inducing cue tested was an effective signal for Bae pathway activation. Based on these findings the authors classified the Bae pathway as a distinct envelope stress response system.

Conquering Stress via Active Efflux

In a screen performed by Baranova and Nikaido,[8] *baeR* overexpression was found to increase resistance of a hypersensitive *acrAB* mutant to novobiocin. The increased resistance was attributed to the fact that cells overexpressing BaeR failed to accumulate the drug. Upon closer inspection

of the region upstream of the *baeSR* locus, the authors discovered that these genes were in fact part of a larger operon encoding the *yegMNOB* multi-drug efflux system.[8,123] After demonstrating that BaeR-mediated activation of *yegM* was responsible for the aforementioned phenotype, the operon was renamed *mdtABCD,* where *mdt* denotes multi-drug transporter.[8] The following year, the Yamaguchi group demonstrated that BaeR overexpression confers increased levels of resistance to β-lactam antibiotics, deoxycholate and low levels of SDS.[100,124] The BaeR-mediated resistance to β-lactams was due to an increase in expression of *mdtABC,* as well as an additional multidrug exporter gene, *acrD.*[100] Further investigation into *mdtA* and *acrD* regulation revealed that BaeR mediated activation of these genes is enhanced when CpxR binds multiple sites upstream of the BaeR binding sites in the *mdtA* and *acrD* promoter regions.[96] This modulation of Bae regulation by the Cpx pathway represents an example of how these two envelope stress response systems cooperatively combat envelope stress. Together, these findings suggest that one way in which the BaeSR two-component system fulfills its function as an envelope stress response system is through upregulation of genes involved in active efflux.

Present Model: Questions Still Remaining

The BaeSR signal transduction pathway is thought to be a prototypical two-component system (Fig. 3). Phosphotransfer from the conserved H1 domain of BaeS to the conserved D1 domain of BaeR has been demonstrated in vitro in the presence of ATP.[125] Based on high homology between the periplasmic inhibitory protein, CpxP and Spy, there has been speculation as to whether Spy inhibits BaeS activity in a similar manner to how CpxP inhibits CpxA[71] (see previous section). This idea is attractive as it would reveal a common mechanism by which envelope stress response systems modulate activity in the absence of an inducing cue. To date, there is no experimental evidence in support of this model.

Activating cues that initiate BaeSR signal transduction include general envelope stresses such as spheroplast formation, overexpression of misfolded PapG pilus subunits and the presence of indole.[75] Increased sensitivity of *baeSR* mutants to myricetin, gallic acid, nickel chloride and sodium tungstate implies that the pathway may also respond to the presence of these antimicrobial compounds.[9] All of the aforementioned inducing cues elicit a cellular response that extends beyond the Bae pathway. For example, overexpression of PapG activates the σ^E, Cpx and Bae signaling pathways; while indole and spheroplast formation activate the Cpx and Bae pathways.[24,75] The identification of more specific inducers could reveal additional functions of the pathway and lead to the identification of new regulon members.

As it stands, the Bae regulon consists of a small but diverse group of genes not limited to those discussed above (*mdtABCD, acrD* and *spy*). Researchers have employed both genetic screens and expression profiling in an effort to complete the regulon. Baranova and Nikaido[8] screened a library of short genomic fragments for promoters that responded to BaeR overproduction. This approach led to the identification of two reputed regulon members, *ygcL* and *yicO,* encoding a protein of unknown function and a putative membrane transport protein respectively. Nishino et al[126] performed microarray analysis of an *E. coli* strain overexpressing BaeR, a *baeSR* deletion mutant and wild type *E. coli* cells exposed to indole. Using the three expression data sets the authors identified *mdtABCD, acrD, spy* and *ycaC* as direct targets of BaeR regulation. The only new regulon member to be uncovered, *ycaC,* encodes a putative membrane localized hydrolase.[126] The fact that the genetic screen and the microarray analyses exposed different genes as possible regulon members suggests that neither approach was exhaustive. This, combined with the knowledge that the more characterized envelope stress responses have extensive regulons, suggests that there are other BaeR regulated genes waiting to be identified.

The Phage-Shock-Protein (Psp) Response

The Phage shock protein response was first discovered by Peter Model's group in *E. coli.*[127] The consensus within the field is that this unique pathway is upregulated in response to dissipation of the proton-motive force and the mislocalization of secretin proteins. In the following sections I

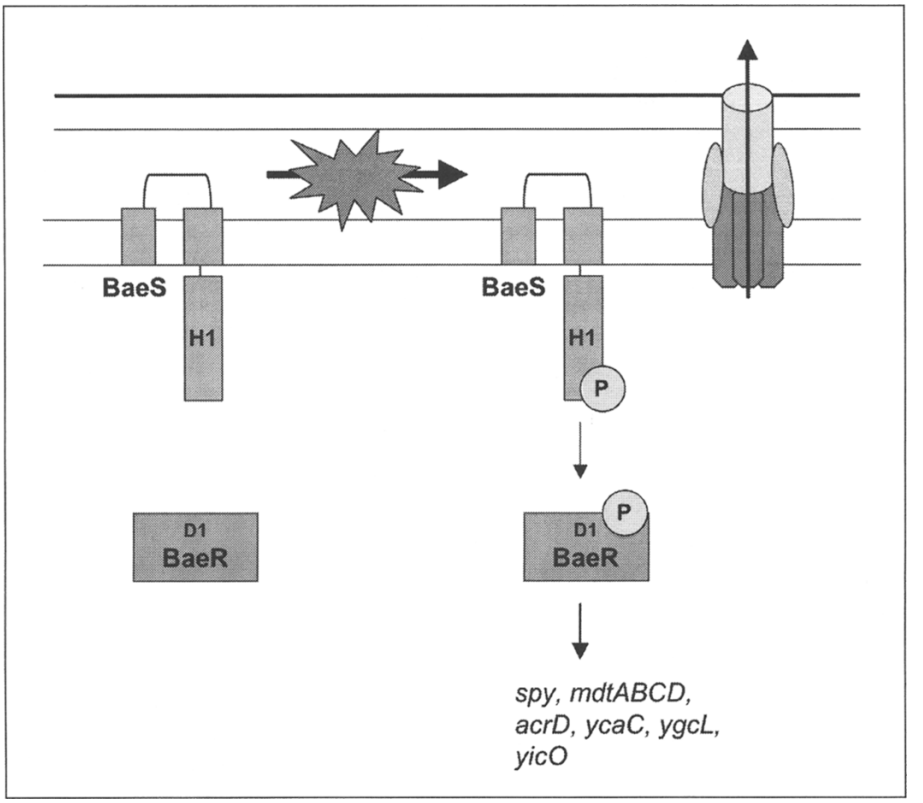

Figure 3. The BaeSR two-component system. Inducing cues such as general extracytoplasmic stresses and the presence of toxic compounds are sensed by the histidine sensor kinase, BaeS. This initiates autophosphorylation and subsequent phosphoryl transfer from BaeS to the cytoplasmic response regulator BaeR. BaeR~P functions as a transcriptional regulator activating expression of genes involved in active efflux and unknown functions. H1= conserved histidine, D1= conserved aspartate

will discuss the working model for Psp signal transduction with emphasis on its predicted activating signals.

Discovery of the Phage Shock Response

The first phage shock protein (PspA) was identified during an examination of cellular proteins from *E. coli* cells infected with filamentous phage.[127] Researchers determined that the elevated level of PspA found in infected cells was a direct result of synthesis of the integral membrane protein encoded by phage gene IV. The phage gene IV protein (pIV) is required at high levels during infection to facilitate virus assembly.[128] Significant amounts of the mature form of this secretin protein become mislocalized during an infection, as the pilot protein that normally targets the secretin to the outer membrane is not encoded on the phage.[128] It is not clear why mislocalized secretin protein specifically induces PspA expression; however, generalized perturbation of the inner and outer membranes was ruled out as the signal.[127] Previous work with λ bacteriophage showed that many of the *E. coli* proteins synthesized during an infection are also induced by heat shock.[129] While exposure to extreme heat shock was found to increase PspA expression, it is not a classic heat shock protein as its expression is not regulated by σ^{32}.[127] Other extracytoplasmic stresses shown to induce PspA expression include exposure to ethanol and hyperosmotic shock.[127]

The observation that Psp expression is induced upon exposure to these general environmental stresses led researchers to categorize *pspA* as a stress responsive gene, in the same class as σ^E and σ^{32} regulated genes.[130]

Working Model of Psp Signal Transduction

Examination of the newly identified locus encoding *pspA* in *E. coli* uncovered an operon encoding five genes, *pspA, B, C, D* and *E.*[131] Characterization of these genes revealed that *pspA* encodes a negative regulator of the *psp* operon, while the *pspB* and *pspC* gene products work together to activate *psp* expression.[130] The *pspE* gene is transcribed as part of the operon under inducing conditions and from its own promoter under non-inducing conditions.[131] For more than a decade, *pspD* was classified as a hypothetical gene.[131] Recently, it was demonstrated that *pspD* is in fact expressed in vivo and that it localizes to the inner membrane.[132] No function has been assigned to either PspD or PspE.

The entire *psp* operon is subject to positive regulation by σ^{54}, with a subset of inducers requiring the presence of IHF for full activation.[130,133] Constitutive activation of the *psp* operon in the absence of the negative regulator PspA and positive regulators PspB and PspC suggested the involvement of an additional positive regulator present in the cell under non-inducing conditions.[130] Transposon mutagenesis on a strain lacking the *pspA, B* and *C* genes led to the identification of the σ^{54} transcriptional activator, PspF.[134] PspF belongs to the enhancer-binding family of proteins and is encoded immediately upstream of the *psp* operon in the opposite orientation.[134] In the absence of stress, PspA interacts with the ATPase domain of PspF and inhibits σ^{54}-mediated transcription.[135,136] Interaction between PspF and PspA has been demonstrated in vivo using the bacterial two-hybrid system.[137] Elderkin et al[137] went as far as to identify the exact residue in PspF that is required for PspA binding and subsequent inhibition.

PspA fractionates with both inner membrane and cytoplasmic proteins, suggesting it can exist as both a peripheral inner membrane protein and as a soluble protein.[131,138] Researchers investigating the ability of PspA to interact specifically with other phage shock proteins were able to crosslink PspA to PspB and PspC.[132] Interestingly, the interaction between PspA and PspB could only be demonstrated if the entire *psp* operon was overexpressed suggesting another Psp protein is needed. Based on the essentiality of *pspB* and *pspC* for the induction of the *E. coli* Psp response by certain inducers, it has been proposed that PspB and PspC interact upon presentation of an activating signal and that this interaction promotes binding to PspA.[130,139] While an interaction between PspB and PspC has not been demonstrated in *E. coli*, it has been shown for the *Yersinia enterocolitica* Psp response[140] (Fig. 4).

Protein-protein interactions appear to be the main mechanism by which Psp signal transduction is achieved. More work is required to determine the dynamics of these interactions under inducing and non-inducing conditions. Many questions remain concerning what takes place upon presentation of an inducing cue. As such, several aspects of the working model for Psp signal transduction are speculative and require further investigation (Fig. 4).

Determining Functionality by Means of Activating Signals

As mentioned above, the original inducers of PspA expression identified were the mislocalization of a phage encoded secretin, pIV and several general extracytoplasmic stresses.[127] Shortly after the Psp response was identified, two separate groups studying protein export in *E. coli* isolated transport defective mutants capable of inducing PspA synthesis.[138,141] Mutation to the signal sequence of the outer membrane porin protein, LamB, causes aberrant localization of the protein. It was hypothesized that elevated levels of PspA functioned to counteract the deleterious effects of this toxic mutation. The authors tested the relevant phenotypes in a Δ*pspA-C::kan* background and found that this was not the case.[141] A separate study found that a collection of precursor PhoE mutants, for which normal biogenesis of this outer membrane protein is defective, activated PspA synthesis in a manner that was dependent on the Sec export system.[138] The requirement of the general secretory pathway for Psp induction suggested that entrance into the export system was generating the activating signal. Work showing that PspA synthesis was activated by addition of

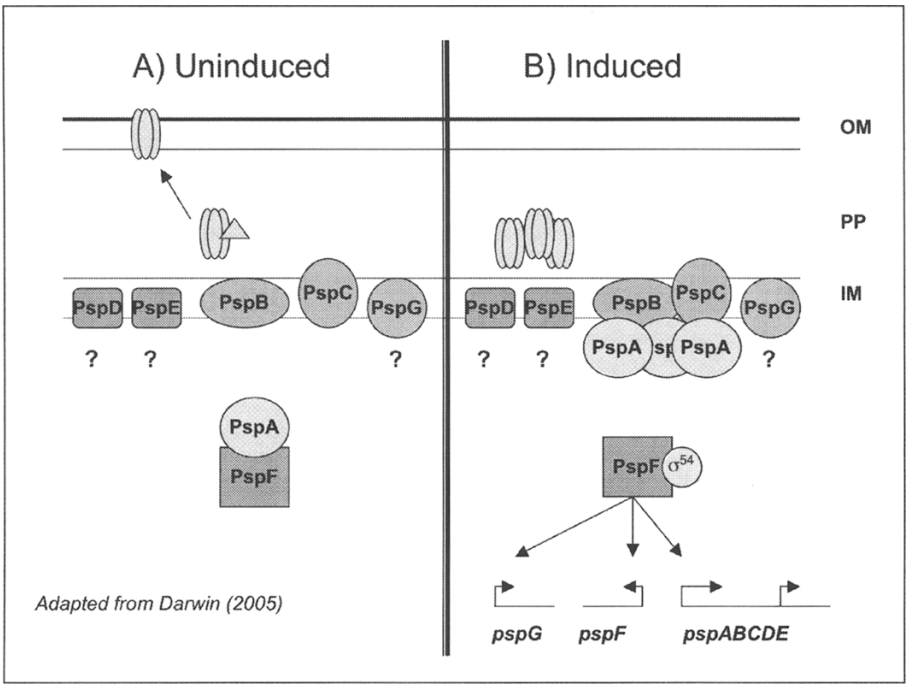

Figure 4. Proposed model for the *E. coli* Phage shock protein response. A) Under normal conditions PspA binds PspF in the cytoplasm and inhibits σ^{54} mediated transcription of the Psp regulon. B) Inducing cues such as mislocalization of secretins or mutations that reduce membrane potential are sensed by PspB and/or PspC in the inner membrane. Interaction between PspB and PspC somehow enables or promotes PspA binding to PspB/C and the subsequent release of PspF. Free PspF initiates *psp* transcription. PspA accumulates at the cytoplasmic face of the inner membrane where it is thought to maintain the PMF. The functions of Psp regulon members PspG, PspD and PapE are unknown.

the proton ionophore, carbonylcyanide chorophenylhydrazone (CCCP),[142] led researchers to investigate the role of PspA in proton motive force (PMF) dependent translocation. They found that PspA was not required for efficient prePhoE transport by a PMF-independent mechanism but was required for PMF-dependent Sec transport, providing the first experimental evidence that the Psp system might respond to dissipation of the proton motive force.[139]

In line with the above observations, the Psp response has also been shown to respond to defects in the Sec secretion system and in YidC, a protein believed to be involved in clearing the Sec translocation channel.[143] YidC depletion was only found to affect secretion when Sec-dependent proteins were overexpressed.[143] This suggests that the Psp response is not required for efficient secretion unless the translocation apparatus becomes saturated. In support of this, PspA has been shown to enhance the transport efficiency of proteins targeted to the periplasm via the Tat pathway when the Tat system becomes saturated, but is not needed for normal Tat pathway function.[144] In both cases it has been proposed that saturation of the translocation apparatus somehow modulates the PMF, which in turn activates the Psp response. While direct activation of the Psp response by PMF dissipation has not been demonstrated experimentally, there are examples where mutations causing a decrease in membrane potential have detrimental effects in the absence of *pspA*.[145,146] It is not clear whether the Psp response is responding specifically to a reduction in membrane potential or simply sensing downstream effects of PMF dissipation.

A physiological role for the Phage shock protein response has been demonstrated in organisms other than *E. coli*. In *Salmonella enterica* serovar Typhimurium, Δ*rpoE* cells in stationary phase growth have elevated PspA levels and an *rpoE pspA* double mutant has reduced survival in stationary phase compared to a single mutant.[145] A decrease in PMF in the double mutant suggests that both genes are involved in PMF maintenance and that PspA can compensate for σ^E.[145] In *Yersinia enterocolitica* a *pspC* mutant is severely attenuated for survival in an animal model, due to YscC secretin production.[147,148] YscC forms the outer membrane channel through which type three secretion substrates are translocated. The Psp response is activated during assembly of a functional type three secretion system in this organism only when YscC is expressed.[148] Thus, in conjunction with, or as a result of, its role in monitoring membrane potential and secretin mislocalization the Psp response facilitates Gram-negative pathogenesis.

Transcriptional profiling of *E. coli* cells overexpressing the main effectors of the Psp response, PspA and the newest member of the pathway PspG,[149] revealed that upon induction of the Psp response cells adjust respiratory gene expression and reduce the expression of high energy, PMF consuming, processes such as motility.[150] This supports a model whereby the Phage shock protein response senses a variety of envelope stresses and responds by activating expression of PspA and PspG, which function to facilitate protein transport and restore membrane potential by down-regulating energetically costly cellular events.

The Rcs Phosphorelay System

The Rcs pathway was first identified during a screen for genes that regulate *E. coli* colanic acid capsular biosynthesis in the mid-1980s.[151] To date, this complex phosphorelay system has been shown to regulate genes associated with envelope integrity and structure and is believed to play a pivotal role in biofilm maturity and enteric pathogenesis. In the following sections we will review what is currently known about the Rcs pathway while clearly demonstrating why it has recently been classified as an extracytoplasmic stress response system.

Discovery and Early History of the Rcs Pathway

The genes regulating capsular biosynthesis in *E. coli* K-12 are encoded by the *cps* operon and are expressed at relatively low levels under standard laboratory conditions. The observation that a mutation in the *lon* locus of *E. coli* results in the overproduction of the colanic acid capsular polysaccharide sent researchers on the hunt for genes involved in the biosynthesis and regulation of this cellular structure. Trisler and Gottesman[152] used insertional mutagenesis in an effort to identify genes responsible for the mucoidy phenotype seen in *lon*⁻ cells. In addition to identifying the six genes encoded in the *cps* operon, they also demonstrated that it is regulated by the ATP-dependent protease, Lon, at the transcriptional level.[152] One year later, the same group identified and characterized three new regulatory genes that affected the expression of several *cps::lac* fusions.[151] The genes were named *rcsA, B* and *C* (for regulator of capsular synthesis), where RcsA and RcsB were classified as positive regulators of *cps* and RcsC was classified (with Lon) as a negative regulator of *cps* expression.[151]

Genetic analysis of *rcsA* in the presence and absence of *lon* revealed that RcsA is targeted for proteolytic degradation by Lon and is a limiting factor in *cps* expression.[153] It was soon demonstrated that RcsB and RcsC can drive *cps* expression independent of RcsA revealing two modes of *cps* regulation.[154] Sequence homology of RcsB and RcsC to environmentally responsive two-component regulators led to the model whereby RcsC functions as a membrane bound sensor kinase and RcsB as a cytoplasmic response regulator.[155] Over the next decade researchers discovered the Rcs pathway was not a typical two-component system dedicated to capsule synthesis, but a unique phosphorelay encompassing numerous accessory proteins and an extensive regulon.

Rcs Phosphorelay Involves Auxiliary Regulators and Accessory Proteins

The RcsC sensor kinase is unique in that it possesses an additional domain at its C-terminal end that shares homology to the N-terminal receiver domain of a response regulator.[155] It was speculated that this RcsC D1 domain facilitated RcsB D2 phosphorylation by mediating transfer

of the phosphoryl group from the H1 domain of RcsC.[155] Further investigation into the proposed Rcs His-Asp-Asp phosphorelay led to the discovery of a unique phosphotransmitter encoded downstream of *rcsB*, YojN.[156] YojN, later renamed RcsD, contains an Hpt (histidine containing phosphotransfer) domain in its C-terminus that is required to transmit signals between RcsC and RcsB.[156] RcsD is a predicted integral membrane protein with a large periplasmic domain that shares limited homology to that of RcsC.[156,157] It has been suggested that RcsD receives signals independent of RcsC; however, no such signal has been uncovered.[12] It is now generally accepted that the RcsC-RcsD-RcsB pathway is a multi-step His-Asp-His-Asp phosphorelay concluding with RcsB~P binding DNA and altering transcription of target genes (Fig. 5).

RcsB is a member of the FixJ subfamily of response regulators and has been shown to function alone or in concert with auxiliary regulators. When acting alone, RcsB binds as a homodimer immediately upstream of the −35 region of the RNA polymerase binding site.[158,159] At a subset of promoters RcsB binds as a heterodimer with RcsA, at a conserved core sequence referred to as the RcsAB box (located 50-100 nucleotides upstream of the transcriptional start site).[160,161] RcsB and RcsA both contain a LuxR-type C-terminal DNA binding motif and function as positive regulators

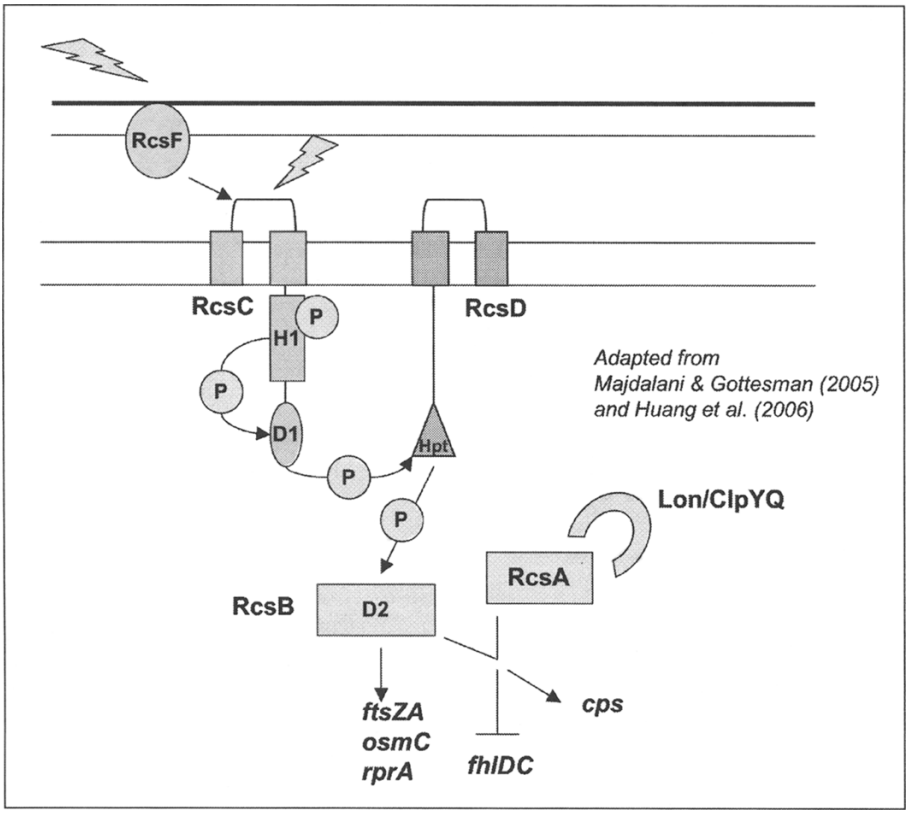

Figure 5. Proposed model for the *E. coli* Rcs phosphorelay system. Activating signals enter the Rcs pathway at the sensor kinase RcsC or at the outer membrane lipoprotein RcsF. RcsC autophosphorylates at an H1 domain and transfers the phosphate to a D1 domain in its C-terminus. The phosphate is then transferred to the Hpt domain of RcsD and finally to the D2 domain in the N-terminal end of the response regulator RcsB. RcsB~P works alone or in concert with RcsA to regulate transcription of the Rcs regulon. RcsA is an unstable protein that is proteolytically degraded by Lon and ClpYQ.

of colanic acid synthesis and *rcsA* autoregulation.[151,162] RcsA stability is controlled by the proteases Lon and ClpYQ.[153,163] RcsA-RcsB heterodimerization is not required for RcsB recognition of the RcsAB box, but significantly stabilizes the RcsB/DNA interaction.[164] It has been proposed that RcsB binding alone at these promoters allows for basal levels of activity, while the presence of the co-inducer RcsA enhances transcription in response to an inducing cue.[164] Another example of this type of cooperative action involves the TviA protein of Vi antigen synthesis in *Salmonella typhi.*[165,166] It is thought that the use of an auxiliary regulator to modulate RcsB activity allows the pathway to differentially control expression of regulon members in response to varied activating cues.

In addition to auxiliary regulators the Rcs phosphorelay also utilizes accessory proteins in order to carry out signal transduction. RcsF was first identified for its ability to activate exopolysaccharide synthesis when overproduced on a multi-copy plasmid.[167] Due to the loss of this function in an *rcsB* mutant, the authors proposed that RcsF was involved in RcsB phosphorylation. It was later determined through epitasis experiments that RcsF is positioned upstream of RcsC in the signaling cascade and that it is involved in signal transduction rather than direct phosphorylation.[168] Evidence showing that RcsF is an outer membrane lipoprotein orientated towards the periplasm supports a model whereby RcsF senses surface cues and transmits the signal to RcsC[168,169] (Fig. 5). It should be noted that not all Rcs inducing cues require RcsF. Overproduction of the DnaJ-like protein, DjlA, activates the Rcs phosphorelay independent of RcsF, suggesting there are two distinct signaling pathways towards RcsC.[169] The use of an outer membrane lipoprotein to transmit signals to a sensor kinase is not unique to this system. As mentioned earlier in this chapter, NlpE transmits a signal to the Cpx pathway upon adherence to hydrophobic surfaces.[7] It appears that lipoproteins may play a vital role in signal transduction.

Another accessory protein shown to influence the pathway is the putative inner membrane protein IgaA of *Salmonella enterica.* IgaA negatively regulates the RcsC-RcsD-RcsB system upon host colonization thereby facilitating *Salmonella* virulence.[170,171] This topic will be discussed further in a later section.

Activating Signals

A commonality among the mutations and general environmental stresses known to activate the Rcs phosphorelay is an association with outer membrane structures and integrity. The majority of the mutations shown to activate Rcs, or the expression of Rcs-regulated genes, are involved in the production of cellular or extracellular polysaccharides. They include *rfa, mdo, tol, pmr* and *surA.* Collectively these genes play major roles in LPS biosynthesis (*rfa*), membrane derived oligosaccharide production (*mdo*), colanic acid synthesis (*tol, pmr*) and proper folding of envelope proteins (*surA*).[169,172-176] In addition to these mutations, the Rcs pathway is also activated by overproduction of certain envelope proteins. As previously mentioned, overproduction of accessory protein RcsF and the membrane anchored chaperone DjlA, activates *cps* expression through RcsC/B.[167,177] The exact signal generated by these proteins is not well understood, however, overproduction of envelope proteins seems to be a common inducer of envelope stress response systems. The environmental conditions shown to influence the Rcs regulon include osmotic shock, dessication, growth on solid media, as well as exposure to the cationic drug chlorpromazine and a subset of β-lactams.[178-183] Each of the aforementioned inducers cause serious stress to the bacterial cell envelope and elicit a response that extends beyond the Rcs pathway. For a more detailed review of this topic see references 11 and 12.

The Rcs Regulon: Determining the Physiological Role of the Pathway

The Rcs regulon further supports the claim that this pathway is somehow involved in monitoring and maintaining cell surface structures. Historically, the Rcs phosphorelay is known to positively regulate colanic acid synthesis in *E. coli* K-12.[151] In line with this finding, the Rcs regulatory proteins RcsB and RcsA have been shown to positively regulate exopolysaccharide production in other organisms, including the plant pathogens *Erwinia amylovora* and *Pantoea stewartii,* as well as the enteric pathogen *Salmonella typhimurim.*[161,184,185] RcsB/RcsA also activate transcription of the *ugd* gene in *Salmonella.*[174,175] While this gene does not encode a component of a capsular

structure it does encode UDP-glucose dehydrogenase, an enzyme required for production of a colanic acid sugar.[186] As previously mentioned, RcsB does not just work in concert with RcsA. In *Salmonella typhi,* RcsB interacts with another positive regulator, TviA, at the *tviA* promoter to initiate Vi antigen production.[165] More coregulation was found between Rcs and the PmrAB two-component system in *Salmonella.*[187] Researchers determined that both systems independently enhance transcription of the *wzz* gene from overlapping regions of its promoter.[187] Wzz is responsible for determining O-antigen chain length in *Salmonella enterica serovar* Typhimurium and so by affecting it expression, these pathways affect susceptibility to host immune responses.[187]

Other positively regulated genes in the Rcs regulon include *ftsA, ftsZ* and *osmC.*[158,188,189] The significance of the Rcs pathway regulating genes involved in cell division and osmoregulation is not clear at this point. A screen for *E. coli* promoters regulated by RcsB uncovered the first example of negative regulation by the Rcs response. Francez-Charlot et al[190] demonstrated that an RcsB-A heterodimer binds to an RcsAB box in the *flhDC* promoter and acts as a negative regulator of flagellin synthesis. A few years later the Dorel group showed that RcsB-A regulates the *csgDEFG* and *csgBA* operons.[191] The negative regulation of flagellin and curlin expression represents a clear overlap between Rcs, the Cpx pathway and the phage-shock-protein response regulons (refer to relevant sections in this chapter). It has also led to the suggestion that the Rcs pathway plays an important role in the transition from early to late biofilm formation, as it down-regulates motility genes and upregulates colanic acid, which functions in biofilm maturity.[11,182,192] The finding that an *rcsB* mutant elaborates premature flagella and an *rcsC* mutant takes longer to display flagella supports this idea.[193] This and other findings also provide support for RcsC phophatase activity.[168,193-196]

It has been demonstrated that the Rcs phosphorelay regulates the stationary phase sigma factor (RpoS) at the translational level.[197] This is accomplished by repressing the synthesis of LrhA, a repressor of the sRNA, RprA, which activates RpoS translation; as well as by directly stimulating RprA activity.[197,198] In addition to affecting RpoS expression, there is overlap between the Rcs and RpoS regulons. One such example is the newly identified periplasmic protein *ydeI.*[157] It was observed that *rcsC, B, D* single mutants were significantly out competed by wild-type cells in a mouse model of infection; *ydeI* was identified as the Rcs-regulated gene responsible for the persistence of *Salmonella* in mice.[157] The authors speculate that *ydeI* somehow increases *Salmonella* resistance to antimicrobial peptides.[157] The Rcs pathway has also been shown to affect pathogenesis in other Gram-negative pathogens. The RcsC-RcsD-RcsB signaling system in enterohaemorrhagic *Escherichia coli* positively and negatively regulates type three secretion by acting through two opposing regulators, GrvA and PchA.[199] In *Yersinia enterocolitica* Biovar 1B, the Rcs phosphorelay positively regulates secretion through the Ysa type three secretion system by affecting expression of genes encoded on the Ysa pathogenicity island.[200]

Since the Rcs phosphorelay seems to play such an integral role in Gram-negative bacterial pathogenesis, it is not surprising that an organism would adapt a way to regulate Rcs activity. As mentioned previously, the IgaA membrane protein of *Salmonella* negatively regulates RcsBC upon host colonization.[170,171] IgaA is only required if the Rcs pathway is active and has proven to be required for *Salmonella* virulence.[170,171] It seems a common feature of envelope stress response systems is to regulate genes important for certain aspects of virulence, but when fully activated, to attenuate virulence.[109,111,201]

In summary, the Rcs regulon suggests that this complex signal transduction pathway is involved in envelope integrity and structure and suggests an important role for the Rcs system in biofilm maturity and Gram-negative pathogenesis.

Acknowledgements

The authors gratefully acknowledge support from the Alberta Heritage Foundation for Medical Research, the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Foundation of Canada.

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