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Envelope stress responses: balancing damage repair and toxicity

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Abstract

The gram-negative envelope is a complex structure, consisting of the inner membrane, periplasm, peptidoglycan, and outer membrane, that protects the cell from the environment. Changing environmental conditions can cause damage, which triggers the envelope stress responses to maintain cellular homeostasis. Here, we review the causes, both environmental and intrinsic, of envelope stress, as well as the cellular stress response pathways that counter these stresses. Furthermore, we discuss the damage to the cell that occurs when these stresses are aberrantly activated either in the absence of stress or to an excessive degree. Finally, we discuss the mechanism through which constant monitoring by a stress response, the σ^E response, prevents cell death from highly toxic unfolded outer membrane proteins. Together, the recent work we discuss has provided insights that emphasize the necessity for proper levels of stress response activation and the extreme consequences that can occur in the absence of proper regulation.

Introduction

The gram-negative envelope is composed of the inner membrane (IM), the periplasm containing a thin layer of peptidoglycan (PG), and the outer membrane (OM)[1]. This envelope provides gram-negative bacteria with a great deal of resistance to environmental insults and toxic molecules including antibiotics. The OM acts as a strong permeability barrier due to its asymmetric organization with lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet. Hydrophilic lateral interactions between LPS molecules bridged by divalent cations and saturated acyl chains make the OM impermeable to both large hydrophilic molecules and hydrophobic molecules[2,3]. OM β-barrel proteins (OMPs) let small hydrophilic molecules, such as nutrients, through the OM[1]. The cell's impermeability is compounded by the presence of efflux pumps that can remove toxic molecules that penetrate the OM[4]. In addition to the barrier represented by the OM, the periplasm contains molecules and proteins involved in protecting the cell from stress, as well as many proteins involved in transport and metabolic functions[1,5]. Furthermore, the PG and OM are load-bearing structures that protect against mechanical and osmotic stress[1,5-7]. The IM serves as a final barrier between the environment and the cytoplasm as well as the location of many important cellular functions[1].

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Due to the importance of the envelope for the maintenance of cellular homeostasis, bacteria have stress responses to respond to damage from the environmental or intrinsic stressors and restore envelope homeostasis. Natural stress is often sudden and transient and cells must respond quickly to survive (Table 1). These stress responses have both overlapping and distinct activating signals and outputs allowing the cell to respond to a wide range of insults (see below). Each envelope stress response is activated through the interaction between sensor proteins and signs of envelope stress. The activation of sensors leads to signaling cascades that cause alterations to gene expression. Generally, these alterations will increase quality control for the stressed component of the envelope while often down regulating the production of the affected envelope component (reviewed in Refs.[8-12]). In addition to these envelope stress responses, some non-envelope stress responses can also alter the envelope (Box 1).

In conditions causing full activation of an envelope stress response, toxic effects are observed. These conditions often result from mutations either within the stress response pathway or in envelope biogenesis pathways. Several recent developments have provided insights to the importance of appropriate levels of stress response activation to avoid damage from excessive activation and to prevent the accumulation of unfolded proteins in the envelope.

In this Review, we provide an overview of the envelope stress response pathways present in *Escherichia coli*, the most extensively studied model system. We discuss the environmental and intrinsic sources of envelope stress that cause their activation. We cover the importance of appropriate levels of stress response activation and the damage caused to the cell by excessive activation. Finally, we discuss how recent insights into stress response activation have highlighted the extreme toxicity of unfolded OMPs.

Sources of Cell Envelope Stress

Environmental changes.

One of the major responsibilities of envelope stress response systems is to protect cells from changing environmental conditions. For instance, a shift to a lower temperature will slow the biosynthesis of envelope components[13], slow the folding of proteins[14], and make the cell's membranes less fluid[15]. By contrast, a shift to high temperature can cause proteins to unfold[16], increase rates of biosynthesis and so overload assembly machinery[17,18], and increase membrane fluidity[15].

In addition to temperature, other changes to the cell's environment, including changing pH, changing osmolarity, and unfavorable redox conditions, also cause stress to the cell envelope. Alterations to pH cause misfolding of proteins as well as disruption of enzymatic reactions. Changing osmolarity can cause rapid uptake or efflux of water from the cell and cause corresponding changes in the physical pressure and tension on the cellular membranes and cell wall, as well as causing changes to solute concentrations[19]. Unfavorable redox conditions, which, among other sources, can be causes by reactive oxygen species, lead to oxidative damage to proteins and membranes, greatly impairing their functions[20]. In addition, many cell envelope proteins, including essential proteins, rely on disulfide bonds to

maintain their structure and alteration of these bonds due to oxidative stress can greatly impair the function of these proteins[20].

An extension of environmental stress leading to envelope damage is stress caused by nutrient limitation. For instance, auxotrophic bacteria may be unable to successfully synthesize portions of their envelope in the absence of key nutrients. Furthermore, micronutrients are essential for synthesis of some envelope components. The large protein complexes of the electron transport chain require numerous iron sulfur clusters and heme groups[21]. Therefore, a lack of environmental iron can cause misfolding of these proteins and disruption of the IM[21]. As another example, a lack of divalent cations in the environment causes disruption of the lateral interactions between LPS molecules in the OM, leading to a loss of OM integrity[22].

Toxic molecules and antibiotics.

In addition to general environmental conditions that disrupt the cell envelope, the environment can also contain toxic molecules capable of damaging the envelope. For instance, exposure to toxic levels of metals (e.g. copper, zinc) damages components of the envelope through oxidative damage or displacement of native metals and leads to stress response activation[23,24]. Furthermore, exposure to antibiotics that either directly target the cell envelope (e.g. inhibit cell wall synthesis[25], OM disruption[26]) or indirectly damage the cell envelope (e.g. protein synthesis inhibitors that cause clogging of the Sec secretion machinery[27]) activates stress responses to attempt to repair the damage caused. Although this exposure can be caused by clinical use of antibiotics, it can also be caused by exposure to organisms that produce antibiotics to increase their competitiveness[28]. In the context of pathogenesis, host barriers to infection can cause envelope damage and stress response activation. Upon host entry, bacteria encounter antimicrobial peptides that disrupt the integrity of both the OM and the IM[29]. In addition, enteric bacteria are exposed to bile salts, which act as detergents and disrupt membranes[30].

Intrinsic sources of stress.

Through the processes of growth and metabolism, the cell can generate toxic molecules and metabolizes (e.g. reactive oxygen species, metabolic intermediates) that are capable of causing damage to the envelope similar to that caused by extrinsic stressors. The effects of these metabolites have been recently reviewed[31]. In addition, translational stress caused by lack of specific amino acids or charged tRNAs or by mutations in the protein translation machinery leads to an increase in mistranslation[32]. The mistranslated polypeptides are likely to misfold and cause unfolded protein stress in the envelope as well as the cytoplasm. When translation stress is exaggerated under laboratory conditions by protein overexpression or by treatment with at least some antibiotics that target translation, it can lead to the mislocalization of cytoplasmic proteins to the envelope[33]. This causes damage to and increased permeability of both the IM and OM[33].

Mutations that cause alterations in envelope biogenesis pathways[18,34-37] can be considered special cases of intrinsic stress and envelope stress responses are often studied in this context. However, this can be misleading because the level of stress caused by mutations are

constant and cells have adapted by the time the experimentalist makes observations. Although it is unlikely that envelope biogenesis pathways evolved to address stress caused by these mutations, they can be used to study the causes of stress response activation as specific alterations to the envelope can be clearly defined in a way that is often not possible with environmental sources of stress, which cause multiple envelope defects.

Overview of Envelope Stress Responses

σ^E Response.

The σ^E system, named for the envelope stress sigma factor (σ^E), responds to the buildup of unfolded OMPs in the periplasm[38,39] as well as to the production of altered forms of LPS[40]. Both decreases in LPS acetylation and the production of deep rough forms of LPS with truncated core polysaccharides have been reported to activate the σ^E response[40,41]. A recent study in *Salmonella enterica* serovar Typhimurium has also demonstrated σ^E activation in response to UVA radiation[42]. It is possible that the sensing of the activating signals differs between the various stresses causing σ^E activation. However, the response of the σ^E system to unfolded OMPs is more clearly understood and perhaps more relevant as activation of σ^E increases expression of σ^H, the heat shock sigma factor[43], underscoring a role in the response to unfolded proteins.

The σ^E response is generated through a proteolytic cascade. When the σ^Esystem is not induced, σ^E is prevented from activating transcription of its target genes by association with an anti-sigma factor, RseA[44] (Figure 1a). RseA, an IM protein, binds directly to σ^E preventing its association with RNA polymerase[45]. To activate the σ^E response, unfolded OMPs in the periplasm bind to an IM protease, DegS, causing a conformational change in DegS that allows it to cleave RseA, removing its periplasmic domain[38]. The cleavage of RseA by DegS can be inhibited by RseB, which binds to the periplasmic domain of RseA[46]. Although the exact mechanism relieving this inhibition in activating conditions remains unclear, it is possible that RseB is displaced through interaction with the acyl chains of LPS[40] or through interactions with unfolded OMPs[46].

In non-activating conditions, a second IM protease, RseP[47], is prevented from cleaving RseA by steric hindrance caused by the periplasmic domain of RseA[48] (Figure 1a). Cleavage of the periplasmic domain of RseA by DegS removes this steric hindrance[48] and allows RseP to carry out a second cleavage of RseA within the transmembrane domain[47], facilitating release of σ^E and a small soluble fragment of RseA into the cytoplasm[49]. The soluble fragment of RseA is removed by cytoplasmic proteases and σ^E is released, allowing it to associate with RNA polymerase and activate its regulon[49].

When the σ^E system is activated, genes are transcribed from σ^E-dependent promoters leading, generally, to the upregulation of OMP folding pathways[50,51]. The regulon includes periplasmic chaperones that maintain OMPs in an unfolded state in the periplasm, members of the β-barrel assembly machine responsible for inserting OMPs into the OM, and periplasmic proteases that degrade misfolded OMPs[50]. In addition, σ^E increases the transcription of two sRNAs, MicA and RybB, responsible for decreasing OMP synthesis[52] and one sRNA, MicL, responsible for decreasing levels of the most abundant lipoprotein

(Lpp)[53]. Together, up regulating the OMP folding apparatuses and quality control factors and decreasing the production of new OMPs leads to the restoration of OMP homeostasis. σ^E also regulates genes involved in LPS biogenesis and transport supporting its role in responding to alterations in LPS structure[50].

Cpx Response.

While the σ^E response is focused on OM stress, the Cpx stress response, named for conjugative plasmid expression[54], seems to be focused on the IM. The unifying signal for Cpx activation is thought to be defects in protein secretion across the IM or to the misfolding of secreted IM and/or periplasmic proteins[10], although the relationship between some Cpx activating stresses and protein misfolding has not yet been determined. Cpx activating stresses include increasing pH[55], changing osmolarity[56], adhesion of the cell to hydrophobic surfaces[57], defects in PG biosynthesis[36,58], exposure to ethanol[59], exposure to copper[60], and changes to phospholipid composition[61], among others[10]. The response to these stresses is transmitted through a canonical two-component system.

CpxA is the sensor histidine kinase of the Cpx two component system and is directly responsible for sensing stress. Based on the structure of CpxA[62] and on mutations in CpxA causing constitutive activity of the Cpx system[63-65], it has been proposed that CpxA can be activated by incorrect folding of its periplasmic sensor domain[10] (Figure 1b). Structural changes in the CpxA sensor domain causes the histidine kinase domain located in the cytoplasm to autophosphorylate[66]. The phosphate group is transferred to the receiver domain of CpxR[66], activating it for transcriptional regulation. As CpxA, like most other kinases, acts as both a kinase and phosphatase, removing CpxA causes activation of CpxR[66]. One of the genes most highly transactivated by CpxR is *cpxP*[67]. CpxP inhibits CpxA activation and acts in a negative feedback mechanism[68]. The transcriptional unit encoding CpxP also encodes an sRNA, CpxQ, which negatively regulates CpxP translation as well as translation of Skp[69,70], a periplasmic chaperone (see section on unfolded OMPs).

It has been known for some time that overexpression of the OM lipoprotein NlpE actives the Cpx stress response[71]. A direct signaling role for NlpE was demonstrated by showing that the protein was required for sensing adhesion to hydrophobic surfaces[57]. Recently, work indicates that the Cpx response plays an important role in combating the stress caused by defects in lipoprotein targeting to the OM. Such defects leave NlpE stuck in the IM where it likely activates CpxA directly (unpublished observations, K.L. May, K.M. Lehman, A.M. Mitchell, and M Grabowicz)[34].

As Cpx is a stress response system that responds to IM stress, one functional result of Cpx activation is the direct or indirect transcriptional repression of the genes for proteins forming non-essential membrane protein complexes[10]. The complexes down regulated include electron transport chain complexes (*nuo, cyo*) and deletion of these operons during membrane stress in a *cpxR* mutant can restore envelope homeostasis[72]. It is likely down regulation of these complexes eases the assembly of essential protein complexes, especially given the increase in chaperone and protease expression caused by Cpx activation[10]. The Cpx response also increases expression of PG modifying proteins, efflux related genes, and

genes related to metal and redox homeostasis[73]. These transcriptional modifications help to restore IM homeostasis.

Rcs Response.

The Rcs, or regulator of capsule synthesis[74], system responds to alterations in LPS charge or fluidity[26,35,37], changes in PG biosynthesis[25], and to defects in lipoprotein trafficking[75]. The system can also be activated by the loss of osmoregulated periplasmic glycans (i.e. membrane-derived oligosaccharides)[37,76]. All of these stresses are signaled through the stress sensor protein RcsF, which is an OM lipoprotein[25,26,37,75]. The response of Rcs is also modulated through effects on the protein stability and folding of one if its response regulators, RcsA (see below).

Unlike the Cpx two-component system, signaling through Rcs is conducted through a more complex phosphorelay (Figure 1c). The sensor protein RcsF is an OM lipoprotein found in a unique conformation where the N-terminal lipidated residue is located in the outer leaflet of the OM and the linker domain is threaded through an OMP, locating the signaling domain of RcsF in the periplasm[77,78]. Recent work has made it clear that this conformation allows RcsF access to sense LPS defects using a series of positively charged residues predicted to interact with the charged residues on LPS[35]. In addition, RcsF threaded through an OMP may be responsible for sensing PG defects. However, it is possible that RcsF senses these defects from the OM but before associating with OMPs[78]. This model has not been thoroughly explored. Rcs signaling in response to lipoprotein trafficking defects are caused by accumulation of RcsF on the IM[75].

In non-activating conditions, Rcs activation is repressed by IgaA, an IM protein that interacts with the histidine kinase, RcsC, or phosphotransferase, RcsD, to prevent RcsD from phosphorylating RcsB, a response regulator[9]. When RcsF receives an activating signal, it is thought to physically associate with IgaA and prevent its repression of signaling[78,79] (Figure 1c). Under these conditions, the histidine kinase, RcsC, can autophosphorylate and then phosphorylate RcsD, which in turn phosphorylates RcsB, activating it for transcriptional regulation[80].

RcsB can regulate transcription either as a homodimer or as a heterodimer with other response regulators, notably RcsA[9]. In contrast to RcsB, RcsA is not regulated by phosphorylation. Instead, it is regulated through proteolysis by the Lon protease[81], through temperature dependent effects on its folding[82], and through transcriptional positive feedback[83]. The most well characterized transcriptional change caused by RcsB homodimers is an upregulation of the RprA sRNA[84]. RprA increases the translation of RpoS[85], the stationary phase sigma factor responsible for cross protection from many stresses[5]. Heterodimers of RcsAB increase expression of genes leading to the production of colanic acid capsule, decrease expression of regulators of flagellar motility, and increase expression of genes involved in a biofilm lifestyle[86-88]. In addition, many members of the Rcs regulon remain genes of unknown function[9]. RcsB has also been reported to form heterodimers with BglJ[89], GadE[90], MatA[91] and DctR[91]; however, these interactions are independent of phosphorylation of RcsB caused by activation of the Rcs response[89-91].

Bae Response.

The Bae stress response, named for bacterial adaptive response[92], can be activated by exposure to toxic molecules including ethanol[59], indole[93], nickel chloride[94], sodium tungstate[94], and zinc[24,95], and by pilin subunit overexposure[93]. The Bae stress response consists of a canonical two-component system, where BaeS is the sensor histidine kinase responsible for phosphorylating BaeR, the response regulator[92] (Figure 1d). When BaeR is phosphorylated, it upregulates a small regulon leading to increases in levels of Spy (a periplasmic chaperone), several efflux pumps, several genes of unknown function, and BaeS and BaeR[96,97]. These effects would make Bae well suited to respond to exposure to toxic molecules from which the cell can be protected by efflux. Indeed, overexpression of BaeR leads to novobiocin and bile salt resistance[97].

Psp Response.

The Psp stress response, named for phage shock protein[98], tends to be activated by extensive disruptions of the IM that result in loss of the proton motive force (PMF)[99-101]. Psp activating IM disruptions tend to be more severe than those required to activate Cpx. These can involve infection by filamentous phage[98], extreme heat shock[98], osmotic shock[98], ethanol exposure[98], organic solvent exposure[102], disruptions of protein secretion[103], and localization of OMPs at the IM[104]. Although it has been thought that Psp is induced directly by changes in the PMF[99-101,105], the inducing signal may be more complicated[106-108]. The Psp transcriptional enhancer, PspF, is prevented from acting in non-inducing conditions through a physical interaction with PspA[109-111] (Figure 1e). The IM proteins PspB and PspC have been proposed to be the sensors of this system[111,112], although at least some signals seem to be directly sensed by PspA[108,113,114]. When they are activated, PspB and PspC bind to PspA releasing PspF[111]. PspF is an enhancer-binding protein that interacts with RNA polymerase containing σ^N (the nitrogen regulation sigma factor) to increase transcription of *pspA, pspB, pspC, pspD, pspE*, and *pspG*[115,116]. In addition to sequestering PspF, PspA acts as an effector in the Psp response by binding to the inner leaflet of the IM and preventing leakage of protons through the membrane[117,118] (Figure 1e). Furthermore, PspB and PspC have been reported to have a direct role in preventing secretin toxicity[100]. PspD and PspG are also considered to be effectors[116,119], although their functions have not been fully characterized. Furthermore, it appears activation of Psp leads to the activation of ArcAB, which represses genes involved in aerobic respiration and increases anaerobic respiration[119].

Extreme Stress Response Activation

RseA deletion is toxic.

The σ^E stress response is essential in *E. coli* and *rpoE* cannot be deleted[120]. However, over-activation of the σ^E stress response is also deleterious. This can be illustrated by the toxicity caused by the deletion of *rseA*, the gene for the anti-sigma factor that represses σ^E's transcriptional activity[121,122]. Deletion of *rseA* causes constitutive full activation of the σ^E system (Figure 2a). Strains lacking RseA are sensitive to compounds that are normally excluded by the OM such as bacitracin, rifampicin, sodium dodecyl sulfate (SDS), and bile salts[123-126]. In addition, these strains lyse within a few days of entry into stationary phase[127].

The death during stationary phase and the sensitivity to chemical insults is caused by defects in OM integrity and can be prevented by addition of divalent cations to increase lateral bridging between LPS molecules[123]. This loss of OM integrity is due in large part to the large decrease in OMP levels caused by the MicA and RybB sRNAs with σ^E activation[123] (Figure 2a). The sensitivity demonstrates that, although the σ^E response is helpful (and in fact essential), too much activation harms the barrier function of the OM and impedes cell growth. These works emphasize that proper levels of response activation are necessary to respond effectively to stress.

PG defects from Cpx over-activation.

Mutations in *cpxA* causing constitutive activation of the Cpx system, known as *cpxA** alleles, cause activation of the Cpx system independent of inducing signals[63-65]. These *cpxA** mutations cause defects in cell division and irregular cell size and shape[128]. This was found to be due to frequent mislocation of the FtsZ ring, and so the site of cell division. The effect is dependent on the strength of the *cpxA** allele, with stronger Cpx induction leading to aberrant cell division at lower temperatures.

More recently, it has been demonstrated that strongly inducing *cpxA** alleles cause growth defects, extending doubling time[129]. In addition, these strains produce both filamented cells and minicells and have defects in cell width. The defective growth, filamentation, and cell width defect could be suppressed by deleting *ldtD*[129], a PG crosslinking enzyme in the Cpx regulon[130]. However, deletion of *ldtD* did not fully suppress the production of minicells[129]. Together, these data demonstrate that, although normal activation of Cpx is helpful in responding to PG stress, too much activation causes defects in PG structure that adversely affect the cell (Figure 2b).

Extreme Rcs activation causes toxicity.

Many studies have demonstrated that RcsF maintained at the inner membrane, through treatment with antibiotics inhibiting lipoprotein processing[35], mutations leading to defects in lipoprotein processing[75,131,132], or mutations to RcsF changing its trafficking[75,133,134], induces constitutive Rcs signaling. An early indication of toxicity occurring due to mislocalization of RcsF at the inner membrane came from investigating the temperature sensitivity of a *pgsA lpp* double mutant[131]. Removal of PgsA is generally lethal[135] due to a lack of phosphatidylglycerol causing a defect in lipoprotein processing[136]. This lethality is suppressed by removing Lpp, a highly abundant OM lipoprotein that is toxic on the IM due to its attachment to PG[136]. However, this strain still undergoes rapid lysis at high temperature (42 °C)[131].

Screening for suppressors of this temperature sensitivity demonstrated viability at high temperature could be restored, without a change in phospholipid levels, by insertions in *rcsF* or by preventing Rcs signaling with deletions in *rcsC* or *rcsD*[131], suggesting that Rcs activation was responsible for the temperature sensitivity. Further work determined that a proportion of RcsF is retained at the IM in a *pgsA lpp* double mutant and that the Rcs signaling and temperature sensitivity could be suppressed by increasing processing of lipoproteins[75] (Figure 2c). A recent study examined the essentiality of components of the

Lol machinery, which transports lipoproteins to the OM[34]. Similar to the results found with *pgsA*, the toxicity caused by depletion of LolB, the OM component of the system, is greatly decreased by deleting *lpp* and *resF* or *rcsB*.

The authors then investigated the members of the Rcs regulon involved in mediating this toxicity[34]. They determined that the toxicity of Rcs activation in the context of LolB depletion was due entirely to the overexpression of *osmB*[34], which encodes a small OM lipoprotein of unknown function[133] (Figure 2c). The reason for the toxicity caused by *osmB* overexpression in the context of a lipoprotein trafficking defect remains unclear, but is thought to be due to IM accumulation of OsmB. Nevertheless, it is evident that aberrant levels of Rcs activation cause toxicity that can, in some cases, be lethal. Interestingly, full activation of Rcs by deletion of *igaA* is also lethal[78,137], although it is not known whether this toxicity involves Lpp or OsmB. Given these examples of toxicity caused by over activation of several different stress responses, caution should be used in interpreting these extreme cases as evidence for, or against, the protectiveness of a stress response in relation to a given stimulus.

Toxicity of Unfolded OMPs

Speed of stress response induction.

Recent studies involving the σ^E response as well as the Cpx response have demonstrated the extreme toxicity of unfolded OMPs. These studies highlight the race between toxicity from the onset of unfolded OMP stress and the induction of stress responses. Several pairs of mutations in OMP biogenesis pathways that cause synthetic lethality or temperature sensitivity have been used to identify mechanisms the cell can employ to respond to the accumulation of unfolded OMPs. The most common pair used is deletion of *bamB*, a non-essential lipoprotein member of the BAM complex that assembles OMPs[138,139], and *degP*, a periplasmic protease that degrades unfolded OMPs[140], which has a synthetic phenotype[17]. Suppressors of this double mutant that restore growth have been found in the EnvZ/OmpR pathway, which responds to osmotic shifts and acid stress[141]. These mutations generally lead to lower levels of OMPs synthesized and so decrease flux through the system. Mutations in the σ^E system that decrease OMP levels have also been found to suppress the Δ*bamB* Δ*degP* double mutant[142]. Loss of function mutations or deletions in *rseA* cause large decreases in OMP levels, while an apparent promoter duplication in the operon encoding *rpoE* and *rseA* caused a slight decrease in OMP levels. Loss of function mutations in *rseA* have also been found to suppress the temperature sensitivity of a strain with deletions in *degP* and *surA*[18], a major periplasmic chaperone involved in the folding of OMPs[143]. In fact, recent work has demonstrated that low level pre-activation of the σ^E stress response due to an activating mutation in DegS can suppress OM defects and lethality caused by an assembly defective OMP as well as synthetic lethality caused by OMP biogenesis mutants[126].

In addition to these mutations that cause pre-activation of the σ^E system, an intriguing point mutation in *rpoE* (RpoE^{S2R}) was found that suppresses the temperature sensitivity of not only the *degP surA* mutant but also two other synthetic lethal pairs in OMP biogenesis[18]. This mutation does not increase basal levels of σ^E activation or alter the function of σ^E. Instead, it increases translation of *rpoE* and transcription of the operon encoding *rpoE* and

rseA. This greatly increases σ^E levels and brings its levels closer to those of RseA. The consequence of this is that the σ^E response is induced more quickly and more strongly in response to unfolded OMP stress[18]. The effectiveness of this suppressor mutation emphasizes the speed with which it is necessary for cells to respond to unfolded OMPs in order to survive. Clearly, these unfolded proteins must have a very rapid and deleterious effect on the cell.

OMP levels are constantly regulated.

The σ^E stress response is essential for viability in *E. coli*[120], *Yersinia enterocolitica* [144], and *Vibrio cholerae*[145] but is non-essential in *Salmonella enterica* serovar Typhiumurium[146], *Haemophilus influenzae*[147], or *Pseudomonas aeruginosa*[148]. Recent work has suggested that the presence of O-antigen on LPS strengthens the OM[6] and that this may be enough to make σ^E non-essential in *Salmonella enterica*[42]. Whether the presence of O-antigen affects σ^E essentiality in *E. coli* remains unclear. However, insight into the reason for σ^E essentiality in *E. coli*, and perhaps the reason why strengthening the OM is necessary in the absence of σ^E, has recently been gained.

In searching for inhibitors of OMP biogenesis, batimastat, a metalloprotease inhibitor[149], was identified as an inhibitor of RseP's protease activity that causes decreased σ^E activity and ultimately death in *E. coli*[150]. When examining the effect of batimastat on the cell, the authors determined that treatment with batimastat caused the accumulation of unfolded OMPs even in the absence of other stress. This accumulation occurred despite the presence of a normal amount of DegP and BamA, an essential member of the Bam complex[150]. These data suggest that even under normal conditions, OMPs are falling off the assembly pathway and that the σ^E response is constantly monitoring and adjusting the rate of OMP production in order to prevent toxicity from these unfolded OMPs. To further support this conclusion, the authors note that batimastat killing is synergistic with mutations in the OMP biogenesis pathway and that deletion of the genes for two abundant OMPs (OmpA and OmpC) confers partial resistance to batimastat[150]. Interestingly, the essentiality of σ^E in *Vibrio cholerae* is suppressed by reducing expression of its major porin, OmpU, suggesting constant monitoring of OMP folding is also necessary in *Vibrio cholerae*[145]. The necessity of constant monitoring of OMP biogenesis and the death that occurs when this monitoring is prevented emphasizes the toxicity of these molecules when they are in an unfolded state.

Unfolded OMPs may disrupt PMF.

Insights into the cause of the toxicity of unfolded OMPs have been gained through investigation of the interaction between a lethal mutation causing LamB (an OMP) to be tethered to the IM and the Cpx system. The mutation in LamB ($LamB^{A23D}$) causes a defect in signal sequence cleavage and severe toxicity to the IM indicated by activation of the Psp stress response[104]. The lethality of this mutant was found to be dependent on the folding competence of LamB. Nevertheless, the toxicity of this mutant can be suppressed by constitutive activation of the Cpx system using CpxA* mutations[63]. Cpx is activated by $LamB^{A23D}$ without CpxA*; however, native levels of Cpx activation are not sufficient for survival. However, until recently, the mechanism of suppression remained unclear.

Work examining the function of an sRNA produced at high abundance by the Cpx system, CpxQ, found that this sRNA is necessary for the suppression of $LamB^{A23D}$ by CpxA* mutations[70]. One of the targets of CpxQ is Skp[69], a periplasmic chaperone that is competent for OMP membrane insertion[151]. Expression of CpxQ causes decreased levels of Skp protein[69,70]. It is, in fact, the decrease in Skp levels that is necessary for the suppression of $LamB^{A23D}$ by CpxA* mutations[70].

These studies combined with the activation of the Psp stress response by $LamB^{A23D}$ suggest a compelling model for the toxicity caused by unfolded OMPs (Figure 3). Folding competent chaperones like Skp seem likely to be allowing unfolded OMPs, which have failed to properly assemble into the OM, to aberrantly fold into the IM. The β-barrel structure of OMPs is not found in the inner membrane because they represent open pores that allow the diffusion of water, ions, and hydrophilic molecules up to 600 Da[152]. The presence of these open pores in the IM would quickly disrupt the PMF and the essential functions of the IM and cause cell death.

Conclusions and Outlook

Bacteria such as *Escherichia coli* have evolved sophisticated mechanisms to protect themselves from the challenging, often hostile, environments that they inhabit. Naively, one may have thought that to maximize protection the cell would have these defense systems full on at all times. Clearly, that is not the case. Not only would this deplete critical cellular resources, but it has also become apparent that aberrant activation of many of these pathways is detrimental to the cell. For one of the stress responses, Rcs, it is clear that the reason for toxicity is the overexpression of *osmB;* however, the function of OsmB remains unclear. For the other stress responses, it has been clarified that the same functions that are normally helpful cause toxicity when present at too high a level and/or in the absence of stress. This paradoxical result could be thought of as analogous to the immune system that normally protects humans from bacterial infections causing death during sepsis.

It is also clear that the envelope stress responses are not off all the time either. This is especially clear for the σ^E stress response; turning this response off is lethal for *Escherichia coli*. In this case, it is clear that OMPs are falling off the assembly pathway all the time, even under normal, unstressed conditions. It seems likely that normal metabolism and normal cell growth cause intrinsic stress that must be combatted and that this is handled by low-level expression of all stress responses. Turning these responses off is not necessarily lethal as it is for the σ^E stress response, but it likely has detrimental consequences in all cases.

Since all of the stress responses must be induced, then clearly highly sensitive detectors are required because the onset of stress begins an existential race between the accumulating damage caused by that stress and the ability of the cell to respond and repair the injury and destruction. How misfolded OMPs are detected by the σ^E stress response has been worked out in detail. However, for the other stress responses the true nature of the inducing signal and how it is sensed are not yet clear. An understanding of the inducing signal would also allow more detailed analysis of induction kinetics. Further investigation of these open

questions will lead to intriguing insights into the interaction between the environment and the envelope.

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Glossary

Outer membrane protein (OMP)
Integral membrane proteins of the OM with a β-barrel structure

β-barrel assembly machine (BAM)
OM protein complex which inserts OMPs into the OM

Efflux pump
PMF or ATP driven transporter that transports toxic molecules out of the cell

MicA
A small RNA that decreases the translation of some OMPs as well as other targets

RybB
A small RNA that decreases the translation of some OMPs as well as other targets

Lpp
A highly abundant OM lipoprotein which cross-links the OM to the PG

Two-component system
A signaling system consisting of an IM sensor histidine kinase which phosphorylate a response regulator that acts as a transcriptional control factor

Regulon
Genes for which a regulator controls transcription

BglJ
A transcriptional regulator possibly involved in the utilization of β-glucosides

GadE
A transcriptional regulator that controls genes related pH homeostasis and efflux

MatA
A transcription factor involved in the switch between a planktonic and an adhered lifestyle

DctR
A transcriptional regulator with poorly defined function

Proton motive force (PMF)
The buildup of protons in the periplasm generated by the electron transport change used to generate ATP as well as directly drive some transport processes

σ^N
A sigma factor involved in controlling the expression of nitrogen-regulated and nitrogen-related genes

Bacitracin
An antibiotic targeting PG biosynthesis by preventing recycling of the isoprenoid lipid carrier used to assemble PG monomers

Rifampicin
An antibiotic targeting transcriptional elongation

Bile salts
Molecules produced as part of bile that act as detergents and help the nutritional absorption of lipids

LdtD
A L,D-transpeptidase that catalyzes DAP^3-DAP^3 cross-links in peptidoglycan

FtsZ ring
Assembly of a ring of FtsZ protein which represents the earliest characterized step in cell division and determines the location of septum

PgsA
Phosphatidylglycerophosphate synthase catalyzing the first committed step in the biosynthesis of acidic phospholipids

Batimastat
A metalloprotease inhibitor which can inhibit activity of RseP

Skp
A periplasmic chaperone that helps prevent the misfolding and aggregation of newly synthesized OMPs

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Display Items

Envelope changes from non-envelope stress responses

Traditionally, envelope stress responses are considered to be stress responses induced by damage to the cell envelope, which respond by altering the processes of envelope biogenesis to combat these stresses. However, stress responses that respond to other signals (e.g. nutritional conditions, environmental stressors) can also change the envelope to aid their response to changing conditions. Some examples of these responses are given below.

- The PhoQP two-component system is responsible for responding to limiting environmental levels of divalent cations[153,154], although other activating signals have been discribed[155-157]. Among its regulon members, some of which are indirect, are genes for LPS-modifying proteins, OMPs, and OM lipoproteins[158-160]. These gene expression changes lead to stabilization of the OM despite the lack of divalent cations to bridge interactions between LPS molecules.
- The EnvZ/OmpR two-component system responds to changes in environmental osmolarity. When osmolarity increases, OmpR changes the ratio of two major OMP porins[161].
- σ^S is the master regulator of stationary phase in γ-Protobacteria. When σ^s levels are increased due to nutrient limitation or another stress, gene expression changes lead to a state of cross-protection from many stresses[5]. Changes occurring to the envelope include increasing levels of periplasmic stress response factors, increasing levels of peptidoglycan, and reducing membrane fluidity[5]. Moreover, we have recently demonstrated that the permeability barrier of the OM is strengthened during stationary phase in an σ^S-dependent manner[162].
- σ^N is the sigma factor responsible transcription of nitrogen-related promoters[163]. Although this is a nutritional response, the σ^E and Psp stress responses both have σ^N-dependent promoters[41,115,116]. In fact, the Psp stress response relies on σ^N for its transcriptional regulation[115,116].

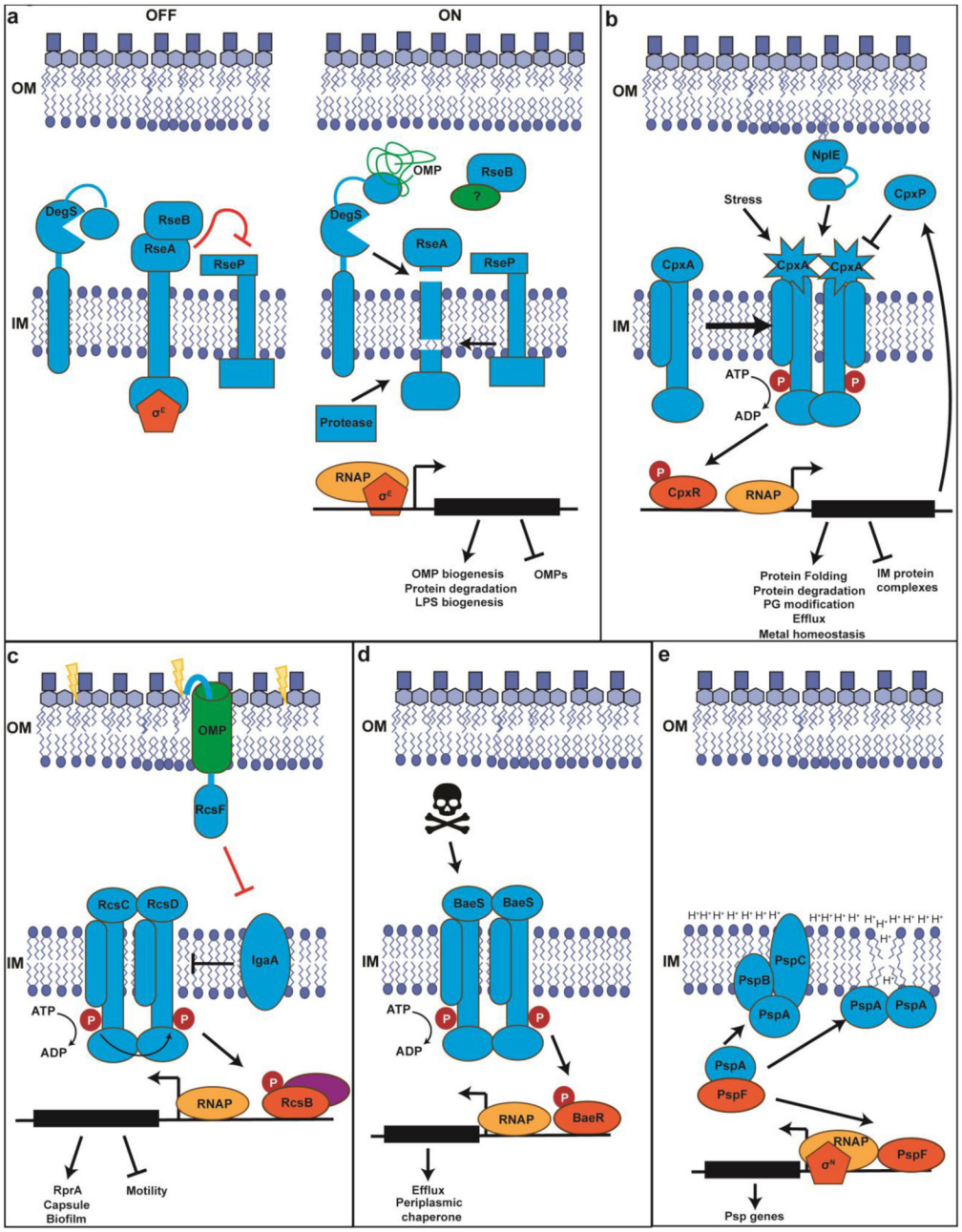

FIG. 1: Overview of envelope stress responses.

(a) The σ^E stress response begins when DegS binds to unfolded OMPs in the periplasm, activating DegS to cleave RseA. Through a series of proteolytic events, σ^E is released into the cytoplasm where it can bind to RNA polymerase (RNAP) and induce its regulon. **(b)** The Cpx stress response is induced by signals including IM protein folding stress and NlpE-dependent signals, causing the autophosphorylation of CpxA. CpxA then phosphorylates CpxR, the response regulator, activating it for transcriptional regulation. **(c)** The Rcs sensor protein, RcsF, senses stresses such as LPS or PG defects and interacts with IgaA to remove inhibition of RcsC, the histidine kinase, and RcsD, the phosphotransferase. RcsC autophosphorylates and then transfers the phosphorylation to RcsD which transfers it to

RcsB, a response regulator. RcsB alone or as a dimer with RcsA acts to regulate transcription of the Rcs regulon. **(d)** The Bae system is activated by exposure to toxic molecules inducing the autophosphorylation of the sensor histidine kinase BaeS. BaeS transfers the phosphorylation to the response regulator BaeR to activate it. **(e)** The Psp system response to severe damage to the IM. This causes PspBC to interact with PspA, allowing PspF to interact with σ^N containing RNA polymerase for transcriptional regulation. PspA also acts as effector by directly stabilizing the IM. See the text for more detail.

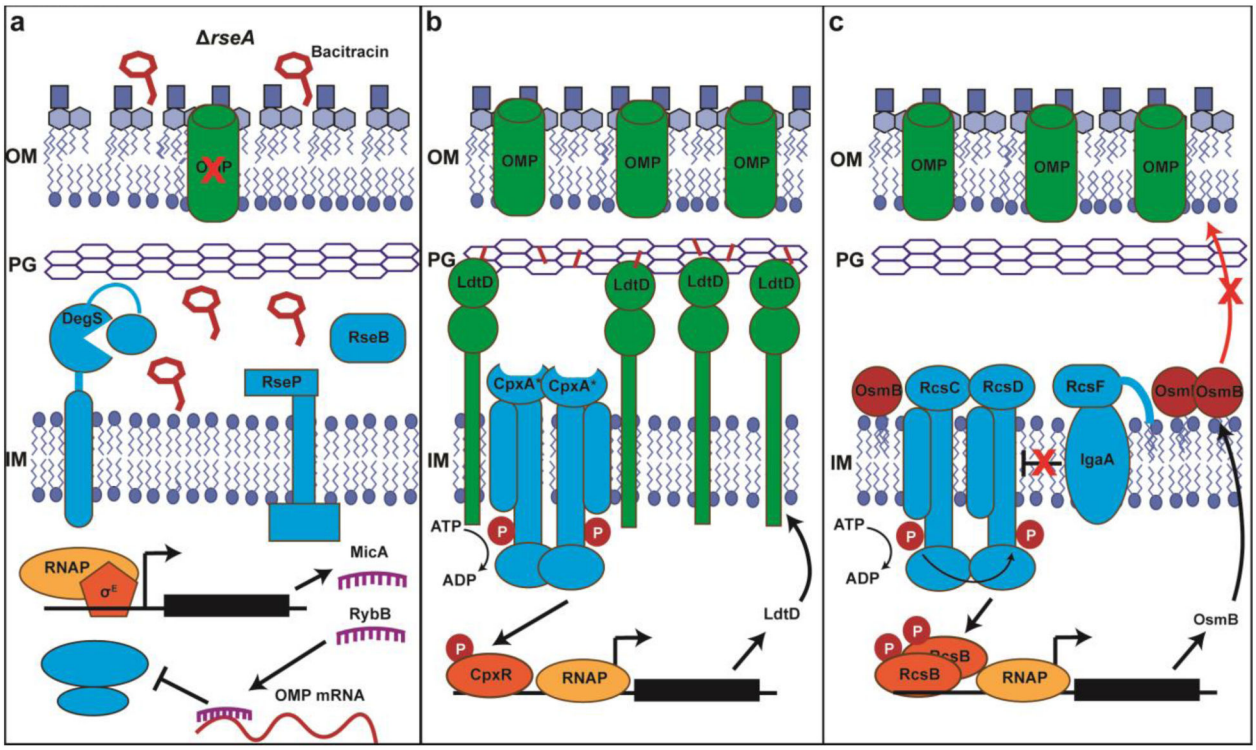

FIG. 2: Constitutive activation of stress responses is harmful.

(a) Deletion of *rseA* causes constitutive activation of the σ^E system and production of high levels of the sRNAs, MicA and RybB. These sRNA down regulate the transcription of OMPs. The loss of OMPs leads to permeability of the OM to antibiotics such as bacitracin, as well as a stationary phase survival defect. **(b)** CpxA* alleles cause constitutive activation of the Cpx system, presumably due to misfolding of the periplasmic domain of CpxA. LdtD, peptidoglycan-modifying enzyme and a member of the Cpx regulon, is produced at high levels, leading to excessive PG cross-linking. This cross-linking leads to growth, cell size, and cell division defects. **(c)** RcsF retained at the inner membrane interacts with IgaA to cause constitutive activation of the Rcs system. This leads to high-level production of OsmB, an inner membrane protein of unknown function. IM mislocalized OsmB is responsible for toxicity of constitutive Rcs activation.

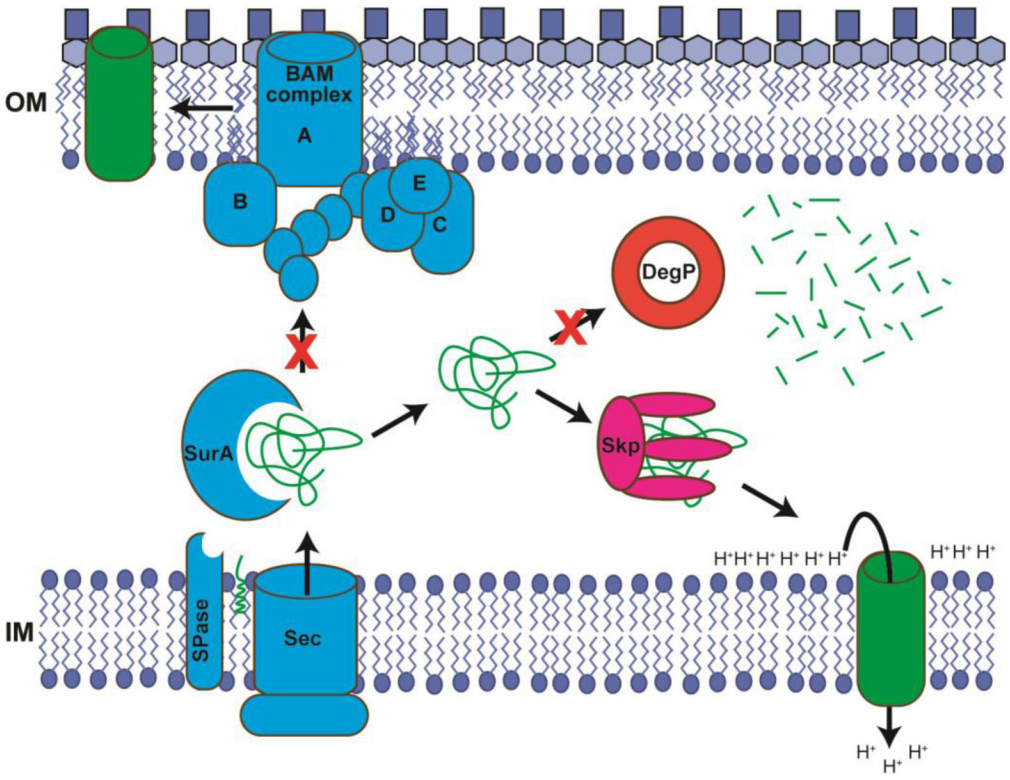

FIG. 3: Toxicity of unfolded OMPs.

After secretion through the SEC complex and cleavage of the signal sequence by signal peptidase (SPase), unfolded OMPs are maintained in an unfolded state in the periplasm by chaperones such as SurA. Then, the OMPs are assembled into the OM by the BAM complex. Unfolded OMPs are degraded in the periplasm by the periplasmic protease DegP. If both of these processes are blocked, unfolded OMPs can build up in the periplasm. Another periplasmic chaperone, Skp, mediates the insertion of the unfolded OMPs into the IM, creating open channels, dissipating the PMF, and causing cell death.

Table 1:

Examples of cell envelope stresses and effects

Category	Stress	Envelope Effect	References[a]
Environmental Stress	Decreased temperature	↓biosynthesis rates ↓ protein folding rate ↓membrane fluidity	13-15
	Increased temperature	Protein unfolding Stress on biogenesis pathways ↑membrane fluidity	15-18
	Changed pH	Protein misfolding Disruption of reactions	10,12
	Decreased osmolarity	Water uptake ↓ solute concentration ↑ membrane tension	19
	Redox stress	Oxidative damage to proteins Oxidative damage to membranes Protein misfolding	20
	Nutrient limitation	Prevention of biosynthesis	21,22
Toxic Compounds/Antibiotics	Metals	Oxidative damage Displacement of native metals	23,24
	Envelope targeting antibiotics	Disruption of target structure (e.g. PG)	25,26
	Translation elongation inhibiting antibiotics	Disruption of SEC-mediated secretion	27
	Antimicrobial peptides	Membrane disruption	29
	Bile salts	Membrane disruption	30
Intrinsic Stress	Translation stress	Protein misfolding	32
	Protein overexpression	Secretion defects Membrane disruption	31
	Mutations	Biogenesis alteration	18,33-36

[a]Representative references demonstrating the effects of the indicated stresses