Biochimica et Biophysica Acta 1843 (2014) 1529–1541

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Everything old is new again: An update on current research on the Cpx envelope stress response☆

Tracy L. Raivio

Department of Biological Sciences, Centennial Centre for Interdisciplinary Science, University of Alberta, Edmonton, AB T6G 2E9, Canada

ARTICLE INFO

Article history:
Received 1 August 2013
Received in revised form 16 October 2013
Accepted 21 October 2013
Available online 31 October 2013

Keywords:
Two-component signal transduction
Post-transcriptional regulation

ABSTRACT

The Cpx envelope stress response (ESR) has been linked to proteins that are integrated into and secreted across the inner membrane for several decades. Initial studies of the *cpx* locus linked it to alterations in the protein content of both the inner and outer membrane, together with changes in proton motive driven transport and conjugation. Since the mid 1990s, the predominant view of the Cpx envelope stress response has been that it serves to detect and respond to secreted, misfolded proteins in the periplasm. Recent studies in *Escherichia coli* and other Gram negative organisms highlight a role for the Cpx ESR in specifically responding to perturbations that occur at the inner membrane (IM). It is clear that Cpx adaptation involves a broad suite of changes that encompass many functions in addition to protein folding. Interestingly, recent studies have refocused attention on Cpx-regulated phenotypes that were initially published over 30 years ago, including antibiotic resistance and transport across the IM. In this review I will focus on the insights and models that have arisen from recent studies and that may help explain some of the originally published Cpx phenotypes. Although the molecular nature of the inducing signal for the Cpx ESR remains enigmatic, recently solved structures of signaling proteins are yielding testable models concerning the molecular mechanisms behind signaling. The identification of connections between the Cpx ESR and other stress responses in the cell reveals a complex web of interactions that involves Cpx-regulated expression of other regulators as well as small proteins and sRNAs. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

1. Introduction

The *cpxRA* locus is found on the chromosome of *Escherichia coli* and numerous other gamma proteobacteria. It encodes a typical two-component system made up of the CpxR response regulator (RR) and the CpxA sensor histidine kinase (HK). In the 1970s and 80s this chromosomal location in *E. coli* was variously known as *cpx* (conjugative plasmid expression), *eup* (energy-uncoupled phenotype) [1,2], *ecfB* (energy coupling factor B) [3], and *ssd* (succinate non-utilizing, high serine deaminase activity) [4,5]. The first phenotypes associated with mutations at the *cpx/eup/ecfB/ssd* locus included alterations in: conjugal plasmid transfer, transport of some substrates, the ability to grow on certain carbon sources, biosynthesis of isoleucine and valine, and resistance to aminoglycoside antibiotics and colicins [2,5–7]. Because the cellular processes affected by this locus were so varied, but most were associated with the membrane, early models proposed that some aspect of energy generation at the IM may be affected. However, biochemical, molecular, and genetic analyses of various *cpx/eup/ecfB/ssd* mutants indicated that they were unaltered in oxygen consumption, ATPase activity, or proton motive force (PMF)[2], but that the protein composition of the inner and outer membranes was altered[8,9]. Thus, it was concluded that the impact of the *cpx/eup/ecfB/ssd* mutations on the varied cellular processes studied must arise from changes in the composition of the envelope [9] and/or an altered regulatory process affecting the coupling of the PMF to transport across the IM[2].

In support of this regulatory hypothesis, in the early 90s Rainwater and Silverman [10] determined that the *cpx, eup, ecfB,* and *ssd* mutations all affected the same locus and it was shown that this locus contained two genes, *cpxR* and *cpxA,* that encoded the RR and HK proteins, respectively, of a two-component signal transduction system (TCST) [11,12]. Also in this decade, the Silhavy group provided the first links between the *cpx* locus and the secretion of aberrantly folded and/or targeted proteins. Mutations mapping to the *cpxA* locus were isolated in studies aimed at identifying protein trafficking factors that act on secreted proteins. The *cpxA** mutations identified could suppress the toxicity associated with grossly misfolded and mislocalized mutant, secreted proteins and were shown to lead to activation of the HK CpxA, ultimately causing up-regulated expression of the periplasmic protease DegP and degradation of the offending proteins [13]. Interestingly, previously isolated *cpx* and *eup* mutations were also shown to lead to "altered function" as opposed to loss of function [10], and it is likely, although unproven, that most or all of the early *eup/ecfB/ssd* mutations were activated *cpxA** alleles. Soon after this connection was made, it was demonstrated that the native CpxA and CpxR proteins could sense the over-expression of the secreted lipoprotein NlpE and mediate elevated DegP

☆ This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

0167-4889/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbamcr.2013.10.018

expression [14], and the protein folding factors DsbA, PpiA, CpxP, and Spy were all shown to be regulated by the Cpx TCS [15–19]. Accordingly, CpxA and CpxR were proposed to regulate an envelope stress response that monitored and mediated adaptation to misfolded, secreted proteins [20–22]. The identity of a plethora of envelope perturbations that act as Cpx activating signals and are expected to result in the misfolding of secreted proteins has shored up this model (see [23] for a recent review). These include (but are not limited to) elevated pH, altered membrane phospholipid composition, the over-expression of misfolded pilus subunits, perturbations in lipoprotein production, osmolarity, adhesion, indole, copper, ethanol, and EDTA [14,16,24–33].

Subsequent analysis of the Cpx envelope stress response around the beginning of the 21st century defined some aspects of signal transduction. Biochemical and genetic experiments indicated that CpxA communicated envelope stress signals to CpxR via the typical phosphotransfer reactions of other TCST systems and that the periplasmic domain of CpxA was necessary to sense activating signals originating in the envelope [34,35]. CpxR was shown to exhibit increased affinity for a specific binding site found upstream of Cpx-regulated genes upon phosphorylation [18,34]. Further, two auxiliary signaling proteins were demonstrated to be involved in regulating the activity of the CpxA HK. Danese and Silhavy identified a small, periplasmic protein – CpxP – encoded directly upstream of the *cpxRA* operon, and showed that it played a role in Cpx-mediated adaptation to alkaline pH as well as the expression of toxic, misfolded envelope proteins [16]. Another role of CpxP is to inhibit Cpx pathway activity. This occurs through an undefined interaction with the periplasmic sensing domain of CpxA, and there is evidence that this inhibition is relieved through the DegP-mediated degradation of CpxP in the presence of inducing signals, possibly in association with misfolded proteins [19,35–38]. In addition to CpxP, the OM lipoprotein NlpE, which over-expression was shown to lead to Cpx pathway activation, was also demonstrated to relay an adhesion signal to the CpxA sensor kinase [30]. The same study also proved that both NlpE and CpxRA were required for efficient adhesion to an abiotic surface [30].

Since several excellent reviews on the Cpx response have been published over the last decade, this chapter will focus on recent studies. For a more extensive examination of older work, please refer to previous reviews [20–22,39,40]. For the most part, recent studies have focused on three main areas: the role of the Cpx ESR in Gram negative gamma proteobacteria (not limited to *E. coli*), including the definition of the Cpx regulon, the characterization of the signaling proteins that regulate the Cpx response, and the definition of connections between the Cpx ESR and other regulatory pathways in the cell. Transcriptomic approaches have provided further explanations and models to explain the diverse phenotypes originally associated with the *cpx* locus. Genetic and structural methodologies have allowed the solution of some of the structures of Cpx signaling proteins and the formulation of more detailed models for the workings of this stress response.

2. Emerging functions of the Cpx ESR; characterization of the Cpx regulated transcriptome

2.1. The regulation of envelope-localized protein complexes

The widely accepted model of the Cpx ESR as a homeostatic mechanism for recognizing and correcting problems with periplasmic protein folding prompted many studies that examined the connection between the Cpx ESR and the expression of envelope-localized, multi-protein structures, especially those involved in pathogenesis [see [23] and references there-in]. Changes in virulence determinant expression in response to ablation or activation of the Cpx ESR have been examined in the human pathogens enteropathogenic *E. coli* (EPEC), uropathogenic *E. coli, Shigella spp., Legionella pneumophila, Salmonella enterica* serovar Typhimurium, *Yersina pseudotuberculosis,* and *Haemophilus ducreyi*. In addition, the impact of the Cpx response on infection by entomopathogenic *Xenorhabdus nematophila* has also been investigated. Two generalizations arise from these studies. First, the expression of envelope-localized virulence factors is influenced in all these cases, but through diverse mechanisms that include changes in the transcription or stability of regulatory or structural genes and their products. These alterations are brought about by either direct effects of CpxR on transcription or through the action of other Cpx-regulated factors. Second, the Cpx response appears to predominantly inhibit the

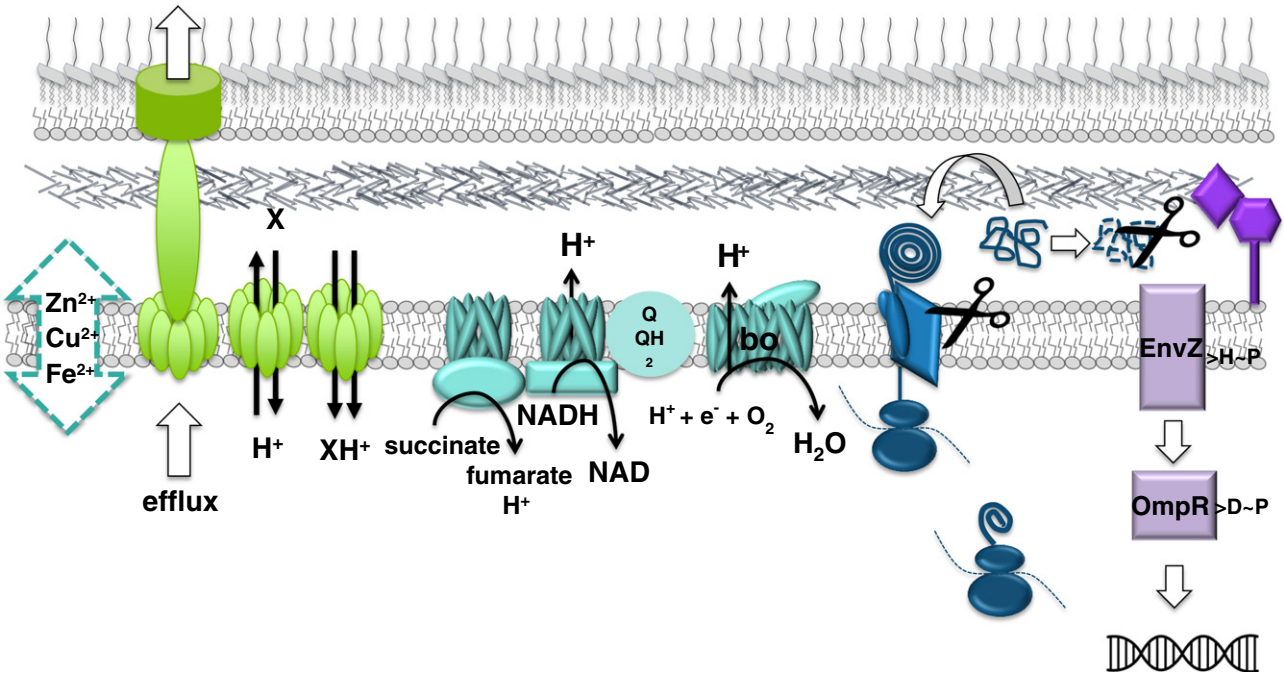

Fig. 1. Conserved features of Cpx-mediated envelope stress adaptation. Activation of the Cpx response leads to changes in the expression of genes involved in inner membrane associated processes, including metal homeostasis, efflux, transport, oxidative phosphorylation, translation, protein secretion, folding, and proteolysis, signal transduction, and cell wall modification.

production of envelope localized protein complexes upon induction (although there are exceptions — the Cpx pathway exerts positive effects on the expression of *L. pneumophila* virulence genes and on colonization and infection by *X. nematophila*). Interestingly, this collection of studies brings us full circle to the original findings of Silverman and colleagues in which *cpx* mutations were found to prevent expression of genes required for conjugative pilus function [1,41]. Clearly, the body of work highlighted in [23] demonstrates that down-regulation of non-essential envelope protein complexes upon activation of Cpx signaling is a general feature of this stress response. Perhaps, this adaptation serves to lighten the load on essential envelope protein complexes.

Although the analysis of interactions between the Cpx ESR and envelope protein complexes pointed to important roles for some of the Cpx-regulated protein folding factors in the assembly and disassembly of these structures [42], surprisingly, detailed examinations of the Cpx regulon using transcriptomic analyses have been published only recently. To date, only four such studies have been published in any detail; two transcriptomic studies of the Cpx response in *H. ducreyi* [43,44], and two examining the Cpx regulon in *E. coli* [24,45]. We have carried out an additional Cpx transcriptome study in *Vibrio cholerae* (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation). None-the-less, some common themes are apparent in these studies that suggest conserved roles for the Cpx response in these (relatively) evolutionarily disparate gamma proteobacteria. Specifically, these studies describe links between the Cpx response and inner membrane functions including energy generation and transport, as well as metals, in addition to the previously known connection to surface exposed structures (Fig. 1).

2.2. The Cpx regulon in E. coli

The first hints that the Cpx regulon may encompass diverse functions beyond protein folding arose from a bioinformatics study by Lin and colleagues in which a weighted matrix describing the CpxR DNA binding site was used to search the genome of an *E. coli* K-12 strain, MG1655 [46]. These authors found CpxR binding sites upstream of expected genes involved in functions previously linked to the Cpx response, including those encoding factors implicated in protein folding stress adaptation, pathogenesis, biofilm formation, and motility. They noted however, that a large number of genes that contained upstream CpxR binding sites could not be reconciled with the published functions of the Cpx response. A second study by Bouloc and Jacq and colleagues, published in 2009, examined the transcriptomes of *E. coli* K-12 strains over-expressing CpxR or lacking the *cpxR* gene [24]. Although this study failed to identify well established Cpx regulon members, it too turned up a list of dozens of genes of diverse function. No enrichment analyses were performed on this data set, but it is clear that regulatory proteins and RNAs were affected, as well as several inner membrane proteins and metabolic enzymes. These studies thus suggested that Cpx-mediated adaptation to envelope stress extended beyond the control of periplasmic chaperones and proteases.

The most recent analysis of the Cpx regulon in *E. coli* confirms this hypothesis and provides new avenues for exploration. Transient induction of the Cpx response lead to strain- and media-dependent changes in 100s of genes [45]. Interestingly, about half of these were down-regulated, in agreement with the (mostly) negative role previously identified for the Cpx response in controlling the expression of envelope localized protein structures [see 2.1 above and [23]]. Functional cluster analysis revealed that Cpx-controlled genes were enriched for those encoding inner membrane proteins, suggesting that this is the main site of Cpx envelope stress sensing and adaptation. All other functional clusters that were enriched for were negatively impacted by Cpx response activation and included genes involved in electron transport and the TCA cycle, oxidative phosphorylation, transporters, and iron or metal binding (Fig. 1). Amongst genes that were changed upon Cpx pathway activation under all four conditions examined in this study, the largest group consisted of "y" genes of unknown function, followed by genes encoding transporters, those involved in cell wall modification, previously identified chaperones and proteases, a group of regulators, and two genes involved in modulating translation. Cumulatively, the results define the Cpx ESR as an inner membrane stress response and confirm that adaptation to stress at this cellular location is conferred by the CpxAR TCS through changes in the expression of genes involved in diverse functions, especially energetics and transport.

2.3. The Cpx regulon in H. ducreyi and V. cholerae

While most work on the Cpx ESR has been carried out on Gamma proteobacteria in the Enterobacteriales order, the only other transcriptome studies aimed at identifying the Cpx regulon were performed in (relatively) more distantly related organisms. Two studies examined the regulon of a putative CpxR homologue in *H. ducreyi,* a member of the Pasteurellales and the causative agent of chancroid [43,44]. In this organism, BLAST analyses identified a *cpxRA* operon, found in a different genomic context and lacking the upstream, divergently transcribed *cpxP* gene that is present in the Enterobacteriales [44]. *cpxR* initially garnered attention in this organism because it was expressed at lower levels in the presence of serum, a condition that leads to increased expression of the identified virulence factors, which include the LspA2–LspB two-partner secretion system [47]. This observation prompted two microarray studies that investigated Cpx-regulated gene expression. Hansen and colleagues first compared the transcriptomes of wild-type *H. ducreyi* to that of its *cpxR* and *cpxA* mutant counterparts and a strain over-expressing CpxR [44]. This work showed that the *H. ducreyi* Cpx system activates expression of a putative fimbrial operon, and represses expression of the LspA2–LspB two-partner secretion system (as previously shown), together with the DsrA serum resistance outer membrane protein and the virulence factor Flp1. CpxR was shown to bind directly to the promoters of these genes to repress their transcription. Interestingly, this study also demonstrated that CpxR did not influence the transcription, or bind to the promoters, of several genes shown to be Cpx-regulated in *E. coli* and linked to protein folding, including *degP*, *dsbA*, *psd*, *secA*, and *ppiD*. This observation led to the conclusion that the Cpx response in *H. ducreyi* has evolved to carry out a different function compared to *E. coli.* This may not necessarily be true however, since it is clear that a conserved function of the Cpx response – to shut down production of envelope localized structures (ie. including virulence factors) – also occurs in *H. ducreyi,* and also because it is becoming increasingly clear from other studies that proteases and chaperones may actually be only one subset of Cpx-regulated genes, and that conserved Cpx functions may rather reflect processes integrally married to the inner membrane, such as energy generation and transport (Fig. 1) [45], N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation, and see Section 3 below].

A second transcriptomic study performed by the Spinola group used RNAseq to compare wild-type *H. ducreyi*, *cpxR,* and *cpxA* mutants at log, transition, and stationary phases of growth [43]. In agreement with other studies that show an integral link between stationary phase and the Cpx ESR (see Sections 4.2 and 4.3), this work showed that the expression of more genes was impacted by alteration of the Cpx signaling pathway in late, stationary phases of growth relative to either log or transition phase growth. This work also reinforced another emerging theme — that inhibition of gene expression is a central part of Cpx-mediated adaptation to envelope stress. Spinola and colleagues showed that approximately 70% of the gene targets they identified were inhibited under conditions where the Cpx response was induced. This work confirmed the observations of the Hansen group that showed virulence factor inhibition by the Cpx response [44,47] and identified additional, established virulence determinants as also being Cpx-repressed. Of more interest, their analysis showed that, in all phases of growth, the functional categories that contained the most Cpx-regulated genes included "cell surface structure and proteins", "generation of precursor metabolites and energy", "hypothetical proteins" and "membrane

transport and uptake". These observations intimate a conserved role for the Cpx response in the regulation of these processes across a wide range of microbes (Fig. 1).

Unpublished work on the Cpx regulon of *V. cholerae* from our group and the Pukatzki lab supports this hypothesis. We have examined genes changed by transient over-expression of the *V. cholerae* CpxR homologue in two different *V. cholerae* serotypes, the O37 serotype strain V52 and the O1 serotype strain N16961, and when grown in two different media (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation). Our preliminary analyses of this work indicates that the functional categories of genes that are most enriched in both strains and media conditions are "hypothetical proteins", "transport and binding proteins", "regulatory factors", and those involved in "energy metabolism" (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation). Thus, in three different Gamma Proteobacteria, from three different orders, the Cpx response appears to be focused on the regulation of energy production and transport, providing strong support for the hypothesis that a conserved function of the Cpx ESR is to modify inner membrane associated processes in response to envelope stress. Interestingly, as in *H. ducreyi*, DegP was not identified as a Cpx-regulated gene in *V. cholerae*, although other protein folding factors, including DsbA, Spy, and YccA were (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation), again suggesting that proteases and chaperones may be a subset of the Cpx regulon that is found only in select organisms. It is also true that cell surface localized virulence determinants requiring envelope-spanning protein structures for assembly are down-regulated by the Cpx ESR in *V. cholerae* (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation), highlighting another apparently conserved function. It will be interesting to determine if, in addition to the demonstrated direct transcriptional repression of some virulence factor genes, some of the diminished expression of these complexes may be indirectly due to the impact of the Cpx response on energy production and transport at the inner membrane.

3. The Cpx inner membrane stress response

The work described above clearly highlights a role for the Cpx ESR in regulating integral inner membrane associated processes. Current insights into the Cpx regulation of inner membrane events provide mechanistic models to explain some of the original observations made on the function of the *cpx* locus, in particular including phenotypes associated with antibiotic resistance and transport.

3.1. The Cpx envelope stress response and antibiotic resistance

It has been known for decades that mutations linked to the *cpx* locus affect resistance to aminoglycosides [2,3,13,48]. In addition, recent publications have linked the Cpx response to β-lactam resistance [49–51] as well as a proposed common mechanism of toxicity exerted by all bactericidal antibiotics [52–54]. The literature confirms a multifactorial role for the Cpx response in influencing resistance to aminoglycosides (Section 3.1.1). Limited evidence suggests that the Cpx response may also impact β-lactam resistance (Section 3.1.2). Although the Cpx response appears to regulate cellular functions that might generally affect resistance to toxic agents, a broad role in resistance to all classes of antibiotics is not supported by current studies [55].

3.1.1. Resistance to aminoglycosides

Resistance to aminoglycoside antibiotics is a trait that has been associated with mutations that map to the *cpx* locus since the 70s and has been used as a phenotype to confirm *cpx* mutations for decades [3,10,13,27,48,56]. Aminoglycosides gain access to the cell in a manner that is dependent on the proton motive force (PMF) and cause mistranslation of proteins at the A-site of the ribosome, leading to the production of aberrant, toxic, membrane proteins [57,58]. At least one aminoglycoside, gentamicin, has been shown to induce the Cpx response [59]. Work from the Silhavy and Akiyama groups suggests a partial mechanism for how activation of the Cpx response may confer resistance to aminoglycosides. These labs have identified a possible role for the gene *yccA*, originally identified as a Cpx regulon member by virtue of its Cpx-mediated up-regulation in response to copper [33]. It turns out that *yccA* encodes a membrane localized protein that inhibits the activity of FtsH [60,61]. FtsH, in turn, is a protease that degrades the SecY component of the general secretion machinery responsible for translocating proteins synthesized in the cytoplasm into the inner membrane and periplasm [61]. Silhavy and colleagues showed that when the SecYEG apparatus is jammed with a mutant protein, SecY is degraded in an FtsH dependent fashion [62]. Overproduction of *yccA* prevented SecY degradation, allowed secretion of the aberrant proteins, and rescued the cell from the toxicity of the jammed translocator apparatus [62]. Importantly, this effect was observed when the aberrant proteins were produced as a result of treatment with antibiotics that targeted the ribosome. It was further shown in *Pseudomonas aeruginosa* that deletion of *yccA*, together with a membrane bound protease *htpX*, (which is Cpx-regulated in *E. coli*), and an additional gene of unknown function, PA5528, conferred sensitivity to aminoglycosides [63]. Altogether, this work suggests that YccA may be involved in the aminoglycoside resistance observed when the Cpx response is induced, by preventing mistranslated proteins from lethally blocking the SecYEG secretion channel. In spite of this, deletion of *yccA* from a *cpxA** strain did not affect aminoglycoside resistance, suggesting that this trait is complex [55]. A reasonable hypothesis is that, as in *P. aeruginosa,* HtpX is also involved, perhaps to degrade mutant inner membrane proteins that may prove to be toxic. It is also possible that the Cpx up-regulated expression of SecA [46], an essential component of the secretion machinery that couples protein translocation into and across the inner membrane with ATP hydrolysis, may influence the aminoglycoside resistance phenotype. Further, recent studies suggest that yet other Cpx controlled loci involved in PMF generation may impact antibiotic resistance by limiting entry of drugs into the cell (see Section 3.2). Clearly, Cpx conferred resistance to aminoglycosides (and possibly other antibiotics) is a complex, multifactorial trait that arises due to the numerous cellular functions impacted by Cpx pathway induction.

3.1.2. The Cpx response and broad-scale antibiotic resistance

In addition to aminoglycoside resistance, the Cpx response was recently proposed to be involved in a common mechanism of cell death exerted by bactericidal antibiotics and other small molecules [52,54,64]. Collins and colleagues proposed that all bactericidal antibiotics, and other toxic molecules, exert their toxic effects through a mechanism that involves a burst of respiratory activity that ultimately leads to the production of harmful oxygen radicals that kill the cell. In support of this model, mutations and conditions that resulted in diminished aerobic respiration or cellular metal concentrations caused a degree of resistance to such antibiotics. Collins et al. also observed that mutations to toxin–anti-toxin modules expected to alter translation, or membrane localized proteolytic regulatory factors HflK and HfkC, impacted resistance to antibiotics and toxic small molecules [52,54]. Further, they showed that genes involved in the cytoplasmic heat shock response as well as inner membrane transport displayed altered expression in the presence of bactericidal kanamycin, as compared to the bacteriostatic drug spectinomycin [54]. Based on these findings, they hypothesized that envelope protein folding may be an important part of the cell death mechanism for kanamycin. In testing this hypothesis, it was found that *cpxA* mutations provided increased resistance to aminoglycosides, as well as to β-lactams and fluoroquinolone antibiotics and it was proposed that the Cpx response was responsible for the induction of a cellular death response in the presence of antibiotics [54].

Several aspects of this model have recently been contested. In particular Imlay and colleagues have shown that antibiotic mediated cell death happens in the absence of oxygen, obviously indicating that killing does not require the production of reactive oxygen species [65].

Further, Mahoney and Silhavy, together with our group, convincingly showed that activation of the Cpx response leads not to a cell death pathway in response to antibiotics, but rather to increased resistance [45,55]. Further, the Silhavy study demonstrated that Cpx response induction, while very protective against aminoglycosides and the small toxic molecule hydroxyurea, had a comparatively smaller effect on resistance to β-lactams and actually increased sensitivity to quinolones [55]. Coupled with the findings of the Imlay group, these results argue strongly against a single mechanism of toxicity for all bactericidal antibiotics and show that the effect of the Cpx response on resistance is drug-specific.

A number of other published findings have implicated the Cpx response in resistance to antibiotics and toxic molecules in addition to aminoglycosides and hydroxyurea. Cpx-mediated activation of the cell wall amidases AmiA and AmiC in *Salmonella enterica* serovar Typhimurium leads to elevated resistance to cationic antimicrobial peptides [66]. Conversely, mutational activation of the Cpx response in *H. ducreyi* leads to sensitivity to antimicrobial peptides [67]. In *Klebsiella pneumonia* elimination of the Cpx response increases sensitivity to β-lactams and chloramphenicol [51], while Cpx response inactivation in *E. coli* leads to sensitivity to a small antibacterial molecule, SM10, isolated for its ability to bind to recombination intermediates [68]. Lastly, we have shown that constitutively active CpxA* mutants are more resistant to some β-lactams (M. Bernal and T.L. Raivio, in preparation), while Hirakawa and colleagues published that over-expression of CpxR leads not only to aminoglycoside resistance, but also increases the ability of *E. coli* to survive deoxycholate toxicity, novobiocin, and β-lactam antibiotics [49,69].

What are the Cpx-regulated cell functions that lead to alterations in antibiotic resistance? While it is clear that the regulation of proteolysis at the inner membrane is a component of resistance to aminoglycosides (see 3.1.1 above), the emerging Cpx regulon coupled with the original literature on the *cpx* locus provide for some additional possible mechanistic models. It is clear that outer membrane permeability changes may affect the ability of some drugs to reach their targets. The earliest publications on the *cpx* locus showed that its mutation altered the outer membrane profile [8], and it has since been shown that the Cpx response regulates the production of the OmpF and OmpC porins at multiple levels [31,45,70–72]. Weatherspoon-Griffin et al. showed that the antimicrobial peptide resistance that occurred upon Cpx response activation was partly due to increased expression of the cell wall amidases AmiA and AmiC and also that deletion of these genes led to increased susceptibility to vancomycin [66]. Since Gram negative cells are normally vancomycin resistant due to the inability of this drug to penetrate the outer membrane, it is likely that Cpx regulation of AmiA and AmiC has a general stabilizing effect on the envelope. Perhaps contributing to this effect is the recently documented Cpx regulation of additional cell wall modifying enzymes, including *slt, ycbB and ygaU* [45]. Induction of the Cpx response leads to elevated resistance to some β-lactam antibiotics in *E. coli* and this effect appears to be partly dependent on *ycbB* and *ygaU* (M. Bernal and T.L. Raivio, in preparation). Whether this is the result of a general effect on the permeability of the outer membrane remains to be determined. Cumulatively, these observations suggest that the general permeability of the outer membrane is altered upon induction of the Cpx response.

The Cpx response has also been shown to regulate the access of toxic molecules to the cell directly through its effect on efflux pump expression. Hirakawa and colleagues linked the deoxycholate resistance of *E. coli* K-12 strains over-expressing CpxR to elevated expression of the AcrD and MdtABC RND efflux pumps [69]. Subsequent studies showed that CpxR ~ P binds directly to the promoters of the *mdtABC* and *acrD* gene clusters in conjunction with another phosphorylated response regulator, BaeR, to increase expression of these pumps during times of envelope stress [73]. The Cpx two-component system was also shown to regulate expression of the KpnEF SMR efflux pump in *K. pneumoniae,* which is involved in resistance to a variety of antibiotics together with hyper-osmotic conditions and bile [50]. Most recently, our group has shown that the Cpx response positively regulates the expression of a number of RND type efflux pumps, together with the common efflux pump outer membrane component TolC in *V. cholerae* (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation). Altogether, these studies indicate that the Cpx response, in addition to limiting permeability of the outer membrane, up-regulates the expression of inner membrane efflux pumps capable of ridding the cell of antibiotics and other toxic compounds. This effect may be the result of a physiologically generated, toxic Cpx signaling molecule (see Section 4.2 below).

In contrast to its effect on the efflux pump category of transporters, the Cpx response negatively regulates a number of inner membrane transporters. This phenomenon may also lead to changes in antibiotic resistance, perhaps by decreasing uptake across the inner membrane, and is discussed below.

3.2. Regulation of transport by the Cpx response

Alterations in inner membrane transport upon Cpx pathway activation have been observed in a number of studies. Some of the earliest descriptions of what we now know were *E. coli* strains carrying *cpxA** mutations revealed that they resulted in diminished uptake of proline and lactose [2,74,75]. More current studies show that when the Cpx response is induced in *E. coli,* transporters are the most enriched category of genes identified with changed expression, behind membrane proteins as a whole [45]. Interestingly, this enrichment occurs only amongst Cpx down-regulated genes. Membrane functions and transport were also altered in *H. ducreyi* by the Cpx response in all nine conditions examined by Spinola and colleagues [43]. Further, transport and binding proteins were the most enriched functional category of genes of known function amongst transcripts that were changed by CpxR overexpression in *V. cholerae,* second in number only to hypothetical proteins (N.A. Acosta. S. Pukatzki, and T.L. Raivio, in preparation). Thus, the Cpx response appears to play a role in altering inner membrane transport in all cases where this has been examined to date (Fig. 1). It remains to be determined if transporters are targeted specifically, or if this reflects a general down-regulation of inner membrane protein expression and/or function.

Although the identification of transporter-encoding genes through transcriptome analyses clearly suggests that the Cpx response may directly regulate the transcription of these genes, other observations indicate that the Cpx-mediated control of inner membrane transport may involve additional levels of regulation. For example, none of the transport genes that showed diminished expression on Cpx pathway induction in *E. coli* under all conditions studied contains a CpxR consensus binding site upstream of the promoter [45]. This could suggest the involvement of other regulators, including Cpx-regulated sRNAs, which have recently been shown to comprise an important part of the Cpx regulatory network (S.L. Vogt, A.D. Evans, R. Guest, and T.L. Raivio, submitted). Alternatively, it is possible that altered expression of some transporters when the Cpx response is induced reflects some type of feedback mechanism triggered as a result of changes in activity. In fact, several early studies provide evidence that the transport of specific substrates across the inner membrane (proline, lactose) is altered upon Cpx pathway induction [2,74].

Activation of the Cpx response in *E. coli* leads to reduced transcript levels for the genes encoding the succinate and NADH deyhdrogenases, and cytochrome bo oxidase of the electron transport chain [45]. Altogether, these changes would be expected to lead to diminished respiration, and perhaps PMF. These changes may also explain some of the antibiotic resistance observed when the Cpx response is activated, since mutations in all of these genes confer resistance to aminoglycosides and hydroxyurea [45]. It is likely, at least for aminoglycosides, that this is through a decrease in the PMF-dependent uptake of the drug [58]. Similarly, it seems possible that an alteration in PMF and/or membrane potential could lead to diminished activity of PMF-

dependent transporters, perhaps indirectly causing a decrease in their transcription through a feedback mechanism. In fact, amongst the transporters that are down-regulated in *E. coli* upon Cpx pathway induction are the PMF-dependent transporters NhaB (Na^+), DctA (dicarboxylates), and TppB (dipeptides), all of which lack a consensus CpxR binding site [45]. Also supporting the hypothesis that inner membrane transport is altered upon Cpx response activation is the early observation that the PMF-dependent processes of proline and lactose transport were altered in *cpxA** mutants, but not the ATP driven transport of glutamine and arginine [2].

Despite this compelling evidence for a Cpx-mediated change in PMF-dependent transport, early studies failed to identify a change in membrane potential or pH gradient in *cpxA** mutants [74,75]. One possibility is that the *cpxA** mutants employed in these seminal experiments were weak alleles that did not activate the response strongly enough to exert an effect on PMF that could be measured with the techniques that were used. It is also possible that the membrane potential/PMF is unaltered on Cpx induction, but the manner in which it is generated and utilized is impacted. In this regard, the genes for an oxalate dependent generation of PMF were up-regulated by the Cpx response in *H. ducreyi* [43], and an uncharacterized putative cytochrome, YceJ, is up-regulated by Cpx in *E. coli* [45].

Alternatively, Plate proposed that the Cpx response might alter not PMF itself, but rather the ability of some transporters to use the PMF [74]. This speculation is supported by the observation that *cpxA** mutants could not use an artificially generated proton gradient to take up substrate after energy starvation [75]. How could such an uncoupling of PMF from transport be effected? Other experiments by Plate and colleagues suggest that the Cpx response may act on a population of some transporters to alter their properties [74]. They found that *cpxA** bacteria contained two populations of lactose transporter, one with wild-type characteristics, and another with altered substrate binding and enzymatic activity [74]. This observation suggests that Cpx pathway activation may serve to alter a subset of transporters to affect their activities. Although how this occurs is unknown, one possibility may be through the Cpx up-regulation of small inner membrane proteins that are capable of associating with and altering transporter function. Storz and colleagues have recently shown that small hypothetical membrane proteins may act to alter the properties of inner membrane transporters, including the PMF-dependent AcrB efflux pump [76], and we have shown that a number of small membrane-associated hypothetical proteins are up-regulated upon Cpx response induction and associated with alterations in membrane properties [45]. The nature of changes in transporter and respiratory gene transcription, translation, and activity, and their overall contributions to the inner membrane adaptations conferred by the Cpx response remain to be determined.

4. Cpx signal transduction

It is well-established that envelope stress signals are transmitted through CpxA to CpxR via the phosphotransfer reactions that are typical of bacterial two-component systems [34,77,78]. Recent advances in studying Cpx signal transduction have centered around the solution of crystal structures for the periplasmic auxiliary signaling molecules CpxP and NlpE, as well as the sensing domain of CpxA. Although we are still far from an answer, the structures have provided for further refinement of models for how signaling might occur. This work has recently been reviewed [23]. As with many two-component systems, the molecular nature of the Cpx-specific envelope stress signal remains an outstanding question. One of the most interesting current topics in Cpx signal transduction is the idea that multiple signals can impact the pathway and that they are sensed at different entry points in the signaling cascade. In particular, it has become clear in recent years that cytoplasmic signals are capable of altering Cpx signaling independent of CpxA [35,79]. Finally, an ever-increasing list of connections between the Cpx response and other cellular signaling pathways is being compiled. Some of these connections form complex feed-forward and feedback-inhibition loops that act to increase the precision and/or magnitude with which the Cpx response affects the expression of adaptive genes.

4.1. The structures of Cpx signaling proteins refine signaling models and predict new ligands

As mentioned in Section 1, auxiliary regulatory factors that regulate CpxA activity have been identified. CpxP is a periplasmic protein with weak chaperone activity that inhibits activation of the pathway [16,19,35,38,80,81], while NlpE is an outer membrane lipoprotein that signals adhesion to a hydrophobic surface [30]. Both CpxP and NlpE act through undefined interactions with the sensing domain of CpxA that may or may not be direct. Recently, structures of both proteins were solved, revealing the presence of putative ligand-binding domains that may be involved in sensing both known and yet-to-be-discovered Cpx inducing signals.

4.1.1. NlpE

The solution of the crystal structure of NlpE revealed that it is a two-domain lipoprotein, with an N-terminal β-barrel bearing similarity to the lipid-binding bacterial lipocalin protein connected to a C-terminal β-barrel domain with a predicted oligonucleotide/oligosaccharide binding (OB) fold [82]. The presence of these predicted ligand binding folds might suggest that changes in lipid or sugar binding upon cell adhesion could be involved in instigating structural changes responsible for sensing adhesion. It has been proposed that the structural changes could involve a propensity of the N-terminal β-barrel of NlpE to unfold. Displacement of one of the β-strands could lead to unfolding and a change in the orientation of the N and C terminal domains of NlpE, perhaps forming an elongated structure capable of interacting with the sensing domain of CpxA at the inner membrane [82].

In addition, it was demonstrated that redox conditions can influence the formation of a disulfide bond in a CXXC motif in the N-terminus of NlpE, which in turn is predicted to impact the rigidity of the loop bearing this motif [82]. Since the Cpx response is induced by copper [33], and conditions that impact disulfide bonded envelope proteins [83], this finding may be relevant for signaling some envelope stress signals that alter the redox status of the periplasm. The CXXC motif is additionally found very near to a serine protease inhibitor motif in NlpE, and so it is possible that redox events involving these cysteine residues could also affect the activity of the protease inhibitor motif and alter proteolytic events in the periplasm [82]. All of these models await further testing.

4.1.2. CpxP

The structure of the auxiliary regulator CpxP was also recently solved [81,84]. CpxP forms a bowl shaped dimer consisting of two monomers that form a bent, hairpin like structure. Strikingly, the concave surface of the bowl is enriched in positively charged residues, while the convex surface is mostly negatively charged, with one hydrophobic stripe. Hunke and colleagues have suggested that the concave, positively charged surface of the CpxP dimer may interact with negatively charged residues in the periplasmic sensing domain of CpxA and that exposed hydrophobic regions of misfolded proteins may titrate CpxP away from CpxA through interaction with the hydrophobic patch on the convex side of CpxP [81]. These conclusions must be viewed cautiously, however, since the CpxP:CpxA interaction was studied only by analyzing binding of purified proteins to spotted oligopeptides on a nitrocellulose membrane and the conclusions regarding the binding of misfolded pilus subunits to CpxP were derived only indirectly from an in vivo assay in which CpxP and the pilus proteins were overexpressed and their stabilities measured. Further, a recent study of the *Vibrio parahaemolyticus* CpxA sensing domain failed to identify an interaction between the purified CpxA sensing domain and CpxP proteins (as

we have also found for the *E. coli* counterparts, G. Thede, J.L. Wong, J.N.M. Glover, and T.L. Raivio, unpublished) and also found that the proposed CpxP binding residues of CpxA identified by Hunke et al. actually formed part of a β strand that was not solvent exposed [85]. Thus, the jury is still out on the question of how CpxP impacts CpxA activity and what type of interaction this involves.

The two most interesting aspects of the CpxP crystal structure derive from its charge and its structural similarities to bacterial metal binding signaling proteins. Surprisingly, there are very few hydrophobic patches on the CpxP dimer, given that this protein is predicted to interact with misfolded proteins [81,84]. Rather, there is a dramatic concentration of positive and negative charges on the concave and convex faces of the CpxP dimer, with the edges of the bowl and a single stripe on the convex surface displaying mostly hydrophobic character [81,84]. Thus, whether and how CpxP might bind to misfolded proteins remains somewhat mysterious. A clue may come from the study of the homologous protein, Spy, which has strong chaperone activity [80]. Conformational studies of Spy in the presence of misfolded proteins, using fluorescent probes, suggest that large conformational changes may be involved in its chaperone activity [80]. Perhaps this is also true for CpxP. Additionally, it is not yet clear what ligand(s) may bind to the oppositely charged faces of the CpxP dimer. Much evidence suggests a direct interaction between CpxA and CpxP [19,38,81], although one has yet to be demonstrated (see above). Perhaps an additional ligand(s) binds to one of these surfaces to allow formation of a ternary signaling complex. Given the recent finding that the Cpx response is intimately associated with regulating events at the inner membrane (see Sections 2 and 3, above), phospholipids are one possibility. Alternatively, or in addition, perhaps CpxP senses a signal that has yet to be identified, although to date, no Cpx inducing signals are known that require the presence of CpxP for sensation [35].

Related to this possibility is the finding that CpxP shares structural similarity with several other periplasmic metal binding proteins involved in sensing and adapting to high levels of potentially toxic metals [84]. These include CnrX, a metal sensing periplasmic protein from *Cupriavidus metallodurans* that binds cobalt and nickel [86,87], as well as ZraP, a *S. typhimurium* periplasmic zinc binding chaperone and signaling protein [88]. Further, the CpxP crystal was isolated in the presence of, and contained, zinc, suggesting the potential for metal binding by CpxP as well [84]. The role of CpxP in metal sensing/binding and protein folding will be an important area of future investigation.

4.1.3. CpxA sensing domain

In addition to NlpE and CpxP, a structure for the sensing domain of the *V. parahaemolyticus* CpxA soluble periplasmic sensing domain has recently been published [85]. This study showed that, similar to several other histidine kinase sensing domains, CpxA utilizes a Per-Arndt-Sim (PAS) domain consisting of a central five-stranded β sheet surrounded by several α helices to sense inducing signals. This group failed to detect an interaction between the purified CpxA sensing domain and the CpxP protein, prompting them to suggest that perhaps an unstructured C-terminal domain that was absent in their interaction assays, and/or an additional component such as phospholipids, was important for CpxP binding [85].

Our group also recently solved the structure of the soluble CpxA sensing domain of *E. coli* in conjunction with a mutational analysis of conserved residues previously implicated in sensing (R.M. Malpica, G. Thede, J.N.M. Glover, and T.L. Raivio, in preparation). We identified a more compact PAS domain structure and showed that mutations that alter sensing affect the edge, connecting loops, and a flanking α helix of the five stranded β sheet. Most strikingly, our genetic analysis has shown that mutation of the CpxA sensing domain almost always leads to activation of the pathway, suggesting that regulation of CpxA activity occurs predominantly through inhibitory signaling events that impact the edge of the β sheet predicted to be localized near the inner membrane (R.M. Malpica, G. Thede, J.N.M. Glover, and T.L. Raivio, in preparation). This model suggests that important inhibitory signaling events occur near the inner membrane, which is satisfying in light of the predominantly inner membrane localized adaptations recently shown to be instigated by the Cpx response in *E. coli, V. cholerae,* and *H.* ducreyi [43,45], (N.A. Acosta, S. Pukatzki, and T.L. Raivio, in preparation). The nature of the inhibitory signal(s), beyond CpxP, that control CpxA remain unidentified.

4.2. Numerous inducing signals enter the Cpx signal transduction pathway at multiple points

Interestingly, for more than a decade, activation of the Cpx response was shown to occur only through mutation of *cpxA.* Silhavy and colleagues identified the first activating signal to affect the wild-type, intact Cpx signaling cascade when the over-expression of the lipoprotein NlpE was shown to suppress the toxicity of secreted, mutant envelope proteins [14]. It was demonstrated that this occurred predominantly through up-regulated expression of the periplasmic protease DegP, and this was dependent on an intact CpxAR two-component signaling system. Since then, a plethora of signals have been identified that lead to activation of the Cpx response. These include elevated pH [16], the over-expression of misfolded pilus proteins [25,29], alterations in membrane phospholipid ratios [27,89], high osmolarity [26], disruptions in disulfide bonding in the periplasm [83], the presence of aminoglycoside antibiotics [59], EDTA [24,35], spheroplasting [19,35], mutation of the gene encoding the inner membrane protein localization factor YidC [90], accumulation of enterobacterial common antigen assembly intermediates [91], deletion of efflux pump components [67], indole [32], mammalian peptidoglycan recognition proteins [59], ethanol [24,92], n-butanol [93], adhesion [30], copper [33], growth [35,79], and assembly of a type IV secretion system [94]. While the sheer number and broad nature of this list make it difficult to make any conclusions regarding the molecular nature of any inducing signal(s), it has long been thought that misfolded proteins are a component of the envelope stress signal. This model is substantiated by the fact that some of the most strongly Cpx-regulated genes in *E. coli* encode periplasmic chaperones (CpxP, Spy, DsbA) and proteolytic factors (DegP, YccA, HtpX) and many of the best studied activating conditions involve the direct generation of misfolded and/or aggregated proteins in the periplasm.

Studies addressing the nature of the inducing signal generated by one specific cue, the over-expression of the pilus protein PapE, indicate that, perhaps counter-intuitively, activation is not achieved simply by aggregation of protein at the periplasmic face of the inner membrane, but rather involves a specific, undefined attribute of the over-expressed protein [95]. Recent Cpx regulon analyses (Section 2) that demonstrate an intimate association between the Cpx response and the inner membrane imply that the envelope stress signal(s) must perturb some aspect of this cellular structure. Interestingly, principal component analysis of the data derived from a comparison of the transcriptomes of the known envelope stress responses in *E. coli* showed that the Cpx response is most closely related to the Psp and Bae envelope stress responses [24]. Current evidence suggests that the Psp response detects insults at the inner membrane that may involve PMF alterations and institutes corrective measures [96], while the Bae response appears to sense toxic compounds and metals and up-regulate predominantly efflux pumps in response [97]. Cumulatively, the above observations suggest that the Cpx signaling pathway may respond to a toxic molecule at the inner membrane that is generated in the presence of misfolded proteins at this location. Intriguingly, it was recently demonstrated that the Cpx response is activated in strains carrying mutations to the gene encoding the common outer membrane component of all efflux pumps in *E. coli, tolC,* as well as in strains carrying certain combinations of efflux pump mutations [98]. Further, in *H. ducreyi,* mutation to the RND efflux gene *mtrC* also leads to Cpx response induction [67]. These two studies support the titillating

hypothesis that the Cpx response may sense an endogenously produced toxic molecule at the inner membrane that could be related to Cpx pathway induction by some of the many previously identified envelope stresses (above).

Classical dogma holds that the sensor kinase serves as the entry point for signals affecting a particular two-component system. Indeed, in all cases in which it has been examined, the envelope stress signals listed above require the sensor histidine kinase CpxA in order to alter the activity of the Cpx response (except growth, see below). This demonstrates that CpxA is clearly a hub for the integration of envelope stress signals [35]. In addition, the activating cue generated upon adhesion to a hydrophobic surface requires, in addition to CpxA, the lipoprotein NlpE (see Section 4.1) [30]. Thus, at least one envelope stress signal depends on a point of entry upstream of CpxA. A limited subset of the Cpx-inducing cues have been investigated to determine whether others might also depend on NlpE, or the other Cpx auxiliary regulator thus far identified, CpxP (Section 4.1) [35]. This study showed that, while signals distinct from adhesion, originating in the envelope, all required CpxA, in no case was either NlpE or CpxP necessary to alter pathway activity. It will be of interest to investigate whether additional, unidentified or untested signals require these proteins for signaling and if other auxiliary regulators in the envelope exist for this purpose.

One relatively unexplored Cpx inducing signal is growth. It was noted early on that the Cpx response was more active in stationary phase [99]. In fact, in some *E. coli* K-12 strains, the *cpxRA* operon is auto-activated in stationary phase in conjunction with the stationary phase sigma factor, σ^S [99]. It has similarly been reported that more genes are influenced by the Cpx response in *H. ducreyi* during stationary phase [43], implying that the pathway may be more active at this growth stage. Wolfe and colleagues have convincingly shown that, under conditions when the culture pH does not become alkalinized in stationary phase, excess carbon leads to an increase in Cpx pathway activity [79]. Intriguingly, this increase appears to be somewhat independent of CpxA and requires a cellular metabolite produced in the presence of the *pta ackA* genes involved in the interconversion of acetyl-phosphate or propionyl phosphate and coenzyme A to acetyl-CoA or propionyl-CoA and phosphate. Additionally, there is some evidence that growth-related acetylation of a specific residue on the alpha subunit of RNAP may be involved in signaling through CpxR as well [100]. Although the mechanism has yet to be fully worked out, the evidence for growth-mediated, CpxA-independent activation of the Cpx response is convincing.

Our group has identified an additional signaling event that appears to utilize CpxR as the entry point. We found that the Cpx response participates in a feedback loop with the small RNA (sRNA) RprA in *E. coli* (S.L. Vogt, A.E. Evans, R. Guest, and T.L. Raivio, submitted). The Cpx response up-regulates expression of the RprA sRNA, while over-expression of RprA, in turn, inhibits Cpx-mediated gene expression. RprA inhibition of the Cpx response occurs in a manner that is independent of CpxP and CpxA, but dependent upon CpxR. Although we do not currently understand how RprA affects CpxR activity, it appears to be independent of all the known targets of RprA, and RprA does not directly affect the levels of CpxR. Further analysis of this regulation will hopefully identify the events responsible for altered CpxR function when RprA is over-expressed, and perhaps yield some insight into the CpxR-dependent growth signal as well, since RprA is tightly linked to growth phase through its regulation of σ^S levels [101].

4.3. The Cpx response as part of the cellular regulatory network

Many recent studies have begun to identify connections between the Cpx envelope stress response and other regulatory pathways in the cell. It is abundantly clear that stresses to the envelope do not trigger individual signaling pathways in isolation. Rather, many envelope perturbations seem to result in the simultaneous induction of numerous responses (for example see [24,92,98,102]). Systems biology approaches and parallel analysis of envelope stress responses in *E. coli* indicate that stress responses are not redundant, but rather sense individual, related cues and in turn lead to unique adaptations [24,92]. In spite of this vertical novelty in responses (ie. general stresses produce unique, related signals that induce specific pathways which lead to different adaptations), we are just beginning to uncover the extensive network of horizontal connections (ie. between pathways) that connect each signaling pathway to many others in the cell. The Cpx response is part of a network that is formed through the control of Cpx signaling activity by other pathways together with the Cpx control of regulatory protein and sRNA expression (Fig. 2). Many of these interactions form complex feed-forward and feed-back circuits that amplify a response or control Cpx signaling.

4.3.1. Cpx-regulated connections to other envelope stress responses

The Cpx response interfaces directly with at least three other envelope stress responses by directly regulating t`he transcription of the signaling proteins involved. The *rpoErseABC* operon encoding σ^E and its regulators was shown to contain a consensus CpxR binding site through bioinformatics, and activation of the Cpx response by mutation or NlpE over-production leads to inhibition of the transcription of this operon (Fig. 2) [31,46]. Further, a large group of genes has been identified that are down-regulated by σ^E while being up-regulated by the Cpx pathway [31]. Whether part of the Cpx activation of these genes involves the repression of σ^E levels has not been addressed, but it seems likely that the expression of one or more of these genes (or other σ^E regulon members) must be detrimental when the Cpx envelope stress response is induced, for yet to be determined reasons.

Another envelope stress response that is impacted by Cpx pathway activation is the Bae response. The BaeSR two-component system regulates the production of at least two multi-drug efflux pumps and a periplasmic chaperone, Spy [32,97]. One of the efflux pumps, encoded by the *mdtABCD* gene cluster, is encoded directly upstream of the *baeSR* genes in an operon. Phosphorylated CpxR binds upstream of the *mdtABCDbaeSR* and *acrD* gene clusters, and in conjunction with active BaeR, up-regulates expression of these genes (Fig. 2) [73]. Thus, not only are the Mdt and AcrD efflux pumps expressed at higher levels upon Cpx response induction, a feed-forward circuit is created in which the levels of the BaeS and BaeR regulators are also elevated, potentially allowing for a further increase in resistance to toxic compounds that may be generated when Cpx inducing cues are present (see Section 4.1 above).

A third envelope stress response that is impacted by Cpx is the high osmolarity stress response regulated by the EnvZ HK and the OmpR RR. As with efflux pump expression, the Cpx pathway induces the EnvZ/OmpR two-component system and its gene targets in multiple ways (Fig. 2). Firstly, phosphorylated CpxR has been shown to work, in a similar way to how it functions with BaeR, by binding to sites upstream of the *ompF* and *ompC* porin genes in conjunction with activated OmpR, to either further repress or activate their expression, respectively [70]. In addition, the Cpx response was recently found to activate the expression of a novel gene, *mzrA,* which encodes a small membrane protein that interacts with the periplasmic domain of the EnvZ HK, ultimately elevating OmpR ~ P levels in an unknown fashion [71,72]. Thus, here too the Cpx response strengthens its potential to exert greater regulatory influence on its downstream targets by controlling expression of not only the adaptive gene(s) in question (*ompF* and *ompC*) but also the activity of their cognate signaling pathway (EnvZ/OmpR).

We recently identified an additional Cpx-regulated feed-forward scheme involving the Cpx-regulated gene *yqaE* and the sRNA CyaR. *yqaE* encodes an inner membrane protein of unknown function that is up-regulated by NlpE over-expression in a Cpx-dependent fashion [45]. The *yqaE* message is degraded in the presence of the sRNA CyaR, whose expression is controlled by the nutritional status of the cell [103]. Microarray and Q-PCR experiments, together with analysis of a *cyaR::lacZ* reporter gene, showed that induction of the Cpx response leads to reduced *cyaR* transcription ([45],S.L. Vogt, A.E. Evans, R. Guest,

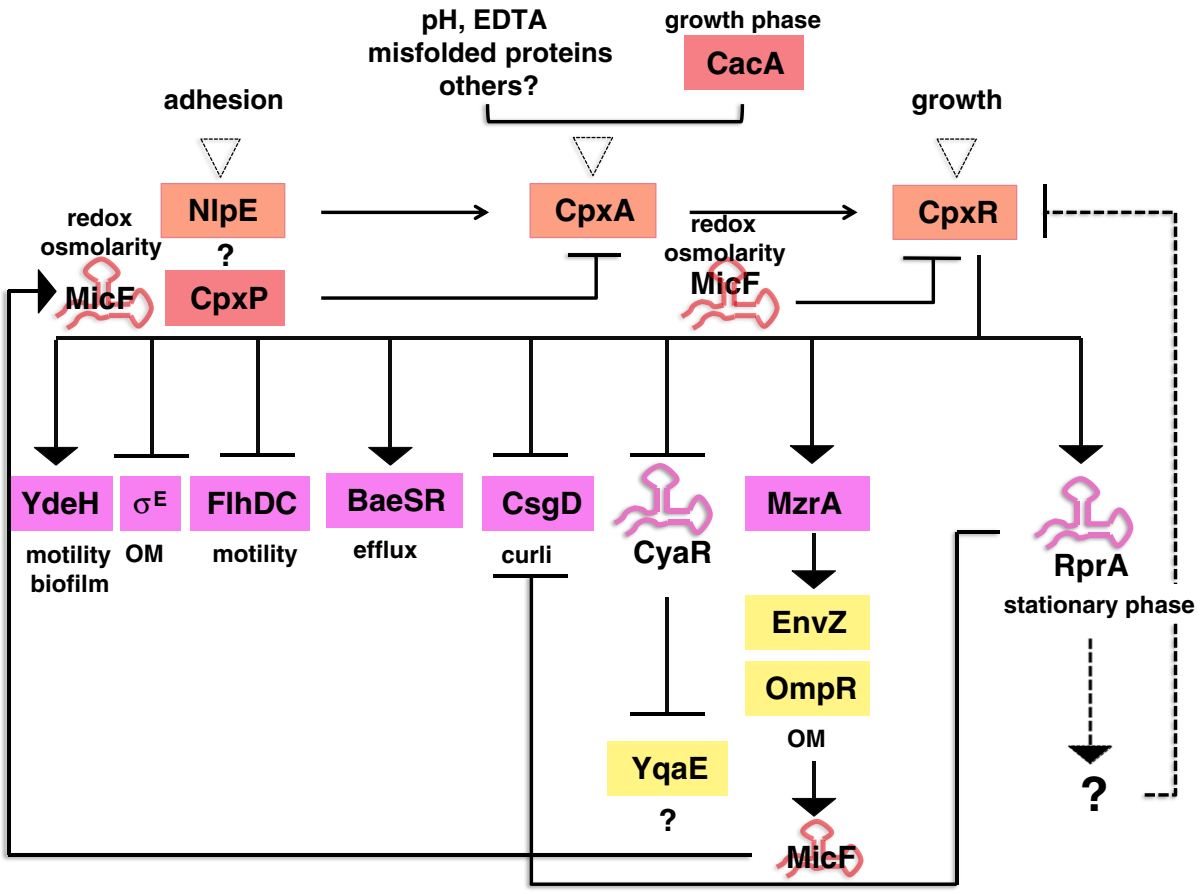

Fig. 2. Signal entry points and connections between the Cpx envelope stress response and other cellular regulatory pathways. Signals enter the Cpx response (top) at one of three known points; NlpE, CpxA, or CpxR (orange boxes near top of figure) and either activate (arrows) or inhibit (lines ending in perpendicular line) activity of the pathway. Other regulatory proteins and sRNAs also affect activity of the pathway by affecting CpxA or CpxR levels or activity (salmon colored boxes and squiggly lines). The Cpx response regulates the transcription of numerous genes encoding regulatory proteins (pink boxes) and sRNAs (pink squiggly lines). The activities regulated by these proteins and sRNAs are listed underneath of them. Additionally, some of these regulators participate in feed-forward and feed-back regulatory loops (CyaR, RprA, MzrA), which are indicated. Dashed lines indicate that intermediate regulators may be involved as a direct interaction has not been demonstrated.

and T.L. Raivio, submitted). Thus, the Cpx response utilizes a coherent feed-forward loop to ensure maximal YqaE expression by down-regulating the inhibitory CyaR sRNA and activating *yqaE* transcription (Fig. 2).

4.3.2. Regulatory circuits connecting envelope stress, biofilm formation, and stationary phase growth

The Cpx envelope stress response also interfaces with a number of regulatory pathways involved in the connected processes of motility, biofilm formation, and stationary phase growth (Fig. 2). At least three regulatory proteins implicated in motility and/or biofilm formation are transcriptionally regulated by the Cpx response. Genes encoding chemoreceptors and parts of the motility apparatus were amongst the first Cpx targets to be identified [46,99]. Lin and colleagues demonstrated that several chemotaxis genes, together with the *motABcheAW* operon encoding parts of the flagellar motor, were negatively regulated by Cpx response activation, CpxR ~ P bound directly to the upstream regions of these gene clusters, and CpxR consensus binding sites could be identified upstream of multiple other genes involved in motility [46,99]. We recently showed that Cpx response activation additionally leads to diminished expression of the *flhDC* operon encoding the master flagellar regulatory complex FlhDC [45]. Thus, yet another feed-forward connection is formed here to reinforce the inhibition of motility — the Cpx response negatively regulates expression of the structural genes themselves, together with their positive regulators. On top of this, *flhDC* is also repressed by OmpR ~ P, and so the positive regulation of EnvZ activity by the Cpx response also likely contributes to inhibition of motility.

The Cpx response positively regulates production of the diguanylate cyclase enzyme YdeH (recently renamed DgcZ [104]) [45]. DgcZ stimulated synthesis of the second messenger cyclic di-GMP is required for the elaboration of a polysaccharide adhesin, poly-GlcNAc, that supports biofilm formation [105,106]. Because a loss of motility and production of poly-GlcNAc are conditions existing in a biofilm, these regulatory connections suggest that the Cpx response could play a role in maintaining this condition. Additionally, transcriptomic studies indicate that there is a large overlap between the Cpx regulon and the gene expression profile found in an *E. coli* biofilm community [107], and a positive association between Cpx response induction and adherence to hydrophobic surfaces has been noted [30,108]. Thus, it seems possible that Cpx control of these biofilm regulatory elements that dictate motility and polysaccharide production could be important in biofilms on hydrophobic surfaces.

The situation may not be this simple, however, as the Cpx response has connections to other regulatory elements that are expected to disfavor biofilm formation. Specifically, Cpx response induction leads to the diminished expression of CsgD, a key positive regulator of biofilm formation. CsgD is the transcriptional activator of the curli biosynthetic genes, which facilitate synthesis of the curli adhesin, an amyloid fiber that contributes to the matrix that surrounds cells in, and helps with formation of, a biofilm [see [109] for a review]. The Cpx response inhibits transcription of CsgD, as well as the structural and biogenesis machinery

CsgBAC and CsgEFG by binding directly to the promoters of the *csgDEFG* and *csgBAC* operons [110,111]. In addition to these negative inputs into curli production, we have shown that the Cpx response stimulates RprA expression, a sRNA that inhibits CsgD expression [45](S.L. Vogt, A.E. Evans, R. Guest, and T.L. Raivio, submitted). Thus, the Cpx response contributes three separate inhibitory inputs into curli expression (Fig. 2). Activation of RprA expression by the Cpx response should have additional negative effects on biofilm formation because RprA negatively regulates production of the diguanylate cyclase YdaM, a positive regulator of CsgD synthesis [112]. Finally, since CsgD is required for the production of cellulose, also involved in biofilm formation, the Cpx response is expected to inhibit biofilm production via this route as well [108,113]. In agreement with the negative impacts of the Cpx response on the CsgD regulatory network required for biofilm formation, *E. coli* strains in which the Cpx response is activated are deficient at forming biofilms [30,108,110].

How can these seemingly contradictory Cpx inputs into biofilm regulatory networks (inhibition of motility and stimulation of poly-GlcNAc formation vs. inhibition of curli and cellulose synthesis) be rationalized? One possibility is that the Cpx response plays a role in biofilm formation only at later stages, after initial establishment of adhesion through curli and cellulose. This speculation would be in line with the fact that RprA, in addition to its effects on curli via CsgD, also stimulates translation of the *rpoS* mRNA encoding the σ^S regulator of stationary phase genes [101,114], which are known to be important in established biofilms [115]. Further, as mentioned in Section 2, the Cpx response is known to be more active in stationary phase. Another model could be that the Cpx response responds to stresses involved in adhering to certain surfaces, or in discrete parts of the biofilm, and exerts an important role in these locales, but not others. Supporting this idea, it is known that biofilms are complex communities containing bacteria in many different growth phases [115], and the Cpx response has been shown to stimulate adherence to hydrophobic surfaces, while inhibiting biofilm formation on hydrophilic surfaces [108]. The precise roles of the observed regulatory connections between the Cpx envelope stress response and the biofilm and stationary phase lifestyles must yet be elucidated.

4.3.3. Cpx regulatory connections mediated by other stress responses

The Cpx pathway not only forges connections to other regulatory pathways through its regulation of their signaling proteins, but its activity is also impacted by other stress responses. The over-expression of the sRNA MicF, which is up-regulated by the osmolarity stress response controlled by the EnvZ/OmpR two-component system, causes reduced expression of both CpxR protein levels and those of a *cpxR::gfp* translational fusion reporter gene [116], presumably by base-pairing with the 5′ region of the *cpxRA* mRNA and reducing translation, as it does for its other targets. We have shown that MicF over-expression also lowers levels of the CpxA protein (S.L. Vogt, A.E. Evans, R. Guest, and T.L. Raivio, submitted). Furthermore, *micF* expression is regulated by a number of regulators in addition to EnvZ/OmpR, including several that are responsible for mediating responses to oxidative stress [reviewed in [117]]. Thus, MicF ultimately completes a feed-back inhibitory loop (Fig. 2). The Cpx response up-regulates EnvZ/OmpR function, leading to elevated MicF expression, and MicF down-regulates levels of CpxA and CpxR. This should keep the Cpx response "in check", ensuring that activation is balanced with the outcome(s). A similar, but simpler feedback inhibitory loop exists with the inhibitor CpxP, which is also up-regulated by the Cpx response [16,19,38]. In spite of this apparent feedback loop, over-expression of MicF does not appear to affect expression of a *cpxP-lacZ* transcriptional reporter (S.L. Vogt, A.E. Evans, R. Guest, and T.L. Raivio, submitted). These results suggest that the approximate two-fold reduction in the CpxR and CpxA proteins that occurs when MicF is over-expressed are not sufficient to alter expression of a promoter that is highly sensitive to CpxR ~ P levels [35]. One possibility is that the MicF-mediated reduction in Cpx signaling proteins may affect only weakly Cpx-regulated promoters, thus providing a mechanism for the EnvZ/OmpR pathway to differentially affect members of the Cpx regulon.

The envelope stress regulated sRNA, RprA, also appears to be involved in inhibiting Cpx pathway activity. In addition to its regulation through Cpx signaling (see 4.3.2), the expression of the RprA sRNA is also activated by the Rcs envelope stress response [see [118] for a review]. We recently showed that RprA over-expression inhibits Cpx pathway activity in a CpxA-independent manner (S.L. Vogt, A.E. Evans, R. Guest, and T.L. Raivio, submitted). Thus, the inhibitory effect of RprA on Cpx activity is not a result of diminished envelope stress based on RprA's inhibition of curli expression. Further, RprA inhibition of the Cpx pathway does not involve decreases in the levels of the CpxR or CpxA proteins, and so a direct effect of RprA on the *cpxRA* transcript cannot be invoked either (S.L. Vogt, A.E. Evans, R. Guest, and T.L. Raivio, submitted). Over-expression of RprA does, however, require the presence of CpxR, but is independent of all of RprA's known targets. Since the only other Cpx signal known to enter the pathway at CpxR is that of growth, it is exciting to postulate that perhaps an unidentified RprA target plays a role in this phenomenon.

A new auxiliary regulator of the Cpx response, CacA, appears to be another link between Cpx and other stress response pathways. CacA was discovered in a multicopy screen for activators of *cpxP::lacZ* expression and its expression was shown to be controlled by the stationary phase sigma factor σ^S [119]. No association between CacA and the CpxA or CpxR proteins was demonstrated, and little other information is available about CacA, but it seems possible that CacA could also be involved in linking Cpx activity to growth phase.

5. Closing remarks

Recent studies of the Cpx envelope stress response have yielded new insights into the phenotypes originally associated with mutations at the *cpxRA* locus, including antibiotic resistance, alterations in inner membrane transport, and the suppression of the toxicity exerted by grossly misfolded and mislocalized secreted proteins. We now know that the Cpx response is intimately associated with events at the inner membrane, impacting protein folding, degradation, and secretion, as well as energy generation and transport. As we gain more knowledge about the hypothetical genes that are Cpx-regulated, we will better understand how these processes are altered by the Cpx response. Knowledge in this arena will facilitate the development of microbes with the ability to function as sensors of harmful chemicals as well as in industrially important production of recombinant proteins and bioremediation agents [93,120–124]. There is a growing appreciation for the complexity involved in controlling these cellular functions. Many adaptations are likely to be regulated at multiple levels (transcriptional, post-transcriptional, activity) by intricate feed-forward and feed-back schemes that often integrate the Cpx response with other cellular signaling networks. Elucidating these circuits has important implications for the design of synthetic networks that can be used to manipulate microbes for our own purposes [92,125,126]. Further investigation into the molecular mechanisms behind the regulation of Cpx-dependent adaptive functions will undoubtedly uncover more regulators and define further connections. At a more local level, the solved structures of the signaling proteins will guide future structure function studies aimed at uncovering signal sensing mechanisms. It will be interesting to see if further auxiliary signaling molecules are identified, and what they tell us about the molecular nature of the inducing cue, the most illusive aspect of our understanding. The ability to study reconstituted signaling pathways, as well as in vivo phosphorylation events, will facilitate these studies [78,127]. Ultimately, a detailed understanding of the Cpx envelope stress response will feed our current understanding of important and wide-spread events involved in microbial life, including biofilm formation, antibiotic resistance, and disease causation. This understanding is already being used to facilitate vaccine development [128,129]

and might one day permit the development of new therapeutics for the treatment of the numerous and wide-spread diseases caused by Gram negative enterobacteriaceae.

Acknowledgements

Work in the Raivio lab is supported by operating grants from the Canadian Institute of Health Research (97819) and the Natural Sciences and Engineering Research Council (RGPIN 238422-2013). TLR is supported by a Senior Scholar Award from Alberta Innovates Health Solutions.

References

[1] J. McEwen, P. Silverman, Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 513–517.
[2] C.A. Plate, Mutant of *Escherichia coli* defective in response to colicin K and in active transport, J. Bacteriol. 125 (1976) 467–474.
[3] S.H. Thorbjarnardottir, R.A. Magnusdottir, G. Eggertsson, Mutations determining generalized resistance to aminoglycoside antibiotics in *Escherichia coli*, Mol. Gen. Genet. 161 (1978) 89–98.
[4] J.F. Morris, E.B. Newman, Map location of the ssd mutation in *Escherichia coli* K-12, J. Bacteriol. 143 (1980) 1504–1505.
[5] E.B. Newman, J.F. Morris, C. Walker, V. Kapoor, A mutation affecting L-serine and energy metabolism in *E. coli* K12, Mol. Gen. Genet. 182 (1981) 143–147.
[6] A. Sutton, T. Newman, J. McEwen, P.M. Silverman, M. Freundlich, Mutations in genes cpxA and cpxB of *Escherichia coli* K-12 cause a defect in acetohydroxyacid synthase I function in vivo, J. Bacteriol. 151 (1982) 976–982.
[7] J. McEwen, P.M. Silverman, Mutations in genes *cpxA* and *cpxB* of *Escherichia coli* K-12 cause a defect in isoleucine and valine syntheses, J. Bacteriol. 144 (1980) 68–73.
[8] J. McEwen, L. Sambucetti, P.M. Silverman, Synthesis of outer membrane proteins in *cpxA cpxB* mutants of *Escherichia coli* K-12, J. Bacteriol. 154 (1983) 375–382.
[9] J. McEwen, P.M. Silverman, Mutations in genes *cpxA* and *cpxB* alter the protein composition of *Escherichia coli* inner and outer membranes, J. Bacteriol. 151 (1982) 1553–1559.
[10] S. Rainwater, P.M. Silverman, The Cpx proteins of *Escherichia coli* K-12: evidence that cpxA, ecfB, ssd, and eup mutations all identify the same gene, J. Bacteriol. 172 (1990) 2456–2461.
[11] J. Dong, S. Iuchi, H.S. Kwan, Z. Lu, E.C. Lin, The deduced amino-acid sequence of the cloned cpxR gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of *Escherichia coli*, Gene 136 (1993) 227–230.
[12] R.F. Weber, P.J. Silverman, The Cpx proteins of *Escherichia coli* K-12: structure of the CpxA polypeptide as an inner membrane component, J. Mol. Biol. 203 (1988) 467–476.
[13] C.L. Cosma, P.N. Danese, J.H. Carlson, T.J. Silhavy, W.B. Snyder, Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope-associated stresses, Mol. Microbiol. 18 (1995) 491–505.
[14] W.B. Snyder, L.J. Davis, P.N. Danese, C.L. Cosma, T.J. Silhavy, Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway, J. Bacteriol. 177 (1995) 4216–4223.
[15] P.N. Danese, T.J. Silhavy, The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*, Genes Dev. 11 (1997) 1183–1193.
[16] P.N. Danese, T.J. Silhavy, CpxP, a stress-combative member of the Cpx regulon, J. Bacteriol. 180 (1998) 831–839.
[17] P.N. Danese, W.B. Snyder, C.L. Cosma, L.J. Davis, T.J. Silhavy, The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP, Genes Dev. 9 (1995) 387–398.
[18] J. Pogliano, A.S. Lynch, D. Belin, E.C. Lin, J. Beckwith, Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system, Genes Dev. 11 (1997) 1169–1182.
[19] T.L. Raivio, M.W. Laird, J.C. Joly, T.J. Silhavy, Tethering of CpxP to the inner membrane prevents spheroplast induction of the Cpx envelope stress response, Mol. Microbiol. 37 (2000) 1186–1197.
[20] T.L. Raivio, T.J. Silhavy, The sigmaE and Cpx regulatory pathways: overlapping but distinct envelope stress responses, Curr. Opin. Microbiol. 2 (1999) 159–165.
[21] T.L. Raivio, T.J. Silhavy, Sensing and responding to envelope stress, in: G. Storz, R. Hengge-Aronis (Eds.), Bacterial Stress Responses, ASM Press, Washington, DC, 2000, pp. 19–32.
[22] T.L. Raivio, T.J. Silhavy, Periplasmic stress and ecf sigma factors, Annu. Rev. Microbiol. 55 (2001) 591–624.
[23] S.L. Vogt, T.L. Raivio, Just scratching the surface: an expanding view of the Cpx envelope stress response, FEMS Microbiol. Lett. 326 (2012) 2–11.
[24] S. Bury-Mone, Y. Nomane, N. Reymond, R. Barbet, E. Jacquet, S. Imbeaud, A. Jacq, P. Bouloc, Global analysis of extracytoplasmic stress signaling in *Escherichia coli*, PLoS Genet. 5 (2009) e1000651.
[25] C.H. Jones, P.N. Danese, J.S. Pinkner, T.J. Silhavy, S.J. Hultgren, The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems, EMBO J. 21 (1997) 6394–6406.
[26] G. Jubelin, A. Vianney, C. Beloin, J.M. Ghigo, J.C. Lazzaroni, P. Lejeune, C. Dorel, CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*, J. Bacteriol. 187 (2005) 2038–2049.
[27] E. Mileykovskaya, W. Dowhan, The Cpx two-component signal transduction pathway is activated in *Escherichia coli* mutant strains lacking phosphatidylethanolamine, J. Bacteriol. 179 (1997) 1029–1034.
[28] H. Miyadai, K. Tanaka-Masuda, S. Matsuyama, H. Tokuda, Effects of lipoprotein overproduction on the induction of DegP (HtrA) involved in quality control in the *Escherichia coli* periplasm, J. Biol. Chem. 279 (2004) 39807–39813.
[29] A.Z. Nevesinjac, T.L. Raivio, The Cpx Envelope Stress Response Affects Expression of the Type IV Bundle-Forming Pili of Enteropathogenic *Escherichia coli*, J. Bacteriol. 187 (2005) 672–686.
[30] K. Otto, T.J. Silhavy, Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 2287–2292.
[31] N.L. Price, T.L. Raivio, Characterization of the Cpx regulon in *Escherichia coli* strain MC4100, J. Bacteriol. 191 (2009) 1798–1815.
[32] R.G. Raffa, T.L. Raivio, A third envelope stress signal transduction pathway in *Escherichia coli*, Mol. Microbiol. 45 (2002) 1599–1611.
[33] K. Yamamoto, A. Ishihama, Characterization of copper-inducible promoters regulated by CpxA/CpxR in *Escherichia coli*, Biosci. Biotechnol. Biochem. 70 (2006) 1688–1695.
[34] T.L. Raivio, T.J. Silhavy, Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system, J. Bacteriol. 179 (1997) 7724–7733.
[35] P.A. DiGiuseppe, T.J. Silhavy, Signal detection and target gene induction by the CpxRA two-component system, J. Bacteriol. 185 (2003) 2432–2440.
[36] D.R. Buelow, T.L. Raivio, Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP, J. Bacteriol. 187 (2005) 6622–6630.
[37] D.D. Isaac, J.S. Pinkner, S.J. Hultgren, T.J. Silhavy, The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP, Proc. Natl. Acad. Sci. U. S. A. 102 (49) (2005) 17775–17779.
[38] T.L. Raivio, D.L. Popkin, T.J. Silhavy, The Cpx envelope stress response is controlled by amplification and feedback inhibition, J. Bacteriol. 181 (1999) 5263–5272.
[39] D.M. MacRitchie, D.R. Buelow, N.L. Price, T.L. Raivio, Two-component signaling and gram negative envelope stress response systems, Adv. Exp. Med. Biol. 631 (2008) 80–110.
[40] N. Ruiz, T.J. Silhavy, Sensing external stress: watchdogs of the *Escherichia coli* cell envelope, Curr. Opin. Microbiol. 8 (2005) 122–126.
[41] J. McEwen, P. Silverman, Genetic analysis of *Escherichia coli* K-12 chromosomal mutants defective in expression of F-plasmid functions: identification of genes cpxA and cpxB, J. Bacteriol. 144 (1980) 60–67.
[42] S.L. Vogt, A.Z. Nevesinjac, R.M. Humphries, M.S. Donnenberg, G.D. Armstrong, T.L. Raivio, The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus, Mol. Microbiol. 76 (2010) 1095–1110.
[43] D. Gangaiah, X. Zhang, K.R. Fortney, B. Baker, Y. Liu, R.S. Munson Jr., S.M. Spinola, Activation of CpxRA in Haemophilus ducreyi Primarily Inhibits the Expression of Its Targets, Including Major Virulence Determinants, J. Bacteriol. 195 (2013) 3486–3502.
[44] M. Labandeira-Rey, C.A. Brautigam, E.J. Hansen, Characterization of the CpxRA regulon in Haemophilus ducreyi, Infect. Immun. 78 (2010) 4779–4791.
[45] T.L. Raivio, S.K. Leblanc, N.L. Price, The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity, J. Bacteriol. 195 (2013) 2755–2767.
[46] P. De Wulf, A.M. McGuire, X. Liu, E.C. Lin, Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in *Escherichia coli*, J. Biol. Chem. 277 (2002) 26652–26661.
[47] M. Labandeira-Rey, J.R. Mock, E.J. Hansen, Regulation of expression of the Haemophilus ducreyi LspB and LspA2 proteins by CpxR, Infect. Immun. 77 (2009) 3402–3411.
[48] L.E. Bryan, H.M. Van Den Elzen, Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria, Antimicrob. Agents Chemother. 12 (1977) 163–177.
[49] H. Hirakawa, K. Nishino, J. Yamada, T. Hirata, A. Yamaguchi, Beta-lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*, J. Antimicrob. Chemother. 52 (2003) 576–582.
[50] V.B. Srinivasan, G. Rajamohan, KpnEF, a new member of the Klebsiella pneumoniae cell envelope stress response regulon is a SMR-type efflux pump involved in broad spectrum antimicrobial resistance, Antimicrob. Agents Chemother. 57 (9) (2013) 4449–4462.
[51] V.B. Srinivasan, V. Vaidyanathan, A. Mondal, G. Rajamohan, Role of the two component signal transduction system CpxAR in conferring cefepime and chloramphenicol resistance in Klebsiella pneumoniae NTUH-K2044, PLoS One 7 (2012) e33777.
[52] B.W. Davies, M.A. Kohanski, L.A. Simmons, J.A. Winkler, J.J. Collins, G.C. Walker, Hydroxyurea induces hydroxyl radical-mediated cell death in *Escherichia coli*, Mol. Cell 36 (2009) 845–860.
[53] M.A. Kohanski, D.J. Dwyer, J.J. Collins, How antibiotics kill bacteria: from targets to networks, Nat. Rev. Microbiol. 8 (2010) 423–435.
[54] M.A. Kohanski, D.J. Dwyer, J. Wierzbowski, G. Cottarel, J.J. Collins, Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death, Cell 135 (2008) 679–690.

[55] T.F. Mahoney, T.J. Silhavy, The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics, J. Bacteriol. 195 (2013) 1869–1874.
[56] I. Debnath, J.P. Norton, A.E. Barber, E.M. Ott, B.K. Dhakal, R.R. Kulesus, M.A. Mulvey, The Cpx stress response system potentiates the fitness and virulence of uropathogenic *Escherichia coli*, Infect. Immun. 81 (2013) 1450–1459.
[57] B.D. Davis, L.L. Chen, P.C. Tai, Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 6164–6168.
[58] B. Ezraty, A. Vergnes, M. Banzhaf, Y. Duverger, A. Huguenot, A.R. Brochado, S.Y. Su, L. Espinosa, L. Loiseau, B. Py, A. Typas, F. Barras, Fe–S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway, Science 340 (2013) 1583–1587.
[59] D.R. Kashyap, M. Wang, L.H. Liu, G.J. Boons, D. Gupta, R. Dziarski, Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems, Nat. Med. 17 (2011) 676–683.
[60] A. Kihara, Y. Akiyama, K. Ito, Different pathways for protein degradation by the FtsH/HflKC membrane-embedded protease complex: an implication from the interference by a mutant form of a new substrate protein, YccA, J. Mol. Biol. 279 (1998) 175–188.
[61] A. Kihara, Y. Akiyama, K. Ito, FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 4532–4536.
[62] J. van Stelten, F. Silva, D. Belin, T.J. Silhavy, Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY, Science 325 (2009) 753–756.
[63] A. Hinz, S. Lee, K. Jacoby, C. Manoil, Membrane proteases and aminoglycoside antibiotic resistance, J. Bacteriol. 193 (2011) 4790–4797.
[64] M.A. Kohanski, D.J. Dwyer, B. Hayete, C.A. Lawrence, J.J. Collins, A common mechanism of cellular death induced by bactericidal antibiotics, Cell 130 (2007) 797–810.
[65] Y. Liu, J.A. Imlay, Cell death from antibiotics without the involvement of reactive oxygen species, Science 339 (2013) 1210–1213.
[66] N. Weatherspoon-Griffin, G. Zhao, W. Kong, Y. Kong, Morigen, H. Andrews-Polymenis, M. McClelland, Y. Shi, The CpxR/CpxA Two-component System Up-regulates Two Tat-dependent Peptidoglycan Amidases to Confer Bacterial Resistance to Antimicrobial Peptide, J. Biol. Chem. 286 (2011) 5529–5539.
[67] S.D. Rinker, M.P. Trombley, X. Gu, K.R. Fortney, M.E. Bauer, Deletion of mtrC in Haemophilus ducreyi increases sensitivity to human antimicrobial peptides and activates the CpxRA regulon, Infect. Immun. 79 (2011) 2324–2334.
[68] S. Yitzhaki, J.E. Rostron, Y. Xu, M.C. Rideout, R.N. Authement, S.B. Barlow, A.M. Segall, Similarities between Exogenously- and Endogenously-Induced Envelope Stress: The Effects of a New Antibacterial Molecule, TPI1609-10, PLoS One 7 (2012) e44896.
[69] H. Hirakawa, K. Nishino, T. Hirata, A. Yamaguchi, Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*, J. Bacteriol. 185 (2003) 1851–1856.
[70] E. Batchelor, D. Walthers, L.J. Kenney, M. Goulian, The *Escherichia coli* CpxA–CpxR envelope stress response system regulates expression of the porins ompF and ompC, J. Bacteriol. 187 (2005) 5723–5731.
[71] H. Gerken, E.S. Charlson, E.M. Cicirelli, L.J. Kenney, R. Misra, MzrA: a novel modulator of the EnvZ/OmpR two-component regulon, Mol. Microbiol. 72 (2009) 1408–1422.
[72] H. Gerken, R. Misra, MzrA–EnvZ interactions in the periplasm influence the EnvZ/OmpR two-component regulon, J. Bacteriol. 192 (2010) 6271–6278.
[73] H. Hirakawa, Y. Inazumi, T. Masaki, T. Hirata, A. Yamaguchi, Indole induces the expression of multidrug exporter genes in *Escherichia coli*, Mol. Microbiol. 55 (2005) 1113–1126.
[74] C.A. Plate, S.A. Seely, T.G. Laffler, Evidence for a protonmotive force related regulatory system in *Escherichia coli* and its effects on lactose transport, Biochemistry 25 (1986) 6127–6132.
[75] C.A. Plate, J.L. Suit, The eup genetic locus of *Escherichia coli* and its role in H+/solute symport, J. Biol. Chem. 256 (1981) 12974–12980.
[76] E.C. Hobbs, X. Yin, B.J. Paul, J.L. Astarita, G. Storz, Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects antibiotic resistance, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 16696–16701.
[77] R. Fleischer, R. Heermann, K. Jung, S. Hunke, Purification, reconstitution, and characterization of the CpxRAP envelope stress system of *Escherichia coli*, J. Biol. Chem. 282 (2007) 8583–8593.
[78] J. Liu, E.J. Thanikkal, I.R. Obi, M.S. Francis, Elevated CpxR P levels repress the Ysc–Yop type III secretion system of Yersinia pseudotuberculosis, Res. Microbiol. 163 (2012) 518–530.
[79] A.J. Wolfe, N. Parikh, B.P. Lima, B. Zemaitaitis, Signal integration by the two-component signal transduction response regulator CpxR, J. Bacteriol. 190 (2008) 2314–2322.
[80] S. Quan, P. Koldewey, T. Tapley, N. Kirsch, K.M. Ruane, J. Pfizenmaier, R. Shi, S. Hofmann, L. Foit, G. Ren, U. Jakob, Z. Xu, M. Cygler, J.C. Bardwell, Genetic selection designed to stabilize proteins uncovers a chaperone called Spy, Nat. Struct. Mol. Biol. 18 (2011) 262–269.
[81] X. Zhou, R. Keller, R. Volkmer, N. Krauss, P. Scheerer, S. Hunke, Structural basis for two-component system inhibition and pilus sensing by the auxiliary CpxP protein, J. Biol. Chem. 286 (2011) 9805–9814.
[82] Y. Hirano, M.M. Hossain, K. Takeda, H. Tokuda, K. Miki, Structural studies of the Cpx pathway activator NlpE on the outer membrane of *Escherichia coli*, Structure 15 (2007) 963–976.
[83] L. Slamti, M.K. Waldor, Genetic analysis of activation of the Vibrio cholerae Cpx pathway, J. Bacteriol. 191 (2009) 5044–5056.
[84] G.L. Thede, D.C. Arthur, R.A. Edwards, D.R. Buelow, J.L. Wong, T.L. Raivio, J.N. Glover, Structure of the periplasmic stress response protein CpxP, J. Bacteriol. 193 (2011) 2149–2157.
[85] E. Kwon, D.Y. Kim, T.D. Ngo, C.A. Gross, J.D. Gross, K.K. Kim, The crystal structure of the periplasmic domain of Vibrio parahaemolyticus CpxA, Protein Sci. 21 (2012) 1334–1343.
[86] G. Grass, B. Fricke, D.H. Nies, Control of expression of a periplasmic nickel efflux pump by periplasmic nickel concentrations, Biometals 18 (2005) 437–448.
[87] G. Grass, C. Grosse, D.H. Nies, Regulation of the cnr cobalt and nickel resistance determinant from Ralstonia sp. strain CH34, J. Bacteriol. 182 (2000) 1390–1398.
[88] C. Appia-Ayme, A. Hall, E. Patrick, S. Rajadurai, T.A. Clarke, G. Rowley, ZraP is a periplasmic molecular chaperone and a repressor of the zinc-responsive two-component regulator ZraSR, Biochem. J. 442 (2012) 85–93.
[89] A. Itou, K. Matsumoto, H. Hara, Activation of the Cpx phosphorelay signal transduction system in acidic phospholipid-deficient pgsA mutant cells of *Escherichia coli*, Biochem. Biophys. Res. Commun. 421 (2012) 296–300.
[90] P. Wang, A. Kuhn, R.E. Dalbey, Global change of gene expression and cell physiology in YidC-depleted *Escherichia coli*, J. Bacteriol. 192 (2010) 2193–2209.
[91] P.N. Danese, G.R. Oliver, K. Barr, G.D. Bowman, P.D. Rick, T.J. Silhavy, Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*, J. Bacteriol. 180 (1998) 5875–5884.
[92] E.J. Clarke, C.A. Voigt, Characterization of combinatorial patterns generated by multiple two-component sensors in *E. coli* that respond to many stimuli, Biotechnol. Bioeng. 108 (2011) 666–675.
[93] B.J. Rutherford, R.H. Dahl, R.E. Price, H.L. Szmidt, P.I. Benke, A. Mukhopadhyay, J.D. Keasling, Functional genomic study of exogenous n-butanol stress in *Escherichia coli*, Appl. Environ. Microbiol. 76 (2010) 1935–1945.
[94] D. Zahrl, M. Wagner, K. Bischof, G. Koraimann, Expression and assembly of a functional type IV secretion system elicit extracytoplasmic and cytoplasmic stress responses in *Escherichia coli*, J. Bacteriol. 188 (2006) 6611–6621.
[95] Y.M. Lee, P.A. DiGiuseppe, T.J. Silhavy, S.J. Hultgren, P pilus assembly motif necessary for activation of the CpxRA pathway by PapE in *Escherichia coli*, J. Bacteriol. 186 (2004) 4326–4337.
[96] N. Joly, C. Engl, G. Jovanovic, M. Huvet, T. Toni, X. Sheng, M.P. Stumpf, M. Buck, Managing membrane stress: the phage shock protein (Psp) response, from molecular mechanisms to physiology, FEMS Microbiol. Rev. 34 (2010) 797–827.
[97] S.K. Leblanc, C.W. Oates, T.L. Raivio, Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*, J. Bacteriol. 193 (2011) 3367–3375.
[98] J.L. Rosner, R.G. Martin, Reduction of cellular stress by TolC-dependent efflux pumps in *Escherichia coli* indicated by BaeSR and CpxARP activation of spy in efflux mutants, J. Bacteriol. 195 (2013) 1042–1050.
[99] P. De Wulf, O. Kwon, E.C. Lin, The CpxRA signal transduction system of *Escherichia coli*: growth-related autoactivation and control of unanticipated target operons, J. Bacteriol. 181 (1999) 6772–6778.
[100] B.P. Lima, H. Antelmann, K. Gronau, B.K. Chi, D. Becher, S.R. Brinsmade, A.J. Wolfe, Involvement of protein acetylation in glucose-induced transcription of a stress-responsive promoter, Mol. Microbiol. 81 (2011) 1190–1204.
[101] N. Majdalani, S. Chen, J. Murrow, K. St John, S. Gottesman, Regulation of RpoS by a novel small RNA: the characterization of RprA, Mol. Microbiol. 39 (2001) 1382–1394.
[102] R. Sikdar, A.R. Simmons, W.T. Doerrler, Multiple envelope stress response pathways are activated in an *Escherichia coli* strain with mutations in two members of the DedA membrane protein family, J. Bacteriol. 195 (2013) 12–24.
[103] N. De Lay, S. Gottesman, The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior, J. Bacteriol. 191 (2009) 461–476.
[104] F. Zahringer, E. Lacanna, U. Jenal, T. Schirmer, A. Boehm, Structure and signaling mechanism of a zinc-sensory diguanylate cyclase, Structure 21 (2013) 1149–1157.
[105] A. Boehm, S. Steiner, F. Zaehringer, A. Casanova, F. Hamburger, D. Ritz, W. Keck, M. Ackermann, T. Schirmer, U. Jenal, Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress, Mol. Microbiol. 72 (2009) 1500–1516.
[106] K. Jonas, A.N. Edwards, R. Simm, T. Romeo, U. Romling, O. Melefors, The RNA binding protein CsrA controls cyclic di-GMP metabolism by directly regulating the expression of GGDEF proteins, Mol. Microbiol. 70 (2008) 236–257.
[107] C. Dorel, P. Lejeune, A. Rodrigue, The Cpx system of *Escherichia coli*, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities? Res. Microbiol. 157 (2006) 306–314.
[108] Q. Ma, T.K. Wood, OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxRA two-component system, Environ. Microbiol. 11 (2009) 2735–2746.
[109] M.M. Barnhart, M.R. Chapman, Curli biogenesis and function, Annu. Rev. Microbiol. 60 (2006) 131–147.
[110] C. Dorel, O. Vidal, C. Prigent-Combaret, I. Vallet, P. Lejeune, Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation, FEMS Microbiol. Lett. 178 (1999) 169–175.
[111] C. Prigent-Combaret, E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, C. Dorel, Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the csgD gene, J. Bacteriol. 183 (2001) 7213–7223.
[112] F. Mika, S. Busse, A. Possling, J. Berkholz, N. Tschowri, N. Sommerfeldt, M. Pruteanu, R. Hengge, Targeting of csgD by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in *Escherichia coli*, Mol. Microbiol. 84 (2012) 51–65.

[113] E. Brombacher, A. Baratto, C. Dorel, P. Landini, Gene expression regulation by the Curli activator CsgD protein: modulation of cellulose biosynthesis and control of negative determinants for microbial adhesion, J. Bacteriol. 188 (2006) 2027–2037.
[114] N. Majdalani, D. Hernandez, S. Gottesman, Regulation and mode of action of the second small RNA activator of RpoS translation, RprA, Mol. Microbiol. 46 (2002) 813–826.
[115] T.F. Mah, G.A. O'Toole, Mechanisms of biofilm resistance to antimicrobial agents, Trends Microbiol. 9 (2001) 34–39.
[116] E. Holmqvist, C. Unoson, J. Reimegard, E.G. Wagner, A mixed double negative feedback loop between the sRNA MicF and the global regulator Lrp, Mol. Microbiol. 84 (2012) 414–427.
[117] N. Delihas, S. Forst, MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors, J. Mol. Biol. 313 (2001) 1–12.
[118] N. Majdalani, S. Gottesman, The Rcs phosphorelay: a complex signal transduction system, Annu. Rev. Microbiol. 59 (2005) 379–405.
[119] A. Kato, H. Hayashi, W. Nomura, H. Emori, K. Hagihara, R. Utsumi, A connecter-like factor, CacA, links RssB/RpoS and the CpxR/CpxA two-component system in Salmonella, BMC Microbiol. 12 (2012) 224.
[120] G. Ren, J.C. Bardwell, Engineered pathways for correct disulfide bond oxidation, Antioxid. Redox Signal. 14 (2011) 2399–2412.
[121] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Industrial biocatalysis today and tomorrow, Nature 409 (2001) 258–268.
[122] H.E. Schoemaker, D. Mink, M.G. Wubbolts, Dispelling the myths—biocatalysis in industrial synthesis, Science 299 (2003) 1694–1697.
[123] Y. Zhou, T. Minami, K. Honda, T. Omasa, H. Ohtake, Systematic screening of *Escherichia coli* single-gene knockout mutants for improving recombinant whole-cell biocatalysts, Appl. Microbiol. Biotechnol. 87 (2010) 647–655.
[124] Y. Zhou, T. Minami, K. Honda, T. Omasa, H. Ohtake, Enhancement of recombinant enzyme activity in cpxA-deficient mutant of *Escherichia coli*, J. Biosci. Bioeng. 110 (2010) 403–407.
[125] J.M. Skerker, B.S. Perchuk, A. Siryaporn, E.A. Lubin, O. Ashenberg, M. Goulian, M.T. Laub, Rewiring the specificity of two-component signal transduction systems, Cell 133 (2008) 1043–1054.
[126] A. Siryaporn, B.S. Perchuk, M.T. Laub, M. Goulian, Evolving a robust signal transduction pathway from weak cross-talk, Mol. Syst. Biol. 6 (2010) 452.
[127] M. Miot, J.M. Betton, Reconstitution of the Cpx signaling system from cell-free synthesized proteins, New Biotechnol. 28 (2011) 277–281.
[128] K. Matsuda, A.A. Chaudhari, S.W. Kim, K.M. Lee, J.H. Lee, Physiology, pathogenicity and immunogenicity of lon and/or cpxR deleted mutants of Salmonella Gallinarum as vaccine candidates for fowl typhoid, Vet. Res. 41 (2010) 59.
[129] K. Matsuda, A.A. Chaudhari, J.H. Lee, Evaluation of safety and protection efficacy on cpxR and lon deleted mutant of Salmonella Gallinarum as a live vaccine candidate for fowl typhoid, Vaccine 29 (2011) 668–674.