MINIREVIEW

Signal integration by the Cpx-envelope stress system

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Abstract

The Cpx-envelope stress system coordinates the expression and assembly of surface structures important for the virulence of Gram-negative pathogenic bacteria. It is comprised of the membrane-anchored sensor kinase CpxA, the cytosolic response regulator CpxR and the accessory protein CpxP. Characteristic of the group of two-component systems, the Cpx system responds to a broad range of stimuli including pH, salt, metals, lipids and misfolded proteins that cause perturbation in the envelope. Moreover, the Cpx system has been linked to inter-kingdom signalling and bacterial cell death. However, although signal specificity has been assumed, for most signals the mechanism of signal integration is not understood. Recent structural and functional studies provide the first insights into how CpxP inhibits CpxA and serves as sensor for misfolded pilus subunits, pH and salt. Here, we summarize and reflect on the current knowledge on signal integration by the Cpx-envelope stress system.

Introduction

The cell envelope of Gram-negative bacteria is composed of the inner membrane, the periplasmic space and the outer membrane and completed by exposed surface structures like flagella, porins, secretion systems and adhesins (Silhavy *et al.*, 2010). These complex structures are composed of several protein subunits, all of which require tight control of their synthesis, export, folding and assembly process for final functional structure formation (Ruiz & Silhavy, 2005). Stresses that interfere with these processes activate the Cpx-envelope stress system (Fig. 1; reviewed in MacRitchie *et al.*, 2008), which responds not only by regulating the expression of folding factors and proteases in the envelope to deal with the misfolded proteins but also by inhibiting the expression of the surface structures (Dorel *et al.*, 2006; Vogt *et al.*, 2010). Because these surface structures include important virulence determinants such as adhesins and secretion systems, the Cpx system contributes to virulence in several Gram-negative species (Raivio, 2005; Rowley *et al.*, 2006).

The Cpx system belongs to the group of two-component signal transduction systems (TCSs) and is made up of the senor kinase (SK) CpxA, the response regulator (RR) CpxR and the periplasmic accessory inhibitor CpxP (Fig. 1; Ruiz & Silhavy, 2005; Buelow & Raivio, 2010), which provides response to additional stimuli (Buelow & Raivio, 2010; Heermann & Jung, 2010; Krell *et al.*, 2010). Three phosphotransfer reactions are involved in controlling the functional state of the Cpx-TCS: (1) the autophosphorylation of a conserved histidine of the SK CpxA, (2) the transphosphorylation of a conserved aspartate of the RR CpxR and (3) the dephosphorylation of phosphorylated RR to return the system back to the prestimulated resting state (Gao & Stock, 2009). Importantly, the balance between phosphorylated and dephosphorylated RRs is crucial not only for the initiation of a specific genetic response to the external stimulus but also for its duration (Stock *et al.*, 2000; Dorel *et al.*, 2006).

It has been suggested that all inducing cues involve misfolded envelope proteins as the actual common stimulus for the Cpx-TCS (Raivio & Silhavy, 2001) and/or dissociation of the inhibitory CpxP from CpxA (Rowley *et al.*, 2006), as well as signal specificity for the Cpx response (DiGiuseppe & Silhavy, 2003; Hunke & Betton, 2003; Ruiz & Silhavy, 2005). However, where and how

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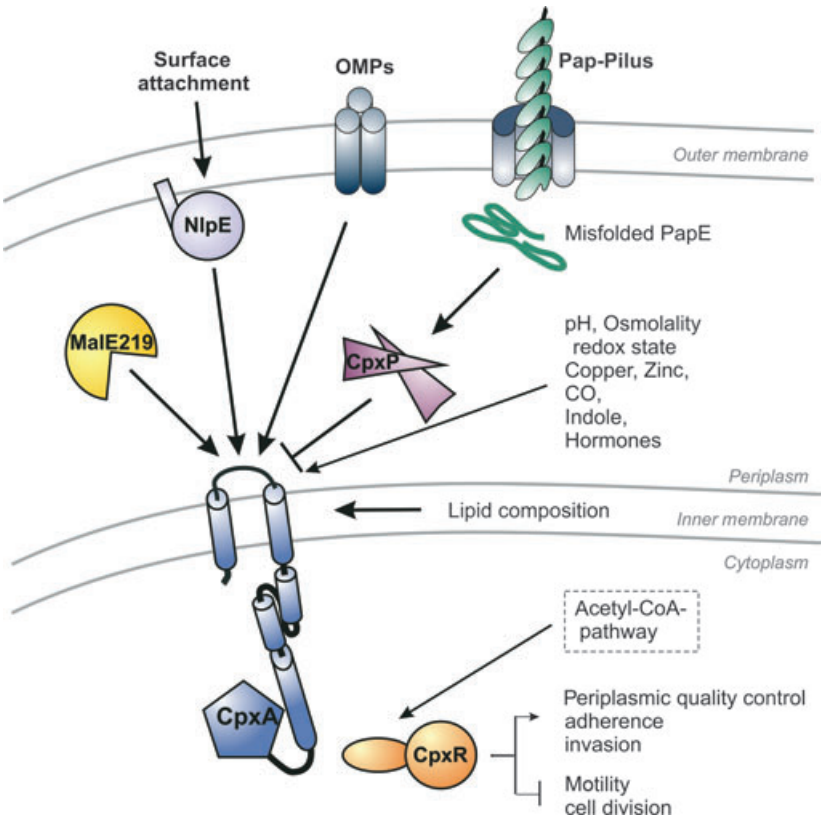

Fig. 1. Signal integration of diverse stimuli by the Cpx-envelope stress system. The Cpx-envelope stress system can be induced dependent or independent of the accessory proteins CpxP and NlpE. CpxP is involved in the sensing of misfolded proteins as PapE, salt and alkaline pH. NlpE is required for the Cpx-dependent adhesion to hydrophobic surfaces. The misfolded variant of the maltose-binding protein MalE219 induces the Cpx-envelope stress system independent from NlpE and CpxP. Activated CpxA transphosphorylates CpxR. Alternatively, intermediates of the acetyl-CoA pathway result in CpxA-independent phosphorylation of CpxR. Phosphorylated CpxR acts as a transcription factor for, among others, genes encoding surface structures and folding factors.

the independent entry points for this signalling system take place has to be addressed.

Structural and functional features of the sensor kinase CpxA and the RR CpxR

The pivotal factor of the Cpx-TCS is CpxA with its central function as a sensor kinase. Sequence alignments revealed that CpxA belongs to class I SK (Grebe & Stock, 1998; Dutta *et al.*, 1999), typically consisting of two transmembrane domains (TMDs) integrating a large periplasmic domain and a cytoplasmic, highly conserved kinase core that acts as a transmitter domain (Fig. 2). The cytosolic domain includes a HAMP domain, which links the second TMD of CpxA with its kinase core (Appleman *et al.*, 2003; Khorchid & Ikura, 2006), which consists of two amphipathic helices joined by a loop (Aravind & Ponting, 1999; Stewart & Chen, 2010). HAMP domains are assumed to act as a link transmitting the signal from the sensor domain to the kinase core (Cheung & Hendrickson, 2010). The kinase core is composed of a DHp domain and a C-terminal CA domain (MacRitchie *et al.*, 2008). The autophosphorylation site of the *Escherichia coli* CpxA is H248 (Fig. 2). The SK acts in a dimeric state (Gao & Stock, 2009), which is achieved by the DHp domains forming a four-helix bundle that constitutes the stem of the kinase core (Casino *et al.*, 2009). The isolated kinase core of CpxA exhibits both kinase and phosphatase activities (Raivio & Silhavy, 1997; Yamamoto & Ishihama, 2005). To understand signal integration by the sensory domain, the analysis of the reconstituted activities of full-length CpxA was indispensable (Fleischer *et al.*, 2007). The sensory domain of most membrane integral SKs is formed by an extracytoplasmic loop (Mascher *et al.*, 2006). Consistent with this, CpxA* gain of function variants with mutations in the periplasmic sensory domain (PSD) are insensitive to certain stimuli *in vivo* (Ruiz & Silhavy, 2005). Mutational analysis revealed that different regions of the PSD impact the kinase activities *in vitro* (Keller *et al.*, 2011). However, the

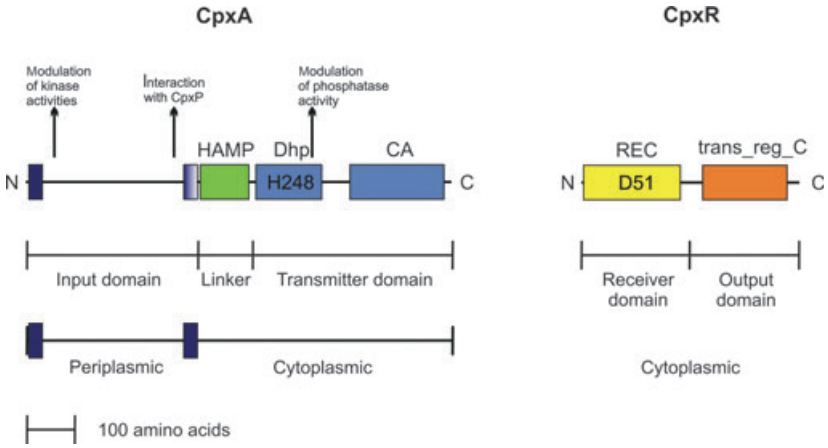

Fig. 2. Domain structure of the sensor kinase CpxA and the response regulator CpxR. The sensor kinases CpxA consists of two conserved domains, the Dhp domain (dimerization and histidine phosphotransfer; light blue) and the CA domain (catalytic; light blue) as conserved domains. The HAMP domain (green) links the signal from the input to the transmitter domain. TMDs are shaded in dark blue. CpxR consist of an N-terminal receiver domain (REC; yellow) fused to a C-terminal DNA-binding output domain (trans_reg_C; orange). The model of CpxA is based on a hydropathy plot (Weber & Silverman, 1988).

PSD of CpxA does not consist of any of the described discrete structural classes (reviewed in Cheung & Hendrickson, 2010), which corresponds to distinct signals that are recognized. In addition to the PSD, the TMD of CpxA might be also involved in signal integration (Mileykovskaya & Dowhan, 1997).

CpxR, the cytosolic, cognate RR of CpxA, belongs to the transcription factors of the OmpR/PhoB subfamily (Fig. 2; Dong *et al.*, 1993; Galperin *et al.*, 2001; Kenney, 2002). CpxR consists of an N-terminal receiver domain (REC) with an aspartate (D51) as the site of phosphorylation and an C-terminal effector domain that mediates the output response as a transcriptional regulator of target genes (MacRitchie *et al.*, 2008). Both domains are linked through a flexible linker region (Tapparel *et al.*, 2006). In its phosphorylated state, DNA binding occurs through a winged helix–turn–helix motif (Galperin, 2006) with 5′-GTAAA(n5)GTAAA-3′ as its consensus recognition sequence (Pogliano *et al.*, 1997). Inactivation of CpxR is achieved either by the phosphatase activity of CpxA or by the Ser/Thr phosphatase PrpA (Missiakas & Raina, 1997; Raivio & Silhavy, 1997).

CpxP: the third component of the Cpx-envelope stress system

The Cpx system consists of an additional third component, the periplasmic, accessory CpxP protein (Fig. 1; Danese & Silhavy, 1998; MacRitchie *et al.*, 2008). As an accessory protein of the TCS (Buelow & Raivio, 2010; Heermann & Jung, 2010), CpxP is also involved in the signalling process (Danese & Silhavy, 1998). Overproduction of periplasmic localized CpxP protein down-regulates the Cpx signalling cascade (Raivio *et al.*, 1999). Thus, as *cpxP* belongs to the Cpx regulon, CpxP acts as a negative feedback regulator for the Cpx pathway (Raivio *et al.*, 1999). In addition, CpxP protein level depends not only on its transcription but also on post-transcriptional mechanisms (Buelow & Raivio, 2005; Isaac *et al.*, 2005; Miot & Betton, 2007).

CpxP has no obligatory function for the induction of the Cpx response (Raivio *et al.*, 1999; DiGiuseppe & Silhavy, 2003). However, the *cpxP* gene was identified as a CpxR target involved in inhibiting the expression of toxic envelope proteins, including misfolded pilus subunits of P-pili that are crucial for uropathogenic *E. coli* (UPEC) during kidney colonization (Jones *et al.*, 1997; Danese *et al.*, 1998; Hung *et al.*, 2001; Isaac *et al.*, 2005). In agreement with its function in quality control for subunits of surface appendages, CpxP is also involved in the early steps of biofilm formation (Beloin *et al.*, 2004; Yang *et al.*, 2008).

Biochemical analysis of the reconstituted CpxAR phosphorylation cascade demonstrated that CpxP, incorporated into the lumen of the proteoliposomes, inhibits the autophosphorylation of CpxA (Fleischer *et al.*, 2007). As the reconstituted system excludes the involvement of other factors, this finding indicates a direct protein–protein interaction between CpxP and CpxA (Fleischer *et al.*, 2007; Zhou *et al.*, 2011). In support of this, peptide library screens showed that the purified PSD of CpxA directly interacts with CpxP (Zhou *et al.*, 2011). Interestingly, the interaction of purified CpxP with peptides derived from the PSD of CpxA depends on negative charges within this domain (Zhou *et al.*, 2011). The crystal structure of CpxP gave further insight into this

interaction (Thede *et al.*, 2011; Zhou *et al.*, 2011). CpxP consists of a dimer, the monomers of which are interwined like 'left hands' (Thede *et al.*, 2011; Zhou *et al.*, 2011). Thereby, each monomer is strengthened by double hydrogen bonds between two highly conserved LTxxQ repeat motifs. Based on the structural and biochemical analysis, CpxP-mediated Cpx inhibition results from an interaction between the concave polar surface of CpxP and the negatively charged sensor domain of CpxA (Fig. 3a; Zhou *et al.*, 2011). The CpxP dimer acts as a patch to shield the CpxA sensor domain from inducing signals, maintaining the SK in an 'off' mode. Moreover, the structure of CpxP provides explanations of how CpxP might act as a sensor for salt (Zhou *et al.*, 2011), pH (Thede *et al.*, 2011) and misfolded pilus subunits (Zhou *et al.*, 2011) for the Cpx system.

Physicochemical and chemical stimuli for the Cpx system

Physicochemical and chemical stimuli inducing the Cpx response include alkaline pH, salt (Raivio & Silhavy, 1997), depletion of the major lipid phosphatidylethanolamine (Mileykovskaya & Dowhan, 1997), attachment to hydrophobic surfaces (Otto & Silhavy, 2002), intermediates of the acetyl-CoA pathway (Wolfe *et al.*, 2008; Lima *et al.*, 2011), low cAMP levels (Strozen *et al.*, 2005), carbon monoxide (Davidge *et al.*, 2009), metals (Lee *et al.*, 2005; Yamamoto & Ishihama, 2006), indole (Raffa & Raivio, 2002), alcohols, acetone and the anaesthetics procaine and phenethyl alcohol (Clarke & Voigt, 2011; Table 1).

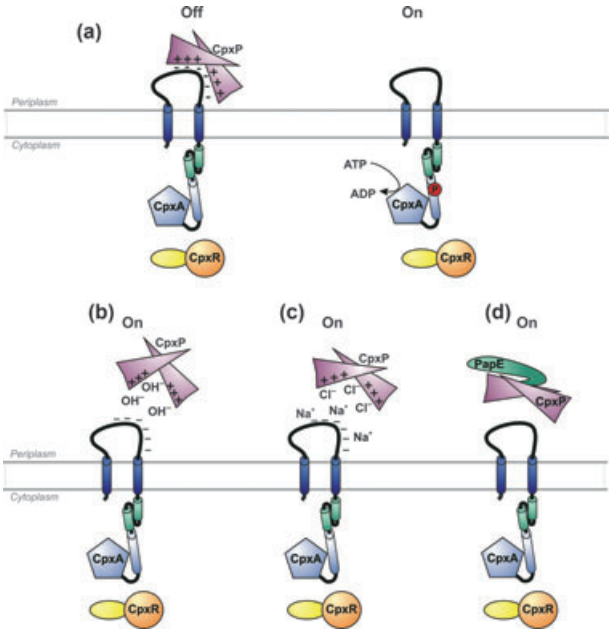

Fig. 3. CpxP-dependent signal integration by the Cpx-envelope stress system. (a) Polar interaction between the inner cavity of the CpxP dimer and CpxA keeps the sensor kinase in an 'off' mode (shown in a monomeric stage). Release of CpxP from CpxA switches CpxA to the 'on' mode (shown in a dimeric stage). (b) Release of CpxP from CpxA is suggested to result from conformational changes in CpxP induced by alkaline pH, (c) a high salt concentration that disturbs the polar interaction between the two proteins, (d) or by competing interaction of CpxP with misfolded P-pilus subunits. The colour scheme for CpxA and CpxR corresponds to the domain colour scheme in Fig. 2.

Although most of these stimuli result in protein misfolding, the specific signal integration of the Cpx-TCs might occur through different mechanisms. Because of their cytosolic localization, stimuli corresponding to variations in central metabolites are thought to affect the expression of CpxR targets in a CpxA-independent way (Strozen *et al.*, 2005; Wolfe *et al.*, 2008; Kinnersley *et al.*, 2009; Lima *et al.*, 2011). Decreased cAMP levels (Strozen *et al.*, 2005), glucose (Kinnersley *et al.*, 2009) and intermediates of the acetyl-CoA pathway (Wolfe *et al.*, 2008; Lima *et al.*, 2011) induce the expression of *degP* and *cpxP*, respectively. For intermediates of the acetyl-CoA pathway, two mechanisms exist: acetyl phosphate is known to act as a direct phosphor donor for CpxR *in vitro* (Raivio & Silhavy, 1997) and *in vivo* (Klein *et al.*, 2007; Groban *et al.*, 2009), and acetyl-CoA promotes the acetylation of RNA polymerase, which is critical for the glucose-dependent induction of *cpxP* transcription (Lima *et al.*, 2011).

In contrast to cytosolic stimuli, phosphatidylethanolamine depletion, indole, alcohols, acetone and phenethyl alcohol are likely sensed by the TMD of CpxA (Mileykovskaya & Dowhan, 1997; Garbe *et al.*, 2000; Rutherford *et al.*, 2010; Clarke & Voigt, 2011). All these stimuli are proposed to modulate the physical properties of the inner membrane (Dombek & Ingram, 1984) and result in conformational changes within the membrane helices of CpxA (Anbazhagan *et al.*, 2010). For phosphatidylethanolamine depletion, two specific mechanisms that result in the activation of CpxA are also conceivable: (1) direct influence by lipids and (2) indirect effects through alteration of a cell envelope component that is modified in a phosphatidylethanolamine-dependent manner such as LPS (Mileykovskaya & Dowhan, 1997). Alternatively, all these stimuli might influence CpxA in an indirect way by inducing misfolding of inner membrane proteins (Shimohata *et al.*, 2002, 2007; Akiyama, 2009).

Another Cpx-inducing signal that modulates the physical properties of the outer membrane is the attachment to hydrophobic surfaces (Otto & Silhavy, 2002). Surface attachment–induced Cpx activation depends on the outer membrane lipoprotein new lipoprotein E (NlpE; Otto & Silhavy, 2002), suggesting that NlpE might serve as a second accessory protein to deliver signalling information to CpxA.

Table 1. Signals inducing the Cpx-envelope stress system assigned to their cellular location

Stimulus	Mechanism/sensed by	References
Cytosol		
Acetyl-CoA level	CpxR Acetylation of the RNA polymerase	Wolfe *et al.* (2008), Lima *et al.* (2011)
cAMP level	CpxR	Strozen *et al.* (2005)
Inner membrane		
Phosphatidylethanolamine depletion	(CpxA)	Mileykovskaya & Dowhan (1997)
Δ*dsbD*	Aberrant disulphide bond formation	Slamti & Waldor (2009)
Δ*yidC*	(protein misfolding)	Shimohata *et al.* (2007), Wang *et al.* (2010)
Δ*ftsH*	(protein misfolding)	Shimohata *et al.* (2002)
YccA11	Inhibition of FtsH	Shimohata *et al.* (2002)
SecY	Accumulation	Shimohata *et al.* (2002)
F_oF_1 ATPase	Accumulation	Shimohata *et al.* (2002)
MalF	?	Mourez *et al.* (1997)
Periplasm		
CpxP	CpxA	Raivio *et al.* (1999), Fleischer *et al.* (2007), Zhou *et al.* (2011)
MalE219	CpxA	Hunke & Betton (2003), Keller & Hunke (2009)
LolA (I93C/F149C)	Aberrant disulphide bond formation	Tao *et al.* (2010)
Outer membrane		
NlpE	CpxA	Snyder *et al.* (1995), Hirano *et al.* (2007)
Lipoproteins	?	Miyadai *et al.* (2004)
OMPs	?	Gerken *et al.* (2010)
P-Pili	CpxP	Jones *et al.* (1997), Isaac *et al.* (2005), Zhou *et al.* (2011)
BFP	(CpxP)	Nevesinjac & Raivio (2005)
Curli	(CpxP)	Prigent-Combaret *et al.* (2001)
Attachment	NlpE	Otto & Silhavy (2002)
Environment		
pH	CpxP/?	Fleischer *et al.* (2007), Thede *et al.* (2011)
Salt	CpxP/?	Fleischer *et al.* (2007), Zhou *et al.* (2011)
Copper	(NlpE or aberrant disulphide bond formation)	Yamamoto & Ishihama (2006), Slamti & Waldor (2009)
Zinc	(CpxP)	Lee *et al.* (2005), Thede *et al.* (2011)
Indole	(protein misfolding)	Raffa & Raivio (2002), Clarke & Voigt (2011)
Alcohols	(protein misfolding)	Clarke & Voigt (2011)

The metals zinc (Lee *et al.*, 2005) and copper (Yamamoto & Ishihama, 2005) are excellent inducers of the Cpx system. Based on the presence of zinc in the CpxP crystal structure (Thede *et al.*, 2011) and the observation that CpxP shares high homology with the metal sensor CnrX (Grass *et al.*, 2000, 2005), it was suggested that CpxP might act as a zinc sensor (Thede *et al.*, 2011). In contrast, it has been suggested that sensing of copper by the Cpx-TCS occurs via NlpE (also known as copper homeostasis protein CutF), because mutation of *nlpE* results in a decrease in copper tolerance (Gupta *et al.*, 1995; Yamamoto & Ishihama, 2006).

Conditions such as alkaline pH and high salt concentrations, which result in activation of the Cpx system, are at least partially CpxP-dependent (Thede *et al.*, 2011; Zhou *et al.*, 2011). Alkaline pH induces a slight structural adjustment to a more compact form of the CpxP dimer that might not precisely fit within the sensor domain of CpxA (Fig. 3b; Thede *et al.*, 2011). High salt concentrations decrease the inhibitory effect of CpxP, most likely by disturbing the polar interactions between the positively charged inner surface of CpxP and the negatively charged sensor domain of CpxA (Fig. 3c; Zhou *et al.*, 2011). On the other hand, CpxA autophosphorylation can be induced by alkaline pH and salts independently of CpxP (Fleischer *et al.*, 2007), suggesting an additional CpxP-independent mechanism for CpxA activation by these stimuli.

Sensing of misfolded proteins by the Cpx-envelope stress system

Several observations support the notion that the Cpx-TCS senses protein misfolding in all regions of the bacterial envelope: the inner membrane, the periplasmic space and the outer membrane (Table 1).

The correct folding and insertion of membrane proteins into the inner membrane depends on

phosphatidylethanolamine, the SecYEG translocase and the YidC insertase (Dalbey *et al.*, 2011). Notably, phosphatidylethanolamine depletion (Mileykovskaya & Dowhan, 1997), mutations in the SecDF-YajC complex that links the SecYEG translocase with the YidC insertase (Shimohata *et al.*, 2007), and YidC depletion (Shimohata *et al.*, 2007; Wang *et al.*, 2010) induce the Cpx response. Moreover, the targeting of membrane proteins or the lack of insertion process does not induce the Cpx response, which suggests a secondary effect resulting from defective assembly machineries culminating in misfolded or misassembled membrane proteins (Shimohata *et al.*, 2007). Consistent with this, conditions that prevent quality control of the inner membrane induce the Cpx-TCS (Shimohata *et al.*, 2002; van Stelten *et al.*, 2009). For example, deletion of the membrane-bound AAA ATPase FtsH, one of the known quality control systems, activates the Cpx system (Shimohata *et al.*, 2002). FtsH expression is proposed to be inhibited by the inner membrane protein YccA (van Stelten *et al.*, 2009), which in turn is under Cpx-control (Yamamoto & Ishihama, 2005). In addition to general conditions that lead to misfolding of inner membrane proteins, some single inner membrane proteins have also been described to activate the Cpx-TCS (Table 1). However, the mechanism for sensing misfolded inner membrane proteins by the Cpx-TCS is currently unknown.

In general, periplasmic proteins are involved in activation of the Cpx-TCS owing to aggregation (Hunke & Betton, 2003), misfolding (Keller & Hunke, 2009) or incorrect disulphide bond formation (Slamti & Waldor, 2009). Variants of the maltose-binding protein that either form aggregates (MalE31) or are misfolded (MalE219) specifically induce the Cpx response (Hunke & Betton, 2003). In a reconstituted system, MalE219 was found to stimulate the phosphotransfer from CpxA to CpxR, indicating that this stimulus is directly sensed by the Cpx-TCS without the necessity of an accessory protein (Keller & Hunke, 2009). The inner membrane protein DsbD (Slamti & Waldor, 2009), part of an enzyme system involved in ensuring proper disulphide bond formation of secreted proteins (Kadokura & Beckwith, 2010), activates the Cpx system in *Vibrio cholerae*, suggesting that incorrect disulphide bond formation of proteins might act as a trigger of the Cpx-TCS (Slamti & Waldor, 2009). Likewise, incorrect disulphide bond formation of a variant of the periplasmic LolA protein (I93C/F149C) might induce the Cpx-TCS in a similar way (Tao *et al.*, 2010). However, LolA acts as a periplasmic chaperone for the lipid tail of outer membrane lipoproteins (Tokuda, 2009). For this process, a hydrophobic cavity of LolA is essential (Tokuda, 2009). Under oxidizing conditions, the hydrophobic cavity of LolA (I93C/F149C) is closed owing to disulphide bond formation between the two introduced cysteine residues (Watanabe *et al.*, 2008). Consequently, outer membrane sorting of lipoproteins is defective for LolA (I93C/F149C; Watanabe *et al.*, 2008) and might be the trigger for the Cpx-TCS (Tao *et al.*, 2010).

Outer membrane lipoproteins are a well-known stimulus for the Cpx system (Snyder *et al.*, 1995; Miyadai *et al.*, 2004; Fadl *et al.*, 2006). NlpE induces the Cpx-TCS, resulting in additional expression of the periplasmic protease DegP (Snyder *et al.*, 1995) and the periplasmic folding factors FkpA and of DsbA (Danese & Silhavy, 1997). Notably, overproduction of NlpE, referred as a specific Cpx stimulus, has been used to identify the Cpx-dependent expression of proposed regulon members (Vogt *et al.*, 2010). Activation of the Cpx-TCS by NlpE depends on lipidation but is independent of anchoring either in the outer or the inner membrane (Miyadai *et al.*, 2004). The structure of the soluble region of NlpE suggests that conformational changes in NlpE might result in direct interaction with CpxA (Hirano *et al.*, 2007). However, although it is clear that NlpE activates the Cpx-TCS in an CpxP-independent manner (Buelow & Raivio, 2010), the mechanism of Cpx-TCS activation by NlpE with respect to the impact of NlpE in sensing surface attachment and copper is unknown.

The Cpx-TCS has also been linked to the sensing of β-barrel outer membrane proteins (OMPs; Gerken *et al.*, 2010). Assembly-defective OMP variants and a defective OMP assembly machinery (Bam-complex) induce the Cpx regulon (Gerken *et al.*, 2010). However, CpxP appears not to be involved in the degradation of misfolded OMPs by DegP nor in the activation of the Cpx-TCS by misfolded OMPs (Gerken *et al.*, 2010).

The impact of the Cpx-TCS in sensing defects during the assembly of adhesive surface structures has been established for type IV bundle-forming pili (BFP) of enteropathogenic *E. coli* (EPEC; Nevesinjac & Raivio, 2005), the curli fimbriae of *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium; Prigent-Combaret *et al.*, 2001) and for the P-pili of UPEC (Jones *et al.*, 1997).

Bundle-forming pili are pivotal for EPEC to form microcolonies and to attach to host cells (Tobe & Sasakawa, 2001). The Cpx-TCS is induced by overexpression of the BFP subunit BfpA and by mature BFP (Nevesinjac & Raivio, 2005). This finding strongly indicates that intermediates other than unprocessed BfpA are also sensed by the Cpx-TCS (Nevesinjac & Raivio, 2005).

The Cpx system controls curli fimbriae expression, which are involved in forming surface amyloidal fibres important for biofilm formation and host cell adhesion (Dorel *et al.*, 1999; Jubelin *et al.*, 2005; Barnhart & Chapman, 2006), and curli overexpression induces the Cpx response (Prigent-Combaret *et al.*, 2001).

P-pili are crucial for kidney colonization by UPEC strains and belong to the group of chaperone-usher pili (CU pili; reviewed in Waksman & Hultgren, 2009). Essential for the formation of CU pili is a periplasmic chaperone that guides the single subunits after release from the SecYEG translocase across the periplasmic space to the usher in the outer membrane. Deletion of the chaperone PapD results in misfolded P-pilus subunits that become toxic for the cell and induces the Cpx response (Jones *et al.*, 1997). Overexpression of CpxP suppresses the lethal phenotype by causing the misfolded pilus subunits to be degraded by DegP (Isaac *et al.*, 2005). Because the induction of the Cpx-TCS by PapG does not depend on the DegP protease (Hung *et al.*, 2001) but rather on CpxP, it was suggested that PapG induces the release of CpxP from CpxA resulting in the activation of the Cpx-TCS (Fig. 3d; Isaac *et al.*, 2005). An elongated hydrophobic cleft on the convex surface of the CpxP dimer might act as a sensory part for pilus subunits (Zhou *et al.*, 2011). However, it remains mysterious which region of pilus subunits is recognized by CpxP. Only two pilus subunits are known to activate the Cpx-TCS: the PapG adhesin and the fibrillum subunit PapE (Jones *et al.*, 1997). It has been suggested that the N-terminal extension of PapE, which is essential for the assembly of pilus subunits, is crucial for recognition of PapE by CpxP (Lee *et al.*, 2004; Isaac *et al.*, 2005). However, PapG is missing an N-terminal extension that is present in the other subunits which are not recognized by the Cpx-TCS (Lee *et al.*, 2004).

New fields in Cpx-envelope stress signalling (inter-kingdom signalling and crosstalk)

Very recently, a crucial role of the Cpx system in inter-kingdom signalling between host and bacteria has been discovered (Karavolos *et al.*, 2011). In *S.* Typhi, exposure to host stress neuroendocrine hormones leads to increased haemolytic activity through the secretion of haemolysin HlyE-containing membrane vesicles (Karavolos *et al.*, 2011). This secretion of membrane vesicles results from the down-regulation of the levels of the outer membrane protein A (OmpA), which is mediated by the up-regulation of transcriptional repressors: the sRNA *micA* and the RNA chaperone Hfq (Karavolos *et al.*, 2011). In the absence of CpxA and CpxR, these repressors are down-regulated, and the level of OmpA is unaffected upon exposure to neuroendocrine hormones, disabling the ability of the pathogen to promote haemolysis-mediated host cell invasion. Thus, the Cpx system could be described as a new adrenergic receptor involved in inter-kingdom signalling.

The ability of the Cpx system to sense misfolded membrane proteins could be involved in antibiotic-mediated cell death of Gram-negative bacteria. Bactericidal-mediated killing of bacteria requires an intact Cpx system together with the Arc redox-responsive TCS (Davis, 1987; Kohanski *et al.*, 2007, 2008). Detection of misfolded proteins activates CpxA followed by putative crosstalk with either the cognate RR CpxR or the non-cognate RR ArcA, which could lead to a lethal stimulation of oxygen radical generation (Ronson *et al.*, 1987; Iuchi *et al.*, 1989; Kohanski *et al.*, 2008; Dwyer *et al.*, 2009).

Conclusion and outlook

We are just beginning to gain insight into the mechanism of signal integration by the Cpx-TCS. It is evident that the Cpx-TCS is capable of responding to misfolded proteins and to physical changes, the key players of this TCS, CpxA and CpxR, but also via different accessory proteins, NlpE, CpxP, extending the signal inputs from all compartments of the cell. However, the underlying mechanisms are only poorly understood. Currently, only models that involve the induction of the accessory CpxP protein in response to alkaline pH (Thede *et al.*, 2011), salt (Zhou *et al.*, 2011) and misfolded P-pilus subunits (Isaac *et al.*, 2005; Zhou *et al.*, 2011) have been developed (Fig. 3). However, many additional questions for the Cpx-specific signal integration mechanism remain to be solved: Do CpxA and the two accessory proteins CpxP and NlpE physically interact? Which conditions disturb these interactions and how? Is NlpE a general accessory protein for changes in and at the outer membrane? Which catalytic activity of CpxA is modulated by NlpE? What is the exact mechanism of detecting changes in lipid composition by CpxA? Are there further accessory proteins that allow integration of specific stimuli into the Cpx signalling cascade, such as QseRS-TCS in the case of neuroendocrine hormones sensing for instance (Novak *et al.*, 2010)? Is the Cpx signalling cascade modulated by scaffolding proteins (Heermann & Jung, 2010) as the influence by metabolic changes indicates? Does the proposed physiological relevant crosstalk with ArcA exist? Despite the many open questions, using MalE219, CpxP, NlpE and PapE as specific modulators of the biochemical activities of the *in vitro* reconstituted Cpx system, we now have the systems and methods at hand to gain a deeper understanding of TCS signal recognition and transmission through and beyond the bacterial membrane.

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