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# Involvement and necessity of the Cpx regulon in the event of aberrant β-barrel outer membrane protein assembly

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## Summary

The Cpx and $\sigma^E$ regulons help maintain outer membrane integrity; the Cpx pathway monitors the biogenesis of cell surface structures, such as pili, while the $\sigma^E$ pathway monitors the biogenesis of β-barrel outer membrane proteins (OMPs). In this study we revealed the importance of the Cpx regulon in the event of β-barrel OMP mis-assembly, by utilizing mutants expressing either a defective β-barrel OMP assembly machinery (Bam) or assembly defective β-barrel OMPs. Analysis of specific mRNAs showed that Δ*cpxR bam* double mutants failed to induce *degP* expression beyond the wild type level, despite activation of the $\sigma^E$ pathway. The synthetic conditional lethal phenotype of Δ*cpxR* in mutant Bam or β-barrel OMP backgrounds was reversed by wild type DegP expressed from a heterologous plasmid promoter. Consistent with the involvement of the Cpx regulon in the event of aberrant β-barrel OMP assembly, the expression of *cpxP*, the archetypal member of the *cpx* regulon, was upregulated in defective Bam backgrounds or in cells expressing a single assembly-defective β-barrel OMP species. Together, these results showed that both the Cpx and $\sigma^E$ regulons are required to reduce envelope stress caused by aberrant β-barrel OMP assembly, with the Cpx regulon principally contributing by controlling *degP* expression.

## Introduction

In *Escherichia coli*, $\sigma^E$ and CpxAR constitute the two major signal transduction pathways that help maintain envelope integrity. In this organism, the $\sigma^E$ pathway is essential while the CpxAR pathway is dispensable. Activation of the $\sigma^E$ pathway is triggered by the accumulation of misfolded β-barrel outer membrane proteins (OMPs) (for reviews, see Alba and Gross, 2004; Ades, 2008). RseA is an inner membrane anti-$\sigma^E$ factor, which sequesters $\sigma^E$ and prevents it from transcribing the downstream target genes (Rhodius *et al.*, 2006). Under stress, the accumulation of misfolded envelope proteins triggers a two-step, DegS-and RseP-mediated degradation cascade of RseA (Ades *et al.*, 1999; Alba *et al.*, 2002; Inaba *et al.*, 2008). This frees $\sigma^E$ to transcribe genes whose products help restore envelope homeostasis by promoting protein folding/degradation or by inhibiting translation through activating the synthesis of small regulatory RNAs (Johansen *et al.*, 2006; Papenfort *et al.*, 2006).

The CpxAR gene products constitute a two-component signal transduction pathway in which CpxA functions as the inner membrane-bound sensor kinase while CpxR acts as a



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cytoplasmic response regulator (Raivio and Silhavy, 1997). Upon receiving the biochemical signals from CpxA, phosphorylated CpxR regulates the expression of a number of genes (De wulf *et al.*, 2002; Yamamoto and Ishihama, 2006; Price and Raivio, 2009). The CpxAR pathway has been reported to be activated in response to diverse environmental signals and aberrant envelope or cell surface structures, including medium pH (Danese and Silhavy, 1998), medium osmolarity (Jubelin *et al.*, 2005), overexpression of certain lipoproteins (Snyder *et al.*, 1995; Miyadai *et al.*, 2004), expression of misfolded P pilus subunits (Jones *et al.*, 1997; Hung *et al.*, 2001), defective protein secretion across the inner membrane (Shimohata *et al.*, 2007), accumulation of enterobacterial common antigen (Danese *et al.*, 1998) and defective membrane lipid composition (Mileykovskaya and Dowhan, 1997; Klein *et al.*, 2009). Many, if not all, of the signals and defects listed above directly or indirectly influence envelope protein folding. Therefore, it is likely that the presence of misfolded envelope proteins activates the CpxAR signal transduction pathway. Indeed, the downstream targets of the CpxAR regulon, *degP*, *dsbA* and *ppiA* are involved in degrading or folding of envelope proteins, thus strongly suggesting a role for the CpxAR regulon in maintaining envelope integrity by sensing and appropriately responding to the folding status of envelope proteins.

Given that both the $\sigma^E$ and CpxAR pathways respond to abnormal envelope biogenesis, it is not surprising that they share at least one very important downstream target gene, *degP*, whose product was originally thought to principally degrade misfolded envelope proteins (Strauch *et al.*, 1989); however, subsequent studies revealed an additional chaperone role for DegP (Spiess *et al.*, 1999; Krojer *et al.*, 2008). It is well established that the $\sigma^E$ pathway specifically responds to misfolded β-barrel OMPs because DegS, one of the two proteases that degrade RseA, recognizes the unique C-terminal sequence of misfolded β-barrel OMPs (Walsh *et al.*, 2003). No systematic study has been conducted to determine whether the CpxAR pathway also plays a role when envelope stress is initiated due to misfolding of β-barrel OMPs. In the absence of such analysis, the defensive role of the CpxAR pathway appears to be reserved only for envelope stresses resulting from the aberrant assembly of cell surface organelles or other stresses not directly related to β-barrel OMPs (Raivio and Silhavy, 1999; Ruiz and Silhavy, 2005; Dorel *et al.*, 2006).

In this study, we investigated the involvement of the CpxAR pathway in the biogenesis of β-barrel OMPs. More specifically, we examined the requirement of the CpxAR pathway in genetic backgrounds: (i) compromised in the proper assembly of β-barrel OMPs due to the presence of a defective β-barrel OMP assembly machine (Bam), (ii) lacking periplasmic chaperones/protease, SurA, Skp and DegP and (iii) expressing assembly defective β-barrel OMPs, OmpF and OmpC. The absence of CpxR in these genetic backgrounds, except Δ*skp*, led to synthetic or conditional lethal phenotypes. In the absence of CpxR, cells failed to increase *degP* expression to a level needed to cope with the envelope stress resulting from the mis-assembly of β-barrel OMPs. Moreover, the conditional lethal phenotype of Δ*cpxR* in these genetic backgrounds was reversed when DegP was expressed from a heterologous plasmid promoter, indicating that the CpxAR pathway plays an important role in the event of aberrant β-barrel OMP assembly by contributing to the steady-state as well as regulated expression of *degP*. Together, our analysis shows that both the $\sigma^E$ and CpxAR signal transduction pathways are required to fully cope with envelope stresses caused by misfolded β-barrel OMPs.

## Results

### Synthetic and conditional lethal phenotypes of ΔcpxR in genetic backgrounds defective in β-barrel OMP assembly

Assembly of β-barrel OMPs depends on two major envelope components: (i) the outer membrane-bound Bam complex comprised of the BamABCDE proteins and (ii) a periplasmic chaperone protein SurA. Another periplamic chaperone, Skp, plays a relatively minor role in β-barrel OMP assembly but its role becomes crucial in the absence of SurA (Rizzitello *et al.*, 2001; Sklar *et al.*, 2007). DegP is the major periplasmic protease (Strauch *et al.*, 1989), which degrades misfolded OMPs (Misra *et al.*, 1991; 2000; CastilloKeller and Misra, 2003). DegP has also been shown to act as a chaperone (Spiess *et al.*, 1999); however, its role in actually promoting the assembly of *E. coli* β-barrel OMPs is not fully understood (Sklar *et al.*, 2007; Krojer *et al.*, 2008).

We asked whether the Cpx regulon plays a role when envelope stress is induced by mutations that directly affect β-barrel OMP assembly. For this, we introduced a Δ*cpxR*::Cm$^r$ null allele in wild type, Δ*bamB*, *bamA66*, *surA*::Km$^r$, Δ*skp* and *degP*::Tn*10* backgrounds and measured cell growth at 30°C, 37°C and 40°C (Table 1; Fig. S1). In the wild type background, disabling of the Cpx regulon produced no growth defects at the three different incubation temperatures. Similarly, the absence of BamB, a non-essential lipoprotein component of the Bam complex (Wu *et al.*, 2005;Vuong *et al.*, 2008), by itself produced no growth defects but growth of the Δ*bamB* Δ*cpxR*::Cm$^r$ mutant became increasingly worse with higher incubation temperatures. At 40°C, the mutant failed to form single colonies; instead, faster growing revertants arose frequently. The introduction of Δ*cpxR* in a *bamA66* background, which expresses a mutant form of BamA in which a single amino acid residue was deleted from the POTRA domain (Bennion *et al.*, unpubl. data), also resulted in a synthetic phenotype, although the growth defect at 37°C and 40°C was not as severe as observed in a Δ*bamB* Δ*cpxR*::Cm$^r$ background (Table 1; Fig. S1). These results showed that some members of the Cpx regulon play an important role when envelope stress is triggered due to a defective Bam complex.

Next, we investigated the significance of the Cpx regulon in genetic backgrounds lacking the periplasmic chaperones SurA, Skp or the protease/chaperone DegP (Table 1). Although the Δ*cpxR*::Cm$^r$ allele could be transduced into a *surA*::Km$^r$ strain, purified transductants produced heterogeneous colonies at all growth temperatures. In contrast, the Δ*cpxR*::Cm$^r$ allele could be readily transduced into a Δ*skp* background and the resulting Δ*skp* Δ*cpxR*::Cm$^r$ double mutant grew just as well as the wild type strain regardless of the incubation temperature (Table 1). Thus, envelope stress generated by the loss of Skp was not sufficient to demand a requirement of the functional Cpx regulon. Lastly, we introduced Δ*cpxR*::Cm$^r$ in a *degP*::Tn*10* background and observed a synthetic phenotype (Table 1; Fig. S1), indicating the involvement of some additional members of the Cpx regulon, other than DegP, in the absence of the major periplasmic protease/chaperone. Together, these results showed that the Cpx regulon is not only involved but plays an important role when envelopes are stressed due to a compromised β-barrel OMP assembly machinery or periplasmic folding/degradation environment.

### Inability to fully activate *degP* expression causes synthetic phenotypes

Having shown that the Cpx regulon is needed when β-barrel OMP assembly is compromised, we set out to determine the status of four transcripts relevant to the β-barrel OMP-mediated envelope stress; namely *degP*, *ompF*, *rseA* and *yfgC*. The *rseA* and *yfgC* genes are positively regulated by $\sigma^E$ (Rhodius *et al.*, 2006), while *rseA* is partially repressed by the Cpx regulon (De wulf *et al.*, 2002). *degP* is positively regulated by both the $\sigma^E$

(Erickson and Gross, 1989) and Cpx (Danese *et al.*, 1995) regulons, while *ompF* is negatively controlled by $\sigma^E$, which lowers *ompF* and *ompC* translation by increasing the synthesis of a small inhibitory RNA, *rybB* (Johansen *et al.*, 2006; Papenfort *et al.*, 2006). Transcription of *ompF* is also inhibited by an activated CpxAR system (Batchelor *et al.*, 2005).

The transcriptional status of these genes was determined by real time, reverse transcription quantitative PCR (RT qPCR) (Fig. 1). For RNA isolation, cultures were gown overnight at 30°C, followed by growth at 37°C for about 2 h to a mid log phase. In a Δ*cpxR*::$Cm^r$ background, *rseA* and *yfgC* transcript levels went up 1.8- and 1.3-fold, respectively, presumably as a result of the de-repression of the *rpoE*($\sigma^E$)*rseABC* operon (De wulf *et al.*, 2002). Interestingly, despite the slight activation of the $\sigma^E$ regulon, *degP* transcript levels went down twofold, reaffirming a positive role for the Cpx regulon in controlling *degP* transcription independently of the $\sigma^E$ regulon (Danese *et al.*, 1995). No appreciable decrease in the *ompF* transcript level was noted in the absence of *cpxR*, indicating that the envelope was not significantly stressed. In *degP*::Tn*10* and *degP*::Tn*10* Δ*cpxR*::$Cm^r$ backgrounds, the *rseA* and *ompF* transcript levels went up (*rseA*) and down (*ompF*) slightly relative to wild type, indicating the existence of mild envelope stress conditions.

In a Δ*bamB* background, the levels of both the *rseA* and *yfgC* transcripts increased by over two- and 1.5-fold, respectively, while the *ompF* transcript level went down almost fivefold, indicating that the cells are experiencing envelope stress (Fig. 1). Consistent with this assertion, *degP* transcript levels also went up over 2.4-fold (Fig. 1). In a Δ*bamB* Δ*cpxR*::$Cm^r$ double mutant, the *rseA* and *yfgC* transcript levels rose slightly further over those present in a Δ*bamB* background, while *ompF* transcript levels remained low. Strikingly, despite the activation of the $\sigma^E$ regulon, the *degP* transcript level remained threefold below that present in a Δ*bamB* background. It is important to note the *degP* transcript level did rise slightly in the Δ*bamB* Δ*cpxR*::$Cm^r$ double mutant over that present in a Δ*cpxR*::$Cm^r$ background (0.8 versus 0.5). Thus, while the activated $\sigma^E$ regulon is capable of elevating *degP* expression under these conditions, the data suggest that without a functional Cpx regulon *degP* expression does not rise to a level needed to cope with envelope stress. We believe that this inability of the Δ*bamB* Δ*cpxR*::$Cm^r$ mutant to increase *degP* expression is one of the reasons for the observed synthetic lethal phenotype at elevated growth temperatures (Table 1; Fig. S1). The effects of Δ*cpxR*::$Cm^r$ in a *bamA66* background were very similar to that observed in a Δ*bamB* background (Fig. 1), thus reaffirming that the inability of Δ*cpxR* cells to sufficiently elevate *degP* expression results in the synthetic phenotype.

The examination of β-barrel OMPs from membranes prepared from the same cultures used for RNA analysis showed that the levels of OmpF and LamB were significantly down in the Δ*bamB* and *bamA66* strains compared with the wild type and *degP*::Tn*10* strains, with the absence of BamB exerting a slightly greater negative effect on OMP levels than the expression of a mutant BamA66 protein (Fig. 2). Consistent with the RT qPCR data, the absence of *cpxR* produced no further negative effect on OmpF and the three other OMPs examined (Fig. 2). Together, these RNA and protein data provided an explanation for the genetic data (Table 1), revealing that despite the fully operational $\sigma^E$ regulon the growth defects are likely caused by the inability of a Δ*cpxR* strain to fully induce *degP* expression when the assembly of β-barrel OMPs is compromised.

## Expression of DegP from a plasmid or the absence of *rseA* reverses the conditional lethal phenotype of Δ*cpxR*

To test whether the expression of DegP from a heterologous plasmid promoter can overcome the growth defects of the mutants listed in Table 1, we transformed selected strains that showed growth defects with an empty vector (pACYC184) and two derivatives

expressing wild type or a protease-deficient $DegP_{S210A}$ variant and monitored growth at 30°C, 37°C and 40°C (Table 2). The expression of wild type DegP from a plasmid promoter fully reversed the growth defects in all cases, except in a *surA*::$Km^r$ Δ*cpxR*::$Cm^r$ background where homogeneous growth was restored but the colony size was smaller compared with other strains. In contrast to wild type DegP, the expression of protease-deficient $DegP_{S210A}$ only partially reversed the growth defects, with no reversal observed in a *surA*::$Km^r$ Δ*cpxR* background. These results indicated that the protease activity of DegP is more crucial than its chaperone activity for lowering stress at elevated growth temperatures.

In a Δ*cpxR* mutant, the $\sigma^E$ pathway should be fully functional and slightly activated due to the absence of the repressive effect of CpxR, yet the activated $\sigma^E$ pathway failed to alleviate growth defects when envelopes were stressed due to a defective Bam complex or periplasmic folding environment. It is possible that when the $\sigma^E$ pathway is fully and constitutively activated due to the absence of its negative regulator, RseA, the collective actions of increased synthesis of factors that promote OMP assembly, degrade misfolded OMPs and inhibit OMP synthesis may be sufficient to overcome the envelope stress of a Δ*cpxR* mutant simultaneously defective in β-barrel OMP assembly. In agreement with our prediction, the introduction of Δ*rseA* restored almost normal growth of *bam* Δ*cpxR* and Δ*degP* Δ*cpxR* mutants at 37°C and 40°C (Fig. S2). Thus, a fully activated $\sigma^E$ pathway can substitute for the CpxAR pathway in cells experiencing envelope stress caused by mis-assembled β-barrel OMPs or the absence of *degP*.

## Comparative analysis of the activation of Cpx and $\sigma^E$ regulons in backgrounds defective in β-barrel OMP biogenesis

We carried out side-by-side assessments of the activation of Cpx and $\sigma^E$ regulons in mutants expressing a defective Bam complex, lacking SurA, or lacking DegP. Wild type and mutant strains were grown overnight at 30°C, and next day after 1:100 dilution growth was resumed at 37°C or 39°C until cultures reached mid to late exponential phase ($OD_{600} \approx 0.6$). RNA prepared from three independent cultures was converted to cDNA and subjected to RT qPCR analyses.

We focused on three key genes, *cpxP*, *rseA* and *yfgC*, which are regulated by Cpx (Danese and Silhavy, 1998), $\sigma^E$/Cpx (De wulf *et al.*, 2002; Rhodius *et al.*, 2006) and $\sigma^E$ (Rhodius *et al.*, 2006) respectively. We also included a Δ*surA* mutant as no colony growth heterogeneity is observed in a Δ*surA* $cpxR^+$ background. At 37°C, expression of *cpxP* was mildly activated in Δ*bamB* and Δ*surA* backgrounds, whereas no significant change in *cpxP* expression was noted in a *bamA66* or Δ*degP* background (Fig. 3, top panel). At 39°C, *cpxP* expression in all four mutant backgrounds increased compared with that at 37°C, indicating a greater demand for the Cpx regulon under conditions of increased β-barrel OMP assembly defects (Fig. 3, bottom panel). In general, fold increase in *rseA* and *yfgC* expression was greater than *cpxP* in all four mutant backgrounds at 37°C; however, this pattern was not obvious at 39°C. Together, these data showed that both the Cpx and $\sigma^E$ regulons are activated in genetic backgrounds conducive for mis-assembly of β-barrel OMPs.

## Lethal effects of Δ*cpxR* in genetic backgrounds expressing an assembly-defective β-barrel OMP

So far we have used Bam or chaperone/protease mutants to underscore the importance of a functional Cpx pathway in overcoming envelope stress. However, these mutants broadly affect OMP biogenesis and in some instances, e.g. *bamA66* and Δ*surA*, may also affect lipid biogenesis indirectly by interfering with the assembly of LptD (Imp), which is involved in the transport of LPS to the outer membrane (Bos *et al.*, 2004). Indeed, defects in LPS biogenesis have been shown to activate the Cpx regulon (Klein *et al.*, 2009). Therefore, to

establish unequivocally that expression of misfolded β-barrel OMPs demands the intact Cpx regulon, we examined the phenotype of Δ*cpxR* in genetic backgrounds expressing individual mutant β-barrel OMPs with known assembly defects. The first group of mutants we used expressed OmpF proteins bearing a C-terminal residue other than the wild type phenylalanine (Misra *et al.*, 2000). It is well established that the C-terminal phenylalanine, which is conserved in most β-barrel OMPs, plays a critical role in their folding and assembly (Struyvé *et al.*, 1991; Misra *et al.*, 2000). Peptides derived from the C-termini of β-barrel OMPs also bind to DegS and initiate a two-step proteolysis of RseA to induce $\sigma^E$ (Walsh *et al.*, 2003). The second group of mutants we used expressed OmpC proteins bearing one or two non-native cysteine residues ($OmpC_{1Cys}$ and $OmpC_{2Cys}$; Misra, 1993; CastilloKeller and Misra, 2003). The formation of a disulfide bond in $OmpC_{2Cys}$ causes the protein to misfold (Misra, 1993; CastilloKeller and Misra, 2003). Expression of both the mutant OmpC and certain C-terminal mutant OmpF proteins demanded the presence of DegP (Misra *et al.*, 2000; CastilloKeller and Misra, 2003).

We transduced the Δ*cpxR* mutation into the mutant OMP backgrounds at 30°C and tested whether the absence of the Cpx pathway interfered with bacterial growth at elevated incubation temperatures. Three of eight different OmpF mutants showed growth defects at 37°C but at 40°C six mutants grew poorly, with $OmpF_{F340R}$ displaying the most severe growth defects at 37°C and 40°C (Table 3; Fig. S3). Similarly, expression of $OmpC_{2Cys}$ in the absence of CpxR inhibited single colony formation at 37°C and 40°C (data not shown). These results showed that the expression of misfolded β-barrel OMPs, and not indirect effects, such as perturbed lipid biogenesis, demands the functional Cpx regulon. As with *bam* mutants, growth defects under these conditions are likely due to reduced synthesis of DegP, since expression of DegP from a plasmid restores normal growth (data not shown).

## Expression of a single misfolded β-barrel OMP species induces the Cpx regulon

Next we asked whether the expression of an assembly-defective mutant OMP activates the Cpx regulon. For this, we introduced plasmids expressing the parental or an assembly defective OmpF under the control of an IPTG-inducible promoter into a strain carrying the *cpxP*::*lacZ* fusion at the lambda attachment site. Cells were grown freshly at 37°C to an early log phase ($OD_{600} \approx 0.3$), at which time cultures were split into three aliquots that received either none, 0.04 mM or 0.1 mM IPTG and growth was resumed for an hour so as to only transiently induce OmpF expression. β-galactosidase activities were measured from three independent cultures of each strain. The results showed that induction of the parental OmpF protein modestly elevated *cpxP* expression (about 1.8-fold; Fig. 4). In contrast, induction of an assembly-defective $OmpF_{F340R}$ protein increased *cpxP*::*lacZ* activity fourfold over that of the control culture not induced with IPTG (Fig. 4), showing that the expression of a single assembly-defective β-barrel OMP species is sufficient to activate the Cpx regulon.

## The absence of cpxP does not exacerbate envelope stress caused by aberrant β-barrel OMP assembly

Isaac *et al.* (2005) previously showed that in the absence of CpxP the toxic effects of overexpression of misfolded PapE and PapG were significantly exacerbated. Based on this and DegP-mediated protein degradation data they concluded that CpxP combats the toxic effects PapE and PapG. We asked whether CpxP similarly combats the toxic effects of misfolded β-barrel OMPs and if so, whether the absence of *cpxP* in defective β-barrel OMP assembly backgrounds would produce a synthetic phenotype. The introduction of a *cpxP* null allele in backgrounds expressing a defective Bam complex, misfolded OmpC or OmpF mutants did not produce a synthetic phenotype at 30°C, 37°C or 40°C (data not shown). These results suggest that CpxP, unlike CpxR or DegP, does not play a significant role in

reducing the toxic effects of misfolded β-barrel OMPs. Because CpxP is shown to act as a negative regulator of CpxA (Raivio *et al.*, 1999), it is possible that de-repression of the CpxAR regulon in a Δ*cpxP* background could potentially mask the negative effect of the loss of CpxP. In this regard, it is interesting to note that the absence of CpxP in a *degP* background allows the strain to form small but homogeneous single colonies at 40°C, thus reflecting a DegP-independent beneficiary effect resulting from the de-repression of the CpxAR regulon.

## Discussion

### Cpx regulon and β-barrel OMP assembly

Genetic and molecular analyses presented here showed the involvement and necessity of the Cpx regulon in the event of aberrant β-barrel OMP assembly. Several lines of evidence supported this conclusion: first, the absence of *cpxR* in mutant *bam* backgrounds, defective in the normal assembly of multiple β-barrel OMPs, caused a conditional synthetic lethal phenotype. Second, expression of a single assembly-defective species of β-barrel OMP in a Δ*cpxR* background caused a conditional synthetic lethal phenotype. Third, the Cpx regulon was activated in genetic backgrounds defective in correct assembly of β-barrel OMPs or expressing a single assembly-defective β-barrel OMP. All of these observations point to the involvement of the Cpx regulon, in addition to the $\sigma^E$ regulon, in maintaining envelope integrity when perturbed due to the mis-assembly of β-barrel OMPs.

We noted that despite the activation of the $\sigma^E$ regulon, the expression of *degP* in a *bam* Δ*cpxR* background was threefold lower compared with isogenic *bam cpxR*$^+$ strains. This suggested that Cpx-mediated expression of *degP* is one of the reasons why the Cpx system is required under these conditions. Consistent with this notion, expression of a protease-active DegP protein from a plasmid reversed the synthetic lethal phenotype of *bam* Δ*cpxR* double mutants. Our results indicated that the Cpx regulon does not need to be activated beyond the wild type level for it to be relevant in reducing envelope stress. This was best exemplified by data obtained from the *bamA66* mutant. When grown at 37°C, *cpxP* expression in the *bamA66* mutant was very similar to that in the wild type strain, indicating no further activation of the Cpx regulon beyond what normally exists in a wild type background. Despite this, elimination of the Cpx regulon in a *bamA66* Δ*cpxR* double mutant caused growth defects, owing to the inability of this mutant to fully induce *degP* expression. Therefore, the basal, unstimulated activity of the Cpx regulon can be sufficient to cope with envelope stress. Together, these results showed that the $\sigma^E$ regulon alone is not sufficient to overcome envelope stress induced by mis-assembled β-barrel OMPs; rather, a concerted effort from both the Cpx and $\sigma^E$ regulons is required (Fig. 5).

Despite our demonstration here that the Cpx regulon is required for effectively combating envelope stress resulting from mis-assembled β-barrel OMPs, we believe that the $\sigma^E$ pathway is the principal responder of stress signals generated under these conditions. One reason for this assertion is that a large number of $\sigma^E$-regulated gene products have been implicated either directly or indirectly in the assembly of β-barrel OMPs or maintaining envelope homeostasis by modulating OMP synthesis. (Fig. 5; Johansen *et al.*, 2006;Papenfort *et al.*, 2006;Rhodius *et al.*, 2006). In contrast, expression of only a handful of genes, including *dsbA*, *ppiA* and *ppiD*, whose products could influence β-barrel OMP assembly, is uniquely controlled by the Cpx regulon (Danese and Silhavy, 1997;Pogliano *et al.*, 1997;Dartigalongue and Raina, 1998;Fig. 5). However, none of these genes are essential for β-barrel OMP assembly, and no data exist for the involvement of PpiA in OMP assembly (Kleerebezem *et al.*, 2004). Moreover, the two major β-barrel OMPs, OmpF and OmpC, lack any natural cysteine residues; therefore, rendering a direct involvement of DsbA in their assembly irrelevant. On the other hand, the further deterioration of growth in a *degP* mutant

in the absence of *cpxR* suggests that at least one other *cpx*-regulated gene product, other than DegP, is needed for normal growth in the absence of the major periplasmic protease/chaperone activity of DegP. In this regard, it is interesting to note that the activated Cpx system has been recently shown to influence another two-component signal transduction comprised of the EnvZ/OmpR, via upregulating the synthesis of *mzrA* (Gerken *et al.*, 2009). The inner membrane product of *mzrA*, whose expression is positively regulated by CpxAR, is shown to directly interact with EnvZ and influence EnvZ's activity in a fashion similar to that of constitutively activated, pleiotropic *envZ* alleles (Gerken *et al.*, 2009). Therefore, the activated Cpx regulon likely triggers a broad defensive response by influencing the expression of a number of genes, including those that are not under direct control of the Cpx regulon.

Connolly *et al.* (1997) first reported that a constitutively activated Cpx regulon can reduce the lethal effects of an *rpoE* null mutation. Here, we have shown that a constitutively activated $\sigma^E$ regulon can overcome the lethal effects of Δ*cpxR* in backgrounds simultaneously defective in the assembly of β-barrel OMPs. This reciprocating behaviour in a large part appears to be due to the involvement of *degP*, which is common to both the regulons. Under normal growth conditions in rich medium and in the absence of a deliberately provoked envelope stress, *degP* expression appears to be evenly controlled by the $\sigma^E$ and Cpx regulons (this study; Danese *et al.*, 1995). We believe that because of this shared responsibility, the activities of both the regulons are needed to effectively combat envelope stress.

## Signals that stimulate Cpx regulon

Signals originating from a wide range of factors, including aberrant synthesis or folding of envelope components, as well as medium osmolarity and pH are known to activate the Cpx regulon (summarized by Ruiz and Silhavy, 2005; Dorel *et al.*, 2006; Price and Raivio, 2009). We have shown here that signals generated by misfolded β-barrel OMPs can also activate the Cpx regulon. However, unlike the $\sigma^E$ regulon, the molecular nature of the signals that activate the Cpx regulon is poorly understood. In the case of $\sigma^E$, a two-step proteolysis of the anti-sigma factor, RseA, by DegS and RseP, leads to the release of $\sigma^E$ from the membrane and subsequent activation. This event begins with the binding of C-terminal-derived β-barrel OMP peptides to the PDZ domain of DegS, activating its proteolytic domain, which then cleaves RseA (Walsh *et al.*, 2003; Hasselblatt *et al.*, 2007). A second proteolytic event is then carried out by RseP, which cleaves RseA near the cytoplasmic phase of the inner membrane (Grigorova *et al.*, 2004).

The signalling pathway that activates CpxA, a membrane-bound receptor kinase, is poorly understood. Although CpxP is not involved in the signalling pathway, the available data suggest that it is a negative regulator of CpxA (Raivio *et al.*, 1999; Buelow and Raivio, 2005). Isaac *et al.* (2005) showed that CpxP acts as an adapter protein, which upon binding to substrate proteins, including misfolded PapE and PapG, not only mediates their degradation by DegP, but also gets degraded in the process. The exacerbation of growth defects of a Δ*cpxP* mutant expressing misfolded PapE and PapG further signifies the importance of CpxP in reducing stress caused by certain misfolded envelope proteins. However, unlike Δ*cpxR* or Δ*degP*, the introduction of Δ*cpxP* does not exacerbate the growth phenotype of the *bam* and OMP mutants used here. Therefore, DegP-mediated reduction in toxicity stemming from misfolded β-barrel OMPs appears to be the result of a direct action of DegP on misfolded β-barrel OMPs without involving CpxP.

The data presented here suggest that misfolded β-barrel OMPs activate the Cpx regulon independent of CpxP. Other envelope stresses resulting from alkaline pH or the production of misfolded PapE and PapG were also reported to activate the Cpx pathway independent of

CpxP (DiGiuseppe and Silhavy, 2003). Mutational data have suggested that the periplasmic domain of CpxA is required for induction of the Cpx pathway by envelope stress (DiGiuseppe and Silhavy, 2003). This then raises the possibility that misfolded envelope proteins, including misfolded β-barrel OMPs, may directly interact with the periplasmic domain of the CpxA kinase to stimulate its activity. Consistent with this notion, a recent *in vitro* study showed a modest 1.6-fold increase in CpxA-mediated phosphorylation of CpxR when proteoliposomes were reconstituted with CpxA and MalE219, a mutant form of MalE known to misfold *in vivo* (Keller and Hunke, 2009). The implication of this finding is that misfolded MalE binds directly to CpxA and stimulates its phosphotransfer activity. In addition to stimulating the phosphotransfer activity of CpxA, it is possible that the binding of substrate proteins to CpxA *in vivo* may displace CpxP from CpxA and this could activate the autokinase activity of CpxA (Fleischer *et al.*, 2007). Clearly, additional work is needed to better understand the molecular nature of stress signals that activate CpxA.

## Experimental procedures

### Bacterial strains and chemicals

All strains used here were derived from *E. coli* K-12 MC4100 and are listed in Table 4. Luria broth (LB) and agar (LBA) were prepared as described previously (Silhavy *et al.*, 1984) and supplemented with ampicillin (50 μg $ml^{-1}$), chloramphenicol (12.5 μg $ml^{-1}$), kanamycin (25 μg $ml^{-1}$), isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.04, 0.1 or 0.4 mM) or arabinose (0.2%) as necessary. SuperSignal West Pico Chemiluminescent substrate was purchased from Thermo Scientific. All other chemicals were of analytical grade.

### DNA manipulations

Standard bacterial genetic methods were carried out as described previously (Silhavy *et al.*, 1984). Primers used for cloning *cpxR* are listed in Table 5. A chromosomal fragment containing *cpxR* was amplified using gene-specific cloning primers. *BspH*I and *Hin*DIII PCR-amplified fragments were cloned into *Nco*I and *Hin*DIII digested pBAD24 (Guzman *et al.*, 1995). The wild-type pACYC*degP* clone was created by restoring the wild-type serine 210 codon in the pACY-C*degP* (S210A) clone using the Quickchange Lightning Site-Directed Mutagenesis (SDM) Kit from Stratagene following the manufacturer's protocol. Primers for the SDM are listed in Table 5.

### RNA isolation and real-time PCR

Total RNA, from 5 ml of log phase cells ($OD_{600}$ ~ 0.6–0.8) grown at 37 or 39°C, was extracted using TRIzol Max Bacterial RNA Isolation Kit (Invitrogen) and further purified with the RNeasy kit (Qiagen). cDNA was synthesized from 10 mg of RNA using 100 pM random hexamer primer (Integrated DNA Technologies) and M-MuLV Reverse Transcriptase (New England Biolabs). After reverse transcription, five units of RNaseH (New England Biolabs) were added and tubes were incubated for 20 min at 37°C followed by purification of cDNA with the QIAquick PCR purification kit (Qiagen). To measure RNA transcripts, 300 nM of each primer and 20 ng of cDNA was added to SYBR Green PCR Master Mix (Applied Biosystems) per each 20 μl of reaction. Gene-specific primers (Table 5) were designed following SYBR Green PCR Master Mix and RT-PCR Reagents manufacturer's protocol. Critical threshold (Ct) values were determined using ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative quantification of target transcripts were calculated according to the $2^{-\Delta\Delta C}$t method (Livak and Schmittgen, 2001) using *ftsL* and *purC* endogenous control genes whose expression did not change by mutations used in this study. Each PCR reaction was performed in triplicate and fold changes in transcript levels along with standard deviations were calculated from at least two experiments ($n \geq 2$).

### Cell fractionation

Overnight cultures were diluted 1:100 and grown to log phase ($OD_{600}$ ~ 0.8). Equivalent amounts of cells, based on $OD_{600}$, were pelleted and resuspended in a lysis buffer (100 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.25 μg $ml^{-1}$ DNase I and 2 mM PMSF) and lysed using a French press. Following a low speed spin to remove unlysed cells, cell lysates were centrifuged for 1 h at 105 000 *g*, 4°C to separate soluble (cytoplasm and periplasm) and insoluble (inner and outer membranes) fractions. Membrane fractions were then subjected to SDS(urea)-PAGE (as described below) followed by Western blot (described below).

### Protein analyses

Membrane pellets were resuspended in 10 mM Tris-HCl pH 7.5 and diluted with SDS-PAGE sample buffer before heating at 95°C for 5 min and analysis by SDS-PAGE. 4 M urea was added to the SDS-polyacrylamide running gel in order to better resolve OmpC and OmpF. Following electrophoresis, proteins were transferred onto Immobilin-P (Millipore) using a mini transblot (Bio-Rad) and incubated in primary antibody for 1.5 h. Primary rabbit antibodies and dilutions used were OmpF/C/A (1:16 000) and LamB (1:10 000). Membranes were incubated with goat anti-rabbit HRP-conjugated immunoglobulin G secondary antibodies for 1 h and developed with SuperSignal West Pico Chemiluminescent substrate for 5 min. Protein bands were visualized with a Molecular-Imager-ChemiDoc-XRS System from Bio-Rad and quantified using Quantity One software (Bio-Rad).

### Enzymatic assays

Overnight cultures grown at 30°C degrees with ampicillin were subcultured (1:100 dilution) without ampicillin and grown at 37°C to early log phase ($OD_{600}$ ~ 0.2–0.3). Cells were split into two separate cultures, one of which *ompF* expression was induced with IPTG, while the other remained uninduced. Cells were grown for an additional hour and β-galactosidase activities determined as described previously (Michaelis *et al.*, 1983; Miller, 1992), using a VersaMax microtiter plate reader (Molecular Dynamics). Assays were performed in quadruplicate and activity was calculated as the rate of *o*-nitrophenyl-β-galactoside (ONPG; Acros Organics) cleavage divided by the cell density in each well.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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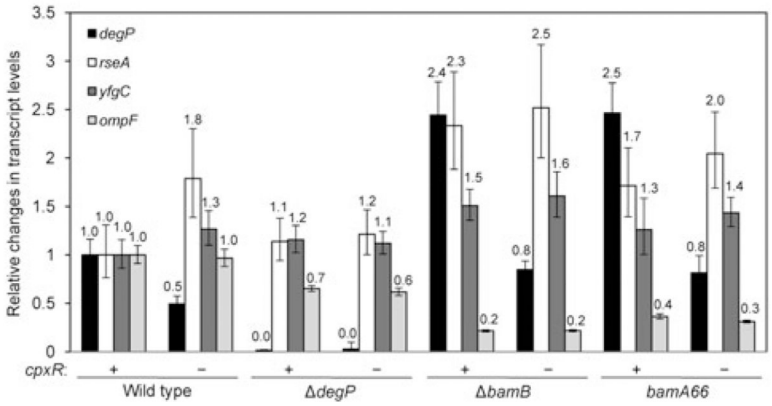


**Fig. 1.**

Evaluation of *degP*, *rseA*, *yfgC* and *ompF* transcripts from wild type, Δ*degP*, Δ*bamB* and *bamA66* strains carrying a wild type *cpxR* or a Δ*cpxR* allele. Cultures were grown at 37°C to mid-log phase and RNA was extracted from approximately $5 \times 10^8$ cells. cDNA synthesized from RNA preparations was subjected to RT qPCR using gene-specific primers. Each PCR reaction was performed in triplicate and fold changes in transcript levels along with standard deviations were calculated from at least two experiments ($n \geq 2$). Comparative critical threshold ($\Delta\Delta C_t$) values were obtained after normalizing data against a housekeeping gene, *ftsL* or *purC*, whose expression did not change in the presence of the various mutations used here.

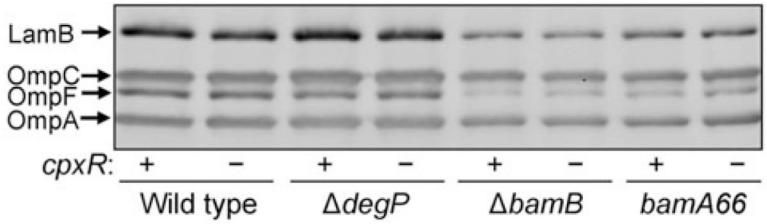


**Fig. 2.**

Analysis of four β-barrel OMPs from wild type and various mutant backgrounds. Envelopes were prepared from the same cultures used for RNA analysis in Fig. 1. OMPs were detected by Western blot analysis using polyclonal antibodies that recognize LamB or OmpF, OmpC and OmpA. Plus and minus signs reflect presence (+) or absence (−) of *cpxR*.

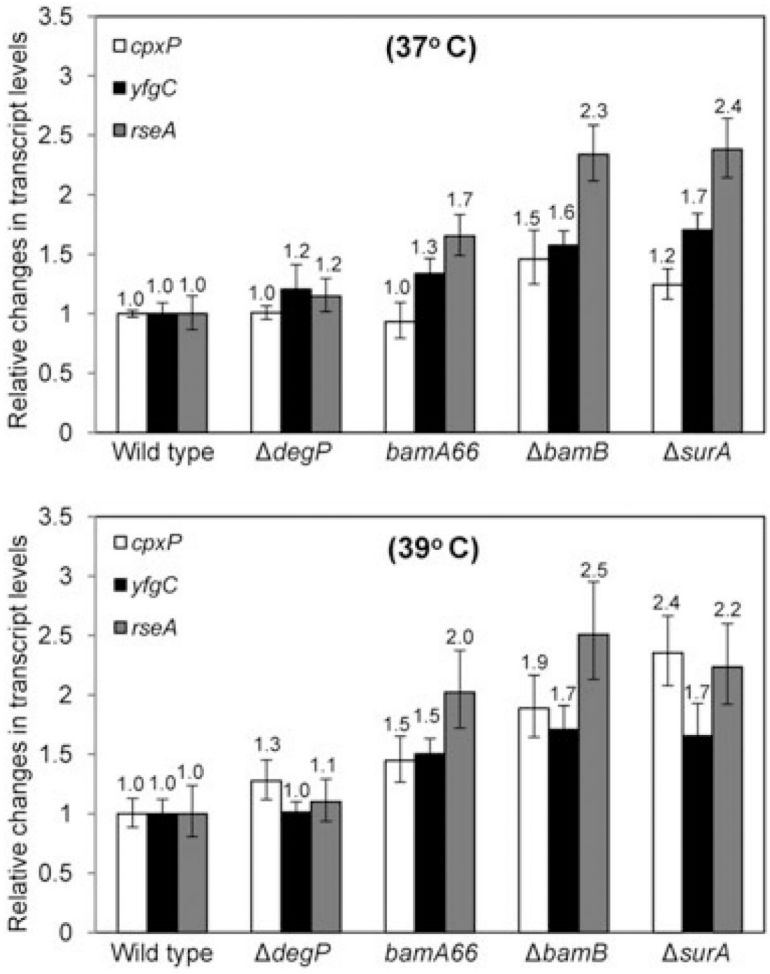


**Fig. 3.**

Evaluation of the status of the *cpxAR* and $\sigma^E$ regulons in wild type and various mutant backgrounds. Relative RNA levels of *cpxP yfgC* and *rseA*, which are regulated by CpxAR, $\sigma^E$ and CpxAR/$\sigma^E$, respectively, were determined by RT qPCR from RNA isolated from cultures grown at mid log phase at 37 and 39°C. RT qPCR analysis was performed as described Fig. 1 legend.

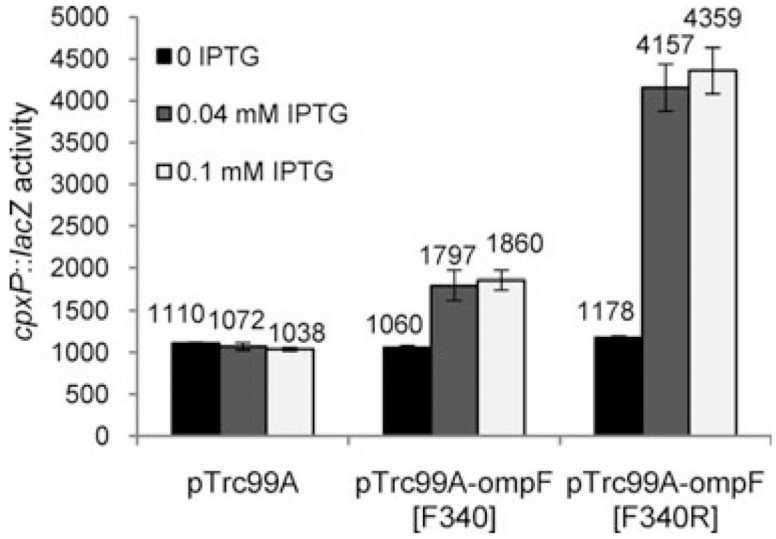


**Fig. 4.**

Activation of the CpxAR regulon by an assembly-defective OmpF protein. Status of the CpxAR regulon was examined through monitoring the activity of a *cpxP::lacZ* fusion located at the lambda attachment site on the chromosome. See the *Experimental procedures* section for details concerning bacterial growth and β-galactosidase assay.

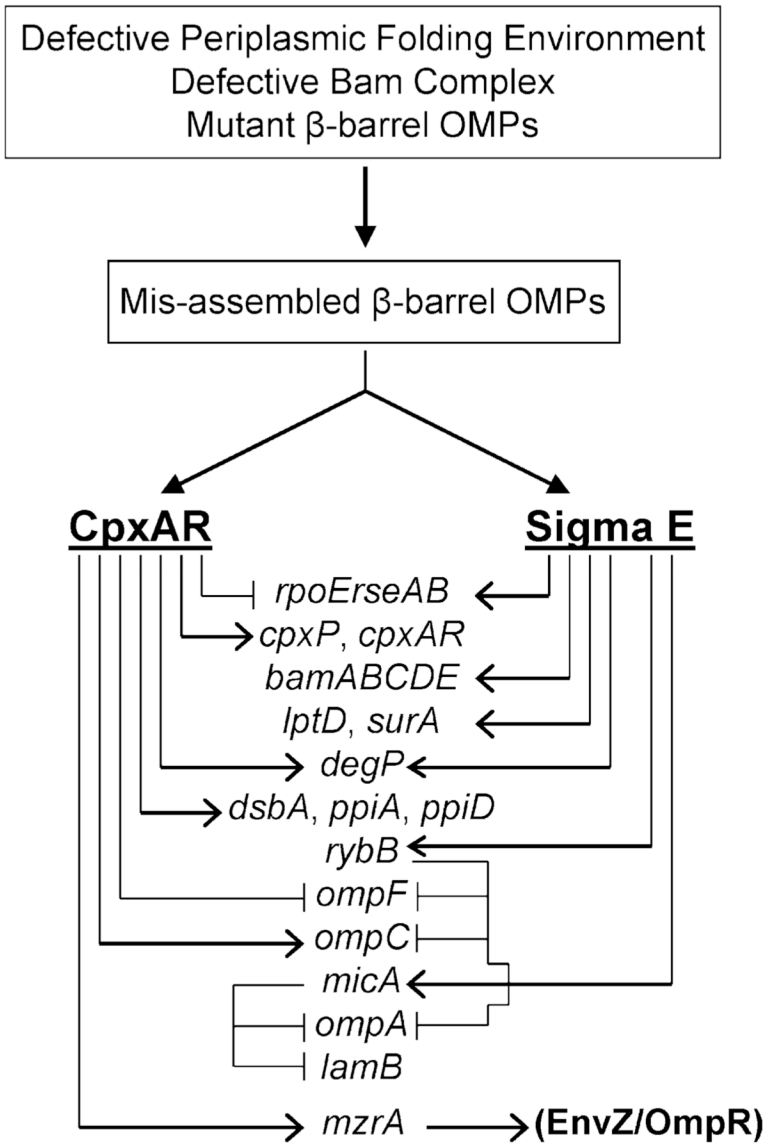


**Fig. 5.**

A cartoon showing selected Cpx- and $\sigma^E$-regulated genes whose products directly or indirectly help reduce envelope stress. Aberrant β-barrel OMP assembly resulting from a defective periplasmic folding environment or Bam complex or alterations in the primary sequence of OMPs stimulates both the Cpx and $\sigma^E$ regulons. Elevated synthesis of proteins, such as the members of the Bam complex, LptD, SurA, PpiD and DsbA directly assists in OMP folding and assembly. No data exist showing a role for PpiA in OMP assembly (Kleerebezem *et al.*, 2004). *degP* is the only gene in this list that is positively regulated by both the Cpx and $\sigma^E$ regulons. The $\sigma^E$-induced synthesis of two small RNAs, *rybB* and *micA*, leads to translation inhibition of various OMPs, thus indirectly assisting in lowering envelope stress (Johansen *et al.*, 2006; Papenfort *et al.*, 2006). Activated CpxR can directly influence transcription of *ompC* and *ompF* (Batchelor *et al.*, 2005), or via increasing expression of *mzrA*, whose inner membrane-localized product directly influences EnvZ's kinase and/or phosphatase activities (Gerken *et al.*, 2009). Besides affecting transcription of *ompC* and *ompF*, the activated EnvZ/OmpR two-component system influences transcription

of a number of genes whose product help reduce stress (Gerken *et al.* 2009 and references therein).

## Table 1

Synthetic phenotypes of Δ*cpxR* in backgrounds lacking or expressing defective component of β-barrel OMP assembly.

| | Growth[a] temperature | | |
|---|---|---|---|
| **Relevant genotype** | **30°C** | **37°C** | **40°C** |
| WT | ++ | +++ | +++ |
| Δ*cpxR*::Cm$^r$ | ++ | +++ | +++ |
| Δ*bamB* | ++ | +++ | +++ |
| Δ*bamB* Δ*cpxR*::Cm$^r$ | + | +/− | −/+ |
| Δ*bamA66* | ++ | +++ | +++ |
| Δ*bamA66* Δ*cpxR*::Cm$^r$ | ++ | + | + |
| *degP*::Tn*10* | ++ | ++ | −/+ |
| *degP*::Tn*10* Δ*cpxR*::Cm$^r$ | ++ | + | − |
| Δ*skp* | ++ | +++ | +++ |
| Δ*skp* Δ*cpxR* | ++ | +++ | +++ |
| *surA*::Km$^r$ | + | ++ | ++ |
| *surA*::Km$^r$ Δ*cpxR*::Cm$^r$ | −/+ | +/− | +/− |

[a] Wild type (WT) and various mutant strains were grown on LBA plates for 16 h at three temperatures shown. Growth resulting in homogeneous large-, medium- and small-sized colonies were scored as +++, ++ and + respectively. Occasionally, medium or small colonies lacked homogeneity, segregating into very small colonies. Due to this behaviour, such colonies were scored as +/− or −/+, reflecting increasing growth defects. Growth was scored as − when no isolated colonies were obtained.

**Table 2**

Expression of DegP from a plasmid reverses the conditional synthetic phenotype of *degP* and double mutants lacking *cpxR*.

| | **Plasmids[a] and Growth[b] temperatures used for complementation** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **pACYC184** | | | **pACYC-*degP* (WT)** | | | **pACYC-*degP* (S210A)** | | |
| **Relevant mutant genotype** | **30°C** | **37°C** | **40°C** | **30°C** | **37°C** | **40°C** | **30°C** | **37°C** | **40°C** |
| *degP*::Tn*10* | ++ | ++ | +/− | +++ | +++ | +++ | +++ | +++ | ++ |
| *degP*::Tn*10* Δ*cpxR*::Cm$^r$ | ++ | +/− | −/+ | ++ | +++ | +++ | ++ | ++ | +/− |
| *bamA66* Δ*cpxR*::Cm$^r$ | ++ | ++ | +/− | ++ | +++ | +++ | ++ | +++ | ++ |
| Δ*bamB* Δ*cpxR*::Cm$^r$ | ++ | + | +/− | ++ | +++ | +++ | ++ | +++ | ++ |
| *surA*::Km$^r$ Δ*cpxR*::Cm$^r$ | +/− | +/− | +/− | + | ++ | ++ | +/− | +/− | +/− |

[a] Leaky expression of DegP or DegP$_{S210A}$ from plasmids was driven by a *lac* promoter in the absence of inducer.

[b] Mutants were grown on LBA plates for 16 h. Growth resulting in homogeneous large-, medium- and small-sized colonies were scored as +++, ++ and + respectively. Small colonies occasionally produced mixed sized colonies and were scored as +/−, with −/+, reflecting a more debilitating growth defect.

**Table 3**

Effects of Δ*cpxR* on growth of strains expressing assembly-defective OmpF proteins.[a]

| | 30°C | | 37°C | | 40°C | |
|---|---|---|---|---|---|---|
| **OmpF Protein** | ***cpxR*$^+$** | **Δ*cpxR*** | ***cpxR*$^+$** | **Δ*cpxR*** | ***cpxR*$^+$** | **Δ*cpxR*** |
| $OmpF_{F340}$ (WT) | ++ | ++ | +++ | +++ | +++ | +++ |
| OmpF- | ++ | ++ | +++ | +++ | +++ | +++ |
| $OmpF_{Y340}$ | ++ | ++ | +++ | +++ | +++ | +++ |
| $OmpF_{L340}$ | ++ | ++ | +++ | +++ | +++ | +++ |
| $OmpF_{A340}$ | ++ | ++ | +++ | +++ | +++ | + |
| $OmpF_{T340}$ | ++ | ++ | +++ | +++ | +++ | + |
| $OmpF_{S340}$ | ++ | ++ | +++ | +++ | +++ | + |
| $OmpF_{P340}$ | ++ | ++ | +++ | ++ | +++ | +/− |
| $OmpF_{H340}$ | ++ | ++ | +++ | ++ | +++ | +/− |
| $OmpF_{R340}$ | ++ | ++ | +++ | + | +++ | −/+ |

[a]Strains were grown on LBA (plus 0.4 mM IPTG) plates for 16 h at temperatures shown. Growth resulting in large-, medium- and small-sized colonies were scored as +++, ++ and + respectively. Small or very small, but mixed-sized colonies were scored as +/− and −/+ respectively. OmpF in these strains was expressed from the lambda attachment site under the control of an IPTG-inducible promoter. No growth defects were observed in the absence of IPTG (see Fig. S2).

## Table 4

Bacterial strains and plasmids used in this study.

| Strain or plasmid | Characteristics | Reference or source |
|---|---|---|
| Strains | | |
| MC4100 | F- *araD139* Δ(*argF lac*)U139 *rspL150 relA1 fibB5301 ptsF25 deoC1 thi*-1 *rbsR* | Casadaban (1976) |
| RAM1292 | MC4100 Δ*ara714* | Werner and Misra (2005) |
| RAM1609 | RAM1292 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1315 | RAM1292Δ*bamB*::scar | Charlson *et al.* (2006) |
| RAM1423 | RAM1292 *bamA66-zad*::Tn*10*(Tc$^r$) | This Study |
| RAM1316 | RAM1292 *degP*::Tn*10*(Tc$^r$) | Charlson *et al.* (2006) |
| RAM1446 | RAM1292 Δ*surA*::Km$^r$ | This Study |
| RAM1610 | RAM1292 Δ*skp*-Tn*10*(Tc$^r$) | This Study |
| RAM1611 | RAM1315 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1612 | RAM1423 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1614 | RAM1316 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1615 | RAM1292 Δ*cpxR*::scar | This Study |
| RAM1616 | RAM1315 Δ*cpxR*::scar | This Study |
| RAM1617 | RAM1423 Δ*cpxR*::scar | This Study |
| RAM1618 | RAM1316 Δ*cpxR*::scar | This Study |
| RAM1619 | RAM1610 Δ*cpxR*::scar | This Study |
| RAM1620 | RAM1609 Δ*surA*::Km$^r$ | This Study |
| RAM1621 | RAM1615 pACYC184 | This Study |
| RAM1622 | RAM1615 pACYC184*degP* | This Study |
| RAM1623 | RAM1615 pACYC184*degP*[S210A] | This Study |
| RAM1624 | RAM1616 pACYC184 | This Study |
| RAM1625 | RAM1616 pACYC184*degP* | This Study |
| RAM1626 | RAM1616 pACYC184*degP*[S210A] | This Study |
| RAM1627 | RAM1617 pACYC184 | This Study |
| RAM1628 | RAM1617 pACYC184*degP* | This Study |
| RAM1629 | RAM1617 pACYC184*degP*[S210A] | This Study |
| RAM1630 | RAM1618 pACYC184 | This Study |
| RAM1631 | RAM1618 pACYC184*degP* | This Study |
| RAM1632 | RAM1618 pACYC184*degP*[S210A] | This Study |
| RAM1633 | RAM1615 pBAD24*cpxR* | This Study |
| RAM1634 | RAM1615 pBAD24*cpxR* Δ*surA*::Km$^r$ | This Study |
| RAM1635–1637 | RAM1621–1623 Δ*surA*::Km$^r$ | This Study |
| RAM1638–1641 | RAM1615–1618 Δ*rseA*::Km$^r$ | This Study |
| RAM1642 | RAM1292 Δ*cpxP*::Km$^r$ | This Study |
| RAM1643 | RAM1315 Δ*cpxP*::Km$^r$ | This Study |
| RAM1644 | RAM1423 Δ*cpxP*::Km$^r$ | This Study |
| RAM1645 | RAM1316 Δ*cpxP*::Km$^r$ | This Study |

| Strain or plasmid | Characteristics | Reference or source |
|---|---|---|
| RAM1646 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A *cpxR*$^+$-zii::Tn*10* | This Study |
| RAM1647 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1648 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340Y] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1649 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340L] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1650 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340A] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1651 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340T] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1652 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340S] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1653 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340P] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1654 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340H] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1655 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340R] *cpxR*$^+$*zii*::Tn*10* | This Study |
| RAM1656 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A Δ*cpxR*::scar- *zii*::Tn*10* | This Study |
| RAM1657 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1658 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340Y] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1659 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340L] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1660 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340A] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1661 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340T] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1662 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340S] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1663 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340P] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1664 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340H] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1665 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340R] Δ*cpxR*::scar-*zii*::Tn*10* | This Study |
| RAM1666–1675 | RAM1646–1655 pACYC184 | This Study |
| RAM1676–1685 | RAM1646–1655 pACYC184*degP* | This Study |
| RAM1686–1695 | RAM1656–1665 pACYC184 | This Study |
| RAM1696–1705 | RAM1656–1665 pACYC184*degP* | This Study |
| RAM1706 | RAM1292 *ompC*$^+$*zei*::Tn*10* pBAD24*cpxR* | This Study |
| RAM1707 | RAM1706 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1708 | RAM1292 *ompC*[1Cys]-*zei*::Tn*10* pBAD24*cpxR* | This Study |
| RAM1709 | RAM1708 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1710 | RAM1292 *ompC*[2Cys]-*zei*::Tn*10* pBAD24*cpxR* | This Study |
| RAM1711 | RAM1710 Δ*cpxR*::Cm$^r$ | This Study |
| RAM1712 | RAM1292 Δ*ompF*::scar *cpxP':lacZ nadA*::Tn*10* pTrc99A | This Study |
| RAM1713 | RAM1292 Δ*ompF*::scar *cpxP':lacZ nadA*::Tn*10* pTrc99A–*ompF*[F340] | This Study |
| RAM1714 | RAM1292 Δ*ompF*::scar *cpxP':lacZ nadA*::Tn*10* pTrc99A–*ompF*[F340R] | This Study |
| RAM1715 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A Δ*cpxP*::Km$^r$ | This Study |
| RAM1716 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340] Δ*cpxP*::Km$^r$ | This Study |
| RAM1717 | RAM1292 Δ*ompF*::scar λInCh-pTrc99A–*ompF*[F340R] Δ*cpxP*::Km$^r$ | This Study |
| RAM1718 | RAM1292 *ompC*$^+$*zei*::Tn*10* | This Study |
| RAM1719 | RAM1292 *ompC*[1Cys]-*zei*::Tn*10* | This Study |
| RAM1720 | RAM1292 *ompC*[2Cys]-*zei*::Tn*10* | This Study |
| RAM1721–1723 | RAM1718–1720 Δ*cpxP*::Km$^r$ | This Study |
| RAM1724–1726 | RAM1718–1720 pBAD24*cpxR* | This Study |

| Strain or plasmid | Characteristics | Reference or source |
|---|---|---|
| RAM1727–1729 | RAM1718–1720 pBAD24*cpxR* Δ*cpxR*::Cm$^r$ | This Study |
| Plasmids | | |
| pACYC184 | Tc$^r$ Cm$^r$; cloning vector | Chang and Cohen (1978) |
| pACYC184*degP* | Cm$^r$; Expresses *degP* under *lac* promoter control | This Study |
| pACYC184*degP*[S210A] | Cm$^r$; Expresses *degP*[S210A] under *lac* promoter control | Spiess *et al.* (1999) |
| pBAD24 | Amp$^r$; cloning and expression vector | Guzman *et al.* (1995) |
| pBAD24*cpxR* | Amp$^r$; cloning and expression vector with *cpxR* | This Study |
| pKD3 | Cm$^r$; Plasmid carrying Cm$^r$ gene cassette | Datsenko and Wanner (2000) |
| pKD4 | Km$^r$; Plasmid carrying Km$^r$ gene cassette | Datsenko and Wanner (2000) |
| pKD46 | Amp$^r$; Red recombinase expression plasmid | Datsenko and Wanner (2000) |
| pCP20 | Cm$^r$ and Amp$^r$; FLP recombinase expression plasmid, temperature sensitive | Datsenko and Wanner (2000) |

## Table 5

Primers used for cloning, deletion and RT-qPCR.

| Purpose | Sequence (5′-3′)[a] |
|---|---|
| For cloning | |
| *cpxR BspHI* Fwd | GCGCATtcatgaATAAAATCCTGTTAGTT |
| *cpxR HinD*III Rev | CAGTaagcttAAGAAGCAGAAACCATCAG |
| For SDM | |
| *degP* [A210S] SDM Fwd | GCAGCGATCAACCGTGGTAACTCTGGTGGTGCGCTGGTTAACCTGAACGGCG |
| *degP* [A210S] SDM Rev | CGCCGTTCAGGTTAACCAGCGCACCACCAGAGTTACCACGGTTGATCGCTGC |
| For RT-qPCR | |
| *rseA* Fwd RTPCR | CCGAGGTGCTCCATTTCG |
| *rseA* Rev RTPCR | GTCGCCGGTTGACGTACTG |
| *yfgC* Fwd RTPCR | ACTCTGCCCTGTTCCGTTATTC |
| *yfgC* Rev RTPCR | TTCGTGCGCCATAACTGAAG |
| *degP* Fwd RTPCR | AACTGAGCGATGGCCGTAAG |
| *degP* Rev RTPCR | ATATCAGAGCGCGGATCTTTG |
| *cpxP* Fwd RTPCR | ATTAAGCCACGCTGCTGAAGTC |
| *cpxP* Rev RTPCR | TCACCCGGATGCCAGTTATC |
| *ompF* Fwd RTPCR | CGATATGCTGCCAGAATTTGG |
| *ompF* Rev RTPCR | CGAAGAAGTCATCGCTGTATGC |
| *ftsL* Fwd RTPCR | TGCATTGCCTGGTGTTATCG |
| *ftsL* Rev RTPCR | TGGCAGCTTCCCAAATCG |
| *purC* Fwd RTPCR | TCGAATTCCGCAATGATACG |
| *purC* Rev RTPCR | GCGATCAAACTGCTCAATGC |

[a]Restriction and mutagenesis sites are shown by lower case and underlined characters respectively.