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cpxP Deletion Confers Resistance to Misfolded PapE-Induced Cytotoxicity Through Enhanced CpxAR Activation in *Escherichia coli*

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CpxAR is a two-component system that is conserved among Gram-negative bacteria. The CpxAR system is thought to respond to envelope stress derived from a variety of stimuli, including pH, salt and protein misfolding. Activation of the CpxAR response results in the transcription of gene targets such as chaperone proteins and periplasmic proteases, which combat envelope stress and re-establish envelope homeostasis. The CpxAR system is negatively by regulated the periplasmic protein CpxP, which has been shown to directly inhibit CpxA. One model suggests that CpxP has an additional role of chaperoning misfolded proteins to the DegP protease for degradation, where both CpxP and the misfolded protein undergo proteolysis. To investigate the role of CpxP in chaperoning misfolded proteins and inhibiting the CpxAR response, we induced expression of misfolded PapE protein in wild type and Δ*cpxP* strains of *E. coli*. Growth rates and activation of the CpxAR system were measured through the use of culture density and a bacterial luciferase reporter driven by the promoter of a CpxAR induced gene, respectively. Our results demonstrate that deletion of *cpxP* confers resistance to PapE-induced cytotoxicity and is correlated with increased activation of the CpxAR response. We propose that the loss of CpxP results in higher CpxAR transcriptional activity and thus higher expression of chaperone proteins and proteases, combating the cytotoxicity associated with misfolded PapE. Together, these results suggest that the primary role of CpxP may be negative regulation of the CpxAR response, while its chaperone activity may have secondary importance, the loss of which can be compensated through redundant mechanisms.

Responding to environmental stress is a fundamental adaptation necessary for the survival of all organisms. Two-component systems are a well-studied and ubiquitous mechanism through which bacteria are able to respond to environmental changes. In broad terms, a sensor kinase located in the bacterial inner membrane responds to a stimulus such as a change in osmolarity, pH or misfolded protein. The sensor kinase then phosphorylates a response regulator, which acts as a transcription factor, driving changes in gene expression (1). Through this system, bacteria have evolved a simple and effective way to integrate and respond to environmental signals.

Since its discovery in 1980, the role of the CpxAR two-component system has been studied in detail (2). However, gaps in current knowledge still persist. The system is conserved across gram-negative bacteria, but has primarily been studied in the context of *Escherichia coli.* The CpxAR system is thought to respond to envelope stress from misfolded proteins in the cellular periplasm (3). This is based on the observation that known stimuli for the CpxAR system cause protein misfolding (3). Examples of CpxAR stimuli include alkaline conditions, overproduction of membrane lipoprotein and the expression of misfolded proteins (4, 5). Upon stimulation, CpxA phosphorylates CpxR, which drives transcription of target genes (3). The target genes of CpxR have diverse functions, including chaperone proteins, proteases and proteins involved in envelope maintenance (6). Key targets of CpxR include, but are not limited to, the *cpxAR* operon, *cpxP,* spheroplast protein Y *(spy), degP,* and periplasmic isomerase A (*ppiA*) (6). Combined, the induction of these genes allows the bacterium to respond to envelope stress and re-establish periplasmic homeostasis.

Like most biological pathways, the CpxAR system is tightly controlled by positive and negative regulatory

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elements, the most important being CpxP. CpxP is a periplasmic protein that has been shown to directly inhibit CpxA (1). Further, one model suggests that CpxP acts as a chaperone in the process of degrading misfolded protein in the periplasm (7). CpxP shuttles the misfolded protein to DegP where both CpxP and the misfolded protein undergo proteolysis (7). This proposed mechanism is summarized in Figure 1. Based on the established role of CpxP in eliminating misfolded protein, we investigated whether *cpxP* knockout strains of *E. coli* were more susceptible to misfolded PapE toxicity. PapE is an adaptor protein that composes the fibrillar tip of the P pilus, which mediates uropathogenic *E. coli* adhesion to the kidney epithelium (8). High concentrations of misfolded PapE are known to be cytotoxic, as they accumulate and form large aggregates (5, 9, 10).

To investigate this question, we utilized wild type and Δ*cpxP* strains of *E. coli* transformed with an IPTG-inducible *papE* plasmid that misfolds in the absence of PapD (5, 9). This is an established system in which to study CpxAR activation (11). In response to increasing induction of misfolded PapE, we measured growth rates, as well as activation of the CpxAR two component system through a *cpxAR::lux* reporter construct (6). Luminescence was used as a readout for CpxAR activation, as it is known that *cpxAR* expression increases with CpxAR activity (6). Surprisingly, our results showed that *cpxP* knockout strains were significantly less susceptible to PapE induced cytotoxicity, which corresponded with increased CpxAR activation. This is consistent with CpxP acting as a negative regulator of CpxAR, but casts doubt on the importance of its chaperone activity.

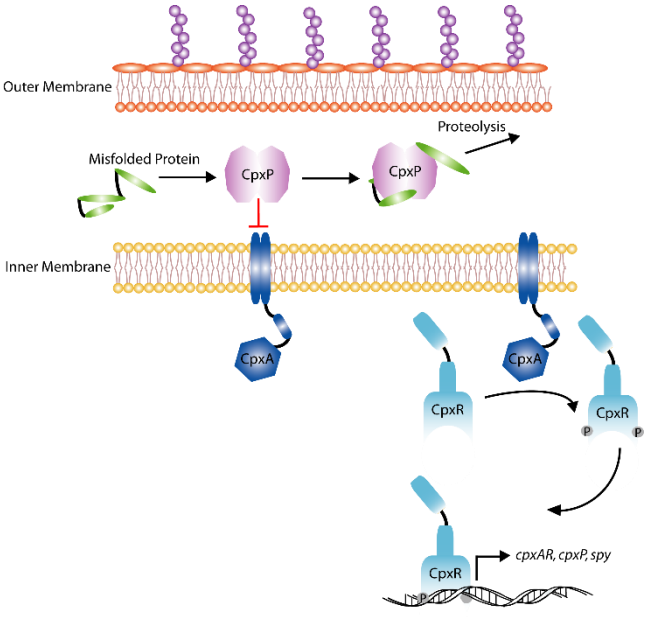

FIG. 1 Overview of the current model of the CpxAR two component system. CpxP inhibits CpxA. Upon stimulation with misfolded protein, CpxP chaperones the misfolded protein to DegP where they undergo proteolysis. This results in CpxA activation, as it is no longer inhibited by CpxP resulting in downstream signalling through CpxR.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* BW25113 wild type and Δ*cpxP* strains were obtained from the Keio collection (12). Bacterial strains used are summarised in Table 1. The *lux* reporter plasmid, pJW1 (*cpxAR::lux*), was provided by Dr. Tracy Raivio at the University of Alberta. The *papE* plasmid, pMMB66, was provided by Dr. Scott Hultgren at the University of Washington. Plasmids used are summarised in Table 2.

Media and stock solutions. Lysogeny broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl) was adjusted to pH7.5 using 1M NaOH, and then autoclaved. Prior to beginning *lux* reporter assays, the strains were diluted in LB that was buffered with one-tenth volume of 1M pH 7.4 sodium phosphate buffer. LB was autoclaved with 1.5% agar for solid media. Kanamycin monosulphate (Sigma-Aldrich, St. Louis, MO) and ampicillin sodium salt (Thermo Fisher Scientific, Waltham, MA) were dissolved in dH_2O as 10 mg/mL and 50 mg/mL stock solutions, respectively, and stored at -20°C. $CaCl_2$ (Thermo Fisher Scientific, Waltham, MA) was dissolved in dH_2O as a 0.1 M solution and stored at 25°C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, Waltham, MA) was dissolved in dH_2O as a 0.1 M stock solution and stored at -20°C. Antibiotic, $CaCl_2$, and IPTG solutions were filter sterilised with a 0.22 µm polyethersulphone filter (VWR International, Radnor, PA).

Overnight bacterial culture for *lux* reporter assays and competent cell preparation. Overnight culture was prepared by inoculating 5 mL of LB, supplemented with 50µg/mL of appropriate antibiotics, with a loop of bacteria and incubating at 37°C for 15-20 hours, with shaking at 150 rpm.

Preparation of competent cells and transformation. Plasmid isolation was performed with a PureLink® Quick Plasmid

TABLE 1 Names and descriptions of strains used across all experiments.

Background Strain	Knockout	Plasmid	Resistance	Reference
BW25113	N/A	N/A	None	(12)
BW25113	N/A	pJW1, pMMB66 (*papE*)	Kanamycin, Ampicillin	This study
BW25113	N/A	pJW1, pMMB66 (Empty Vector)	Kanamycin, Ampicillin	This study
BW25113	Δ*cpxP*	N/A	None	(21)
BW25113	Δ*cpxP*	pJW1, pMMB66 (*papE*)	Kanamycin, Ampicillin	This study
BW25113	Δ*cpxP*	pJW1, pMMB66 (Empty Vector)	Kanamycin, Ampicillin	This study

TABLE 2 Names and descriptions of plasmids used across all experiments.

Backbone Vector	Gene	Resistance	Induction	Reference
pMMB66	None (empty vector)	Ampicillin	IPTG	(7)
pMMB66	*papE*	Ampicillin	IPTG	(7)
pJW1	*cpxAR::lux*	Kanamycin	Constitutive	(6)

Miniprep kit (Thermo Fischer Scientific, Waltham, MA). Construction of double transformants proceeded in a sequential manner as per standard protocol (13). Briefly, cells were made chemically competent by inoculating 50mL of LB with a 1/200 dilution of overnight culture and grown at 37ºC, with shaking at 150 rpm, to an OD_{600} between 0.3-0.4. The bacterial culture was incubated on ice for 10 minutes, and then 25 mL aliquots were centrifuged at 10000 rpm for 5 minutes at 4ºC. Pellets were resuspended in 12.5 mL of ice-cold (4ºC) 0.1 M $CaCl_2$ solution and incubated on ice for 30 minutes. Cells were centrifuged at 10000 rpm for 5 minutes at 4ºC and the corresponding pellets were resuspended in 0.5 mL of $CaCl_2$ solution and incubated at 4ºC for 20 hours. 100 µL aliquots of competent cells were mixed with 100 pg of plasmid and incubated at 4ºC for 30 minutes. In a 42ºC water bath, cells were heat-shocked for 45 seconds and incubated on ice for 1 minute. Cells recovered in 1.2 mL of LB for 1 hour at 37ºC with shaking at 150 rpm. Cells were plated on solid LB with appropriate antibiotics (50 µg/mL kanamycin for pJW1, 50 µg/mL for pMMB66, and both antibiotics at 50 µg/mL for double transformants) and grown overnight at 30ºC.

***E. coli* growth curves.** A 250 µM IPTG solution was prepared in LB and 100 µL dispensed into a 96-well black wall, clear bottom plate (Corning, Corning, NY). IPTG was then serially diluted 1:2 with LB to 31.25 µM. Double transformed overnight cultures (WT *E. coli* BW25113 pJW1 (*cpxAR::lux*), and pMMB66 *papE* or empty vector; Δ*cpxP E. coli* BW25113 pJW1 (*cpxAR::lux*), and pMMB66 *papE* or empty vector) were diluted 1/200 in 10 mL of kanamycin and ampicillin containing LB media (50 µg/mL of each antibiotic). 100 µL of each bacterial strain was dispensed in triplicate into the IPTG-containing 96-well plate. Final concentrations of IPTG ranged from 125 µM to 15.6 µM. Cells were grown at 37ºC in a Synergy H1 plate reader (Biotek, Winooski, VT) for 4 hours with OD_{630} measurements taken every 15 minutes. OD_{630} was used instead of OD_{600}, in accordance with BioTek application notes (14).

***cpxAR::lux* reporter assay.** The experimental set-up followed the same procedure described above for the bacterial growth curves; however, for this experiment the final IPTG concentrations ranged from 2.28µM to 150µM (serially diluted 1:2 from 300µM). After plate set-up was complete, cells were incubated at 37ºC for 4 hours before measuring luminescence and OD_{630} in a in a Synergy H1 plate reader (Biotek, Winooski, WT). Luminescence was normalized to OD_{630} when analyzing the data.

IPTG dose response. The experimental set-up followed exactly as described above for the *cpxAR::lux* reporter assay, except that *cpxAR::lux* single transformants were used instead of *cpxAR::lux* with *papE*/empty vector.

Statistical analysis. Due to the fact that each experiment was only performed once with all treatments plated in triplicate, each of the three measurements was treated as an independent event. Statistical analysis was guided by the data distribution and number of groups, primarily utilizing one or two was analysis of variance (ANOVA) to determine statistical significance.

RESULTS

CpxP deficient *E. coli* are resistant to PapE induced cytotoxicity. To elucidate the mechanism behind CpxP-mediated inhibition of the CpxAR system, we used wild type and CpxP deficient *E. coli* and induced misfolded protein cytotoxicity via expression of PapE without its chaperone protein (5). PapE expression was controlled through a *tac* promoter; therefore, treatment with increasing concentrations of IPTG would correspond to increasing PapE expression (15). Susceptibility to PapE induced cytotoxicity was determined through a growth curve with OD_{630} measured at 15 minute intervals. An empty vector lacking the *papE* insert was used as a control in wild type and *ΔcpxP* cells, which demonstrated no observable growth defects in response to increasing IPTG treatment (Fig. 2B and 2D). The wild type strain showed dose-dependent cytotoxicity from IPTG-induced PapE expression (Fig. 2A). In contrast, there is no observable cytotoxicity at any concentration of IPTG for *ΔcpxP* cells (Fig. 2C). 31.2µM of IPTG was the lowest concentration to result in a statistically significant decrease ($p < 0.0001$) in growth (Fig. 2A**)**. At 125µM IPTG, PapE-induced cytotoxicity is lethal to all wild type cells, while CpxP deficient *E. coli* maintain comparable growth to vehicle treated cells (Fig. 2A and 2C). At the 4 hour time point, IPTG has an EC_{50} of 46.04µM for wild type cells transformed with the *papE* vector (Fig. 3). As wild type *E.*

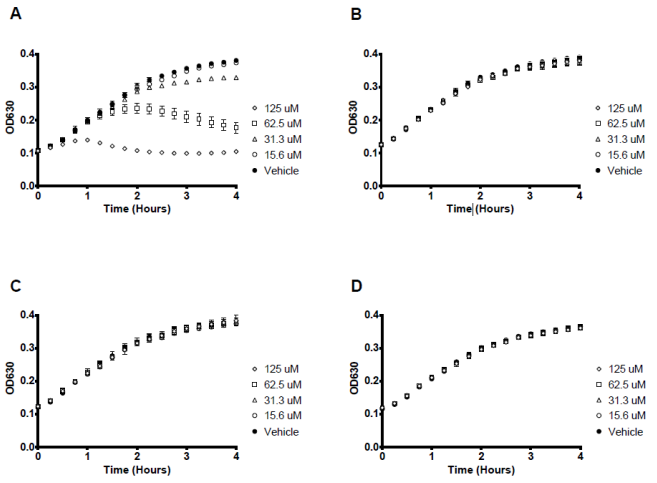

FIG. 2 Bacterial growth curves at 15 minute intervals for 4 hours using OD_{630} in response to increasing concentrations of IPTG. Treatments were plated in triplicate; error bars represent the standard deviation. Statistical analysis utilized two-way ANOVA. **(A)** Wild type *E. coli* transformed with *papE* plasmid. Significant effect of IPTG

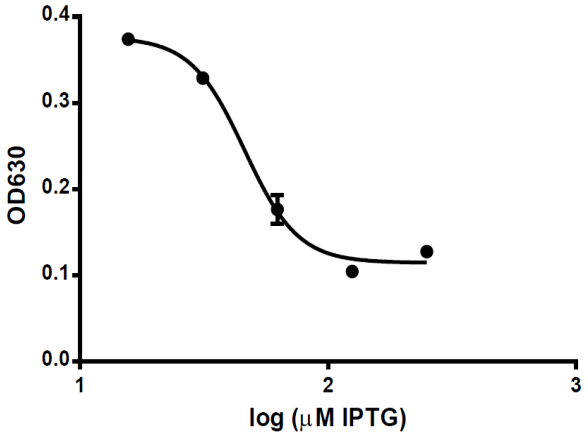

FIG. 3 Dose-response curve of IPTG treatment inducing PapE-induced cytotoxicity on wild type *E. coli.* Dose-response curve used the same data from Figure 2 at the 4 hour time point. Error bars represent standard deviation within triplicates.

coli exhibit a dose-dependent response to IPTG treatment while *ΔcpxP E. coli* do not, these results strongly suggest that CpxP deficient *E. coli* are resistant to PapE induced cytotoxicity.

CpxP deficient *E. coli* trends toward higher CpxAR activation. Since CpxP deficient *E. coli* were unaffected by PapE induced cytotoxicity, we hypothesized that the loss of CpxP resulted in increased activation of the CpxAR response. Downstream targets of CpxAR include chaperones and proteases, which can combat misfolded PapE related cytotoxicity (6). The loss of CpxP would increase baseline CpxAR activity relative to wild type *E. coli*. To investigate this hypothesis, we used a bacterial luciferase reporter controlled by the *cpxAR* promoter to measure transcriptional activity of the CpxAR response. In wild type *E. coli* transformed with the *papE* plasmid, we observed dose-dependent induction of *cpxAR::lux* that peaked at 18.25μM of IPTG before rapidly decreasing, likely due to PapE-induced cytotoxicity. Transcriptional activation driven by the CpxAR system was significantly higher ($p < 0.001$) at 18.25μM IPTG in *papE* transformed wild type *E. coli* compared to the empty vector negative control (Fig. 4A). At high concentrations of IPTG (150μM) *cpxAR::lux* activity was significantly ($p < 0.001$) lower in *papE* transformed cells in comparison to the negative control, which can be attributed to cell death (Fig. 4A). These results indicate that the CpxAR system is being successfully induced in response to misfolded PapE expression.

In *ΔcpxP E. coli,* no significant dose-dependent increase of *cpxAR::lux* activity was observed in response to increasing IPTG concentrations (Fig. 4B). For empty vector transformed cells, *cpxAR::lux* activity was consistently higher in *ΔcpxP E. coli* (2200-2800AU) in comparison to wild type *E. coli* (~1500AU) (Fig. 4C). This indicates higher basal levels of CpxAR activity in *ΔcpxP E. coli.* This elevated CpxAR activity across all concentrations of IPTG lends support to the hypothesis that *ΔcpxP E. coli* is resistant to PapE-mediated cytotoxicity due to enhanced CpxAR activity. Unexpectedly, *cpxAR::lux* activity was consistently higher in *ΔcpxP E. coli* transformed with empty vector compared to cells transformed with the *papE* vector, though this may be an artefact of the experimental system. Taken together, these data indicate a plausible mechanism to explain why *ΔcpxP* do not succumb to the same toxic effects of misfolded PapE that are lethal to wild type cells.

IPTG treatment decreases CpxAR activity. To ensure that IPTG itself did not have any impact on CpxAR activation, we assessed CpxAR activity in *cpxAR::lux* single transformants after treatment with IPTG. As

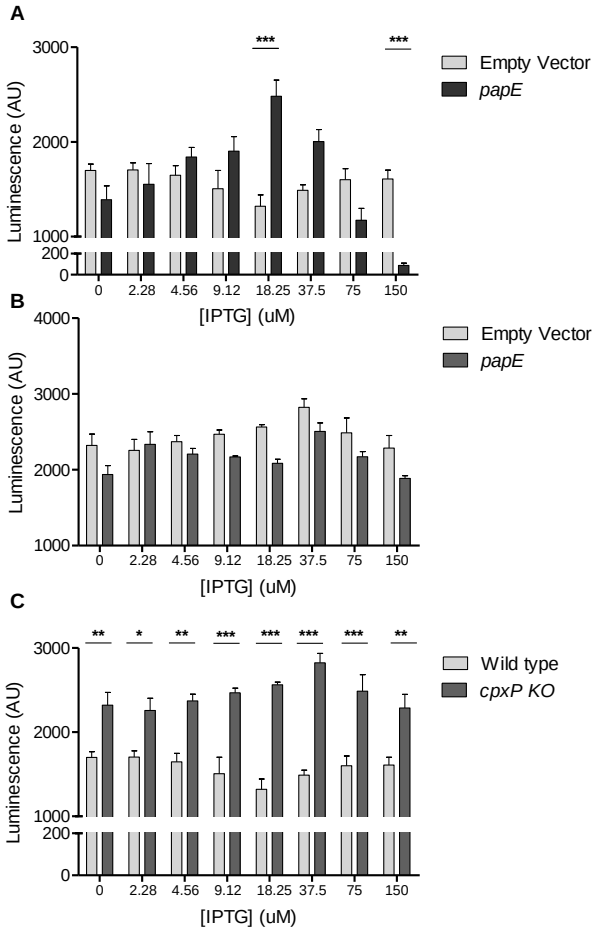

FIG. 4 CpxAR activation in response to IPTG-induced PapE expression. *E. coli* transformed with *cpxAR::lux* reporter construct and either *papE* or empty vector. Luminescence values were normalized to the OD_{630} for each treatment. Error bars are based on the standard deviation across triplicates. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test across all treatment groups. *** = $p < 0.001$. A. Wild type *E. coli*. B. Δ*cpxP E.coli*.C. Wild type and Δ*cpxP E. coli* both transformed with *papE.*

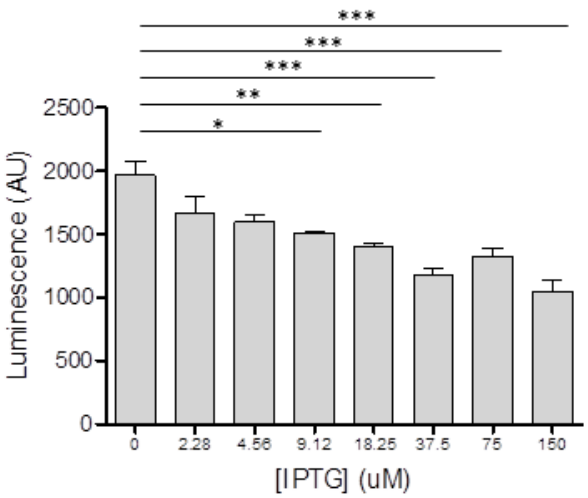

FIG. 5 Effect of IPTG on CpxAR activation in *cpxAR::lux* transformed wild type *E. coli.* Luminescence was normalized to OD_{630} for all treatments. Error bars represent the standard deviation across triplicates. Statistical analysis was based on one-way ANOVA with a Bonferroni post-test comparing all groups. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

concentrations of IPTG increased, there was a clear trend in decreasing CpxAR activity (Fig. 5). This result demonstrates that IPTG alone can decrease CpxAR activity which may have unknown implications within our experimental model.

DISCUSSION

The purpose of this study was to understand the role of the CpxP periplasmic protein within the CpxAR two-component system response to cellular stress. In these studies, cellular stress was induced by the expression of the misfolded pilus protein, PapE, under the control of an IPTG inducible promoter. Previous studies have demonstrated an important role of CpxP in degrading misfolded periplasmic protein; thus, we hypothesized that CpxP deficient *E. coli* would be more susceptible to misfolded PapE-induced cell death (7). Surprisingly, our results suggested the opposite; CpxP deficient *E. coli* were highly resistant to PapE-induced cell death. Our explanation for this phenomenon was that the deletion of *cpxP* would result in higher basal levels of CpxAR activation, allowing cells to more rapidly respond to misfolded periplasmic protein, avoiding PapE-induced cytotoxicity. CpxP deficient *E. coli* avoid the lag phase of the response experienced by wild type cells, wherein CpxP must be bound by misfolded PapE to relieve inhibition on CpxAR. This hypothesis was supported by the observation that Δ*cpxP E. coli* have elevated baseline CpxAR activity compared to wild type cells. CpxAR activity in wild type *E. coli* is consistently lower than the Δ*cpxP.* Taken together, these data provide a plausible mechanism: CpxP inhibits CpxA, and thus upon deletion of CpxP, cells more readily respond to misfolded protein stress.

The enhanced ability of Δ*cpxP* to resist PapE-induced cytotoxicity is surprising given the putative chaperone role of CpxP to eliminate misfolded periplasmic protein (7). Our results show that increased CpxAR activation resulting from the loss of CpxP overcomes the decline in fitness caused by the loss of chaperone activity. Otherwise, Δ*cpxP* should show greater susceptibility to PapE-induced cytotoxicity, due to an inability to eliminate misfolded periplasmic PapE. In the work by Isaac *et al.* that first suggested the chaperone role of CpxP, they found that Δ*cpxP E. coli* was more susceptible to PapE cytotoxicity compared to similarly treated wild type *E. coli* (7). The reason for the discrepancy between our results is unclear. It could be strain specific phenomenon. In our experiments, we utilized BW25113 strains, while Isaac *et al.* used MC4100 derived strains. Though this may initially appear as a minor difference, the importance of this discrepancy is rooted in the fact that different *E. coli* strains have been shown to have differing responses to *cpxP* deletion (16). Between different strains, redundant systems may be better or worse at overcoming the lack of CpxP chaperone activity (16). Other factors such as cell growth measurements, IPTG concentration, incubation time and temperature matched those used by Isaac *et al.* Though the mechanism of CpxP proteolysis via DegP is well studied, the details regarding CpxP chaperone activity remain clouded (17).

Alternatively, we cannot rule out the possibility that our experimental system is not working as anticipated. We never directly measured PapE expression in the two strains, and thus we cannot say for certain that PapE is being expressed at equal levels. In order to conclude that the Δ*cpxP* is in fact more resistant to PapE-mediated cytotoxicity, we should first confirm that both strains are expressing equal levels of *papE*. This could be done through quantitative PCR, to ensure equal expression of *papE.* It would be difficult to assess whether PapE expression is equal at the protein level due to differing efficiencies of degrading the protein between strains. However, comparison of protein level expression could still be pursued through a western blot using a FLAG-tagged PapE.

We postulate that the reason for the resistance to PapE-induced cytotoxicity is enhanced activation of the CpxAR system in the absence of CpxP, as shown in Figure 4. CpxP has been shown to directly interact with and inhibit CpxA; misfolded PapE disrupts this interaction leading to activation of CpxAR (18).

Overexpression of CpxP has been shown to limit the protein stress response through increased CpxAR inhibition (19). Thus, it seems like a natural extension that *cpxP* deletion would enhance the response. Further, activating mutations in CpxA that lead to elevated CpxAR activity have been shown to result in resistance to misfolded protein toxicity (20). Resistance to misfolded protein toxicity in the constitutively activate CpxA strain is likely the result of increased expression of chaperone and protease molecules, most notably DegP, that can breakdown misfolded protein in the periplasm (20). This same mechanism likely holds true in our experimental system.

In our experimental system, wild type cells would not begin to initiate a protein stress response until *papE* is transcribed, the mRNA translated and misfolded protein begins accumulating in the periplasm, at which point CpxAR activity increases, and transcription of proteases and chaperones begins. The result is a significant lag between the start of PapE accumulation and the cellular response to the misfolded protein. In contrast, Δ*cpxP* cells likely have a high concentration of proteases and chaperones in the periplasm, due to elevated basal CpxAR activity. Thus, there is no lag between PapE reaching the cytoplasm and its rapid proteolysis. This likely prevents the accumulation of PapE, bypassing its toxic effects. We did not show elevated expression of chaperones and proteases induced by CpxAR, which would be a natural extension of our work.

Finally, we show that IPTG on its own suppresses CpxAR activation in wild type cells transformed with *cpxAR::lux*. The results of this experiment were surprising given that we did not anticipate IPTG to have any impact on cells that lacked an IPTG-inducible promoter. This suggests that there may be off target effects of IPTG. However, given that our experiments compared between strains treated with equal concentration of IPTG, we anticipate that this phenomenon does not affect our findings.

In conclusion, we have shown evidence that supports the hypothesis that CpxP acts as a negative regulator of CpxAR, and that CpxP deficient *E. coli* are resistant to misfolded PapE toxicity. Results from the luciferase assay suggest that CpxAR activity in wild type cells decreases in the presence of higher concentrations of misfolded PapE, while cpxAR activity increases in cells without CpxP expression. Further research is needed to fully understand the role of CpxP in responding to envelope stress and to tease apart the relationship between CpxAR activity and misfolded periplasmic protein accumulation.

FUTURE DIRECTIONS

One major shortcoming of this study is the fact that PapE expression was never confirmed in the wild type or Δ*cpxP* double transformants. We recognize the possibility that the *papE* plasmid carried by the Δ*cpxP* strain may have accumulated mutations that prevent PapE expression. This can often happen when a toxic protein is expressed from a plasmid; bacteria that accumulate mutations that prevent the expression of the toxic compound are selected for over those expressing the toxic version, resulting in the loss of expression of the toxic protein. This may explain the remarkable resistance of the Δ*cpxP* strain, independent of the loss of CpxP activity. We suggest that PapE expression be confirmed at both the mRNA and protein levels. This can be done at the mRNA level using quantitative PCR to measure mRNA expression of the *papE* plasmid. At the protein level, we suggest immunoblotting cellular lysates of induced and uninduced cells, as well as cells without the *papE* plasmid using an anti-PapE antibody. If expression of PapE is confirmed in the induced wild type and Δ*cpxP* strains carrying the *papE* plasmid, this will provide further evidence that Δ*cpxP* resistance is caused by an enhanced mechanism to eliminate misfolded protein.

The use of Δ*cpxA* and Δ*cpxR* strains would be useful in further confirming our proposed mechanism of resistance to PapE-induced cytotoxicity. Repeating the experiments that we have discussed in this paper using these knock-out strains will provide valuable support for our proposed model. Both of these knock-out strains should be highly susceptible to PapE-mediated cell death, due to an inability to activate the CpxAR system.

This study has provided evidence that cells without CpxP are able to more effectively deal with misfolded PapE. However, evidence that increased CpxAR activity is directly involved in this process was inconclusive. In order to prove causality, we suggest that further investigations be pursued using a Δ*cpxA,* Δ*cpxP* strain. If our model is correct, the loss of CpxA activity should increase susceptibility to PapE toxicity. Additionally, rather than determining CpxAR activity by monitoring the activity of one gene, multiple different CpxAR-influenced genes should be explored including: *cpxP, spy, degP* and *rpoE-rseABC*.

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