Cpx Signal Transduction Is Influenced by a Conserved N-Terminal Domain in the Novel Inhibitor CpxP and the Periplasmic Protease DegP

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In *Escherichia coli*, envelope stress can be overcome by three different envelope stress responses: the σ^E stress response and the Bae and Cpx two-component systems. The Cpx envelope stress response is controlled by the sensor kinase CpxA, the response regulator CpxR, and the novel periplasmic protein CpxP. CpxP mediates feedback inhibition of the Cpx pathway through a hypothetical interaction with the sensing domain of CpxA. No informative homologues of CpxP are known, and thus it is unclear how CpxP exerts this inhibition. Here, we identified six *cpxP* loss-of-function mutations using a CpxP-β-lactamase (CpxP′-′Bla) translational fusion construct. These loss-of-function mutations identified a highly conserved, predicted α-helix in the N-terminal domain of CpxP that affects both the function and the stability of the protein. In the course of this study, we also found that CpxP′-′Bla stability is differentially controlled by the periplasmic protease DegP in response to inducing cues and that mutation of *degP* diminishes Cpx pathway activity. We propose that the N-terminal α-helix is an important functional domain for inhibition of the Cpx pathway and that CpxP is subject to DegP-dependent proteolysis.

Constantly changing environments are a critical situation to which all bacteria must adapt in order to survive. In gram-negative bacteria, the envelope, consisting of the outer membrane, periplasm, and inner membrane, is in constant contact with the environment. *Escherichia coli* has at least three regulatory pathways, the σ^E stress response and the CpxRA and the BaeSR two-component systems, which are activated by and mediate adaptation to different envelope stresses (1, 2, 30, 34).

The Cpx signal transduction pathway is a typical two-component system with a membrane-bound histidine kinase, CpxA, and a cytoplasmic response regulator, CpxR (15, 42). The activating cues of the Cpx pathway include alterations in extracellular pH (9, 27), accumulation of enterobacterial common antigen intermediate lipid II (7), overexpression of NlpE (38), overexpression of P pilus subunits in the absence of their periplasmic chaperone PapD (18), and overexpression of the enteropathogenic *Escherichia coli* type IV bundle-forming pilus subunit BfpA (28). Each of these activating cues is expected to lead to the accumulation of misfolded and/or mislocalized proteins associated with the envelope, which are likely a component of the activating signal for the Cpx pathway.

When activated, CpxA acts as a histidine autokinase (33). The phosphorylated CpxA then transfers the phosphate to a conserved aspartate on CpxR (33). Phosphorylated CpxR has enhanced ability to bind to consensus sequences and increase transcription of the Cpx regulon (8, 29, 33), which contains numerous envelope protein folding and degrading factors, and a variety of other genes whose roles in responding to envelope stress are not understood (13, 34). Among the envelope protein folding and degrading factors induced are the periplasmic

endoprotease DegP (6, 25), two peptidyl-prolyl-isomerases, PpiA and PpiD (11, 26, 29), and DsbA, the major periplasmic disulfide oxidase (3, 8, 20, 29). Along with increased transcription of the protein folding and degrading factors, a small periplasmic inhibitor protein, CpxP, is also expressed at elevated levels, together with the *cpxRA* genes (9, 31, 32). Thus, a major role of the Cpx response appears to be maintaining envelope proteins under adverse conditions.

cpxP was first identified as a pH-regulated locus which encodes a periplasmic protein that helps overcome extracytoplasmic protein-mediated toxicity (9). Danese and Silhavy (9) identified *cpxP* as a *lacZ* operon fusion that was up-regulated by NlpE in a CpxA-dependent manner. Furthermore, CpxP is involved in signal transduction, since overexpression of CpxP causes a three- to fivefold reduction in Cpx-mediated gene expression via the periplasmic sensing domain of CpxA (31, 32). An inner membrane-tethered maltose-binding protein–CpxP fusion protein can maintain Cpx inhibition in the presence of spheroplasting, a strong Cpx-activating signal, while a maltose-binding protein-CpxP fusion localized to the periplasm does not, suggesting that the CpxA-CpxP interaction is direct (31). Currently it is thought that in the absence of envelope stress, CpxP interacts with the sensing domain of CpxA, maintaining the pathway in an off state. Upon activation, CpxP inhibition would be relieved, allowing induction of the response. However, CpxP is not required for signal transduction, since in either the absence of, or presence of overexpression of, CpxP, the Cpx pathway can still be induced further (14, 32). Thus, the hypothesized role of CpxP is in fine-tuning the response.

In this study we address the question of how CpxP-mediated inhibition might occur and be relieved. Since CpxP has no informative homologues, we set out to identify possible functional domains in CpxP that are important for signal transduc-

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tion. Using a translational CpxP′-′Bla fusion construct, we identified a highly conserved, predicted α-helix in the N-terminal domain of CpxP that affects both the inhibitory function and stability of the protein. Diminished levels of some of the loss-of-function mutants relative to the wild-type CpxP′-′Bla protein suggested that proteolysis might affect CpxP-mediated inhibition. Indeed, we noted that the levels of the mutant CpxP′-′Bla proteins could be returned to, or elevated above, normal in the absence of DegP. DegP proteolysis is likely important for controlling CpxP levels in response to inducing cues since *degP* mutation simultaneously abrogates the disappearance of CpxP′-′Bla and diminishes pathway activation at elevated pH. We propose that the predicted N-terminal α-helix is important for the CpxA-dependent inhibition of the pathway and that CpxP levels are controlled by DegP-dependent proteolysis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The stains and plasmids used in this study are listed in Table 1. All strains were constructed using standard genetic techniques (36). PCR primers are described in Table 2.

Media, antibiotics, and growth conditions. All strains were grown on Luria-Bertani (LB) agar (36) or lactose-MacConkey agar (Difco), which is an indicator medium that permits measurement of β-galactosidase levels, at either 37 or 30°C. Liquid cultures were grown in LB broth with aeration. Strains were maintained with the appropriate selection, 100 μg/ml ampicillin, 100 μg/ml chloramphenicol, 25 μg/ml kanamycin, or 25 μg/ml tetracycline. All antibiotics were purchased from Sigma.

Mutagenesis of pPB. Random *cpxP* point mutants were isolated by passaging pPB through DNA repair-deficient strain XL-1 Red (Stratagene), according to the manufacturer's specifications. Twenty independently derived samples of mutagenized pPB were collected after passaging through XL-1 Red and were transformed into TR50. Transformants were grown at 37°C for a maximum of 20 h on lactose-MacConkey medium containing ampicillin. Lac$^+$ Amp^r derivatives were isolated and characterized further.

DNA sequence analysis. Mutated pPB plasmid DNA was extracted using a QIAprep Spin mini prep kit (QIAGEN), according to the manufacturer's protocol. The mutated *cpxP* genes were amplified using the CpxP*Bla* and CpxP5′*Kpn* primers (Table 2). The PCR products were purified using a QIAGEN PCR purification kit (QIAGEN), according to the manufacturer's directions. Sequencing was carried out using the protocol for the DYEnamic ET terminator cycle sequencing premix kit (Amersham) provided by the Molecular Biology Service Unit (University of Alberta).

β-Galactosidase assay. Single colonies of each bacterial strain to be assayed were inoculated into 2-ml overnight cultures of LB broth containing the appropriate antibiotics at 37 or 30°C with aeration. The next day the cultures were diluted 1:50 into fresh medium and grown at 37°C to late log phase (optical density at 600 nm = 0.6). Induction of the Cpx response by elevated pH was accomplished by adding 1 M sodium phosphate buffer of the appropriate pH (35) to the medium to a final concentration of 100 mM. β-Galactosidase activity was measured using the microtiter plate assay (37) with slight variation to the protocol; 5 μl of cell mixture was added to 195 μl of Z-buffer due to high expression of β-galactosidase in the strains. The final calculation was then multiplied by 10 to account for the dilution. Each assay was performed in triplicate. Error bars represent the standard deviation.

Western blot analysis. Overnight cultures of the appropriate strains grown in LB plus antibiotics at 30°C or 37°C with aeration were subcultured 1:50 and grown at 37°C for 4 h. pH induction of the Cpx response was accomplished using the same procedure described for the β-galactosidase assays above. The optical density at 600 nm of the cultures was measured and whole-cell extracts were prepared by pelleting volumes that were adjusted to correspond to 1 ml of the culture that reached the lowest optical density at 600 nm. Cell extracts were resuspended in 50 μl of 2X sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading dye (35). Lysates were boiled for 5 min and 10 μL was electrophoresed on a 10% SDS-PAGE minigel system (Bio-Rad) (24). Proteins were transferred to nitrocellulose membrane at 10 V overnight (41). The membrane was labeled as previously described by Raivio et al. (32). β-Lactamase monoclonal antibody (1:1,000 dilution) (QED BioScience Inc.) or bac-

terial alkaline phosphatase polyclonal antibody (1:20,000 dilution) (Research Diagnostics, Inc.) were used as primary antibodies. A secondary anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase (Sigma) was used at a 1:10,000 dilution. Proteins were visualized using the Immun-Star alkaline phosphatase substrate pack chemiluminescent kit (Bio-Rad).

Site-directed mutagenesis of pCpxPU to make pCpxPD$_{61}$EU and pCpxPQ$_{55}$PU. The D$_{61}$E and Q$_{55}$P mutations were transferred to the pCpxP vector as follows. First, the genomic region surrounding *cpxP* was PCR amplified using the CpxPBam and CpxPKpn primers and cloned into the BamHI and KpnI sites of pUC18 to construct pCpxPU. Next, the D$_{61}$E and Q$_{55}$P mutations were introduced into pCpxPU using the primers D-61-E1 and D-61-E2 (Table 2) for pCpxPD$_{61}$EU and the primers Q-55-P1 and Q-55-P2 (Table 2) for pCpxPQ$_{55}$PU using the Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with slight variations to the protocol as follows; 2 μL of 10 mM deoxynucleoside triphosphates was used in the extension reaction for 6.5 min at 68°C, 1 μL of DpnI (5 U/μL) was used to digest unmutated template DNA for 2.5 h, One Shot Top10 chemical competent cells (Invitrogen) were used in place of the XL-1 Blue competent cells, 5 μL of DpnI-treated DNA was added to the Top10 cells, and LB was used in place of NZY$^+$ broth.

RESULTS

Inhibition of the Cpx pathway is specific to CpxP overexpression. Raivio et al. (31, 32) have shown that overproduction of CpxP represses the Cpx regulon in a CpxA-dependent manner. From these findings it was proposed that CpxP acts as a periplasmic inhibitor by interaction with the sensing domain of CpxA. Alternatively it is possible that CpxP overexpression may lead to pathway inhibition by aiding in envelope protein folding, thus relieving envelope stress and causing down-regulation of the Cpx response in this fashion (14). If this is true, then overproduction of other Cpx-regulated members may also down-regulate the pathway.

To test this hypothesis, we overexpressed numerous members of the Cpx regulon: a periplasmic endoprotease, DegP (21, 25), a major disulfide oxidase, DsbA (3, 20), a peptidyl-prolyl-isomerase, PpiA (26), a small periplasmic protein of unknown function, Spy (17), and CpxP (9). Overexpression of CpxP, PpiA, and Spy was confirmed by SDS-PAGE analysis of isopropylthiogalactopyranoside (IPTG)-induced periplasmic extracts (data not shown). DegP and DsbA overexpression was confirmed by complementation of temperature sensitivity (40) and dithiothreitol sensitivity (10), respectively, of *degP* and *dsbA* null mutants transformed with pDegP or pDsbA (data not shown).

To see what effect overexpression of these factors may have on Cpx-regulated gene expression we used a *cpxP*′-*lacZ*$^+$ fusion in single copy on the chromosome. When CpxP is overexpressed there is a fivefold reduction in Cpx-mediated gene expression, as expected (Fig. 1, compare lanes 1 and 2). In contrast, overexpression of DegP, DsbA, PpiA, or Spy had no effect on Cpx-mediated gene expression (Fig. 1, compare lanes 1 with 3 to 6). We conclude that CpxP functions uniquely as an inhibitor of the Cpx response.

Isolation of *cpxP* mutants. Since CpxP has no informative homologues, we sought to identify functional domains in CpxP that are important for signal transduction. The strongest phenotype available to monitor CpxP function is the inhibition of Cpx-regulated gene expression upon CpxP overexpression (14, 31, 32). Thus, we looked for *cpxP* loss-of-function mutations that prevented Cpx pathway inhibition upon overexpression of a CpxP–β-lactamase (CpxP′-′Bla) translational fusion protein encoded on plasmid pPB. Overexpression of the CpxP′-′Bla

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150* (Str^r) *relA1 flbB5301 decC1 ptsF25 rbsR*	5
TR50	MC4100 λRS88 (*cpxP'-lacZ$^+$*)	33
TR757	MC4100 λRS88 (*cpxP'-lacZ$^+$*) *degP*::Tn*10*	This study
TR930	TR50(pBBR1MCS)	This study
TR932	TR50(pPB)	This study
DB4	TR757(pPB)	This study
DB8	TR50(pCpxP)	This study
DB10	TR50(pPB1)	This study
DB12	TR50(ptrc99A)	This study
DB30	TR50(pPB3)	This study
DB31	TR50(pPB2)	This study
DB32	TR50(pPB4)	This study
DB33	TR50(pPB5)	This study
DB40	TR50(pPB6)	This study
DB58	TR50(pDegP)	This study
DB59	TR50(pDsbA)	This study
DB60	TR50(pPpiA)	This study
DB61	TR50(ptrc99:Spy)	This study
DB70	TR757(pPB1)	This study
DB71	TR757(pPB2)	This study
DB72	TR757(pPB3)	This study
DB73	TR757(pPB4)	This study
DB74	TR757(pPB5)	This study
DB75	TR757(pPB6)	This study
DB115	TR50(pCpxPU)	This study
DB116	TR50(pCpxPD$_{61}$EU)	This study
DB117	TR50(pCpxPQ$_{55}$PU)	This study
DB127	TR50(pCpxPD$_{61}$E)	This study
DB128	TR50(pCpxPQ$_{55}$P)	This study
Plasmids		
pBBR1MCS	Expression vector with a multiple cloning site following a *lac* promoter (Cam^r)	22
pCxpP	*cpxP* overexpression vector (Amp^r)	32
pCpxPD61E	*cpxPD$_{61}$E* gene amplified from pCpxPD$_{61}$EU using the CpxP*Kpn* and CpxP5'*Eco* primers and cloned into the Kpn and EcoRI sites of p*trc*99A (Amp^r)	This study
pCpxPD$_{61}$EU	Site-directed mutagenesis (SDM) was carried out on pCpxPU using the D-61-E1 and D-61-E2 primers; refer to Materials and Methods for complete protocol (Amp^r)	This study
pCpxPQ55P	*cpxPQ$_{55}$P* gene was amplified from pCpxPQ$_{55}$PU using CpxP*Kpn* and CpxP5'*Eco* and cloned into p*trc*99A using the Kpn and EcoRI sites (Amp^r)	This study
pCpxPQ$_{55}$PU	SDM was carried out on the pCpxPU using the Q-55-P1 and Q-55-P2 primers; refer to Materials and Methods for complete protocol (Amp^r)	This study
pCpxPU	*cpxP* gene and flanking upstream region was amplified using CpxP*Bam* and CpxP*Kpn* and cloned into pUC18 BamHI and Kpn sites (Amp^r)	This study
pDegP	*degP* open reading frame was PCR amplified using the primers DegP*Kpn* and DegP*Bam* and cloned into the KpnI and BamHI sites of p*trc*99A (Amp^r)	This study
pDsbA	*dsbA* gene was amplified using the DsbA*Kpn* and DsbA*Bam* primers and cloned into the KpnI and BamHI sites of p*trc*99A (Amp^r)	This study
pPB	CpxP'-'Bla translational fusion overexpression vector; the *cpxP* gene was amplified from MC4100 using CpxP5*Kpn* and CpxPBla primers, and cloned into pBBR1MCS using KpnI and BamHI; β-lactamase gene from pUC19 was amplified using BlaCpxP and Bla3*Bam* primers, digested with BamHI and cloned in-frame downstream of *cpxP* (Amp^r Cam^r)	This study
pPB1	pPB encoding a CpxPD$_{61}$E'-'Bla mutation (Amp^r Cam^r)	This study
pPB2	pPB encoding a CpxPQ$_{55}$P'-'Bla mutation (Amp^r Cam^r)	This study
pPB3	pPB encoding a CpxPR$_{60}$Q'-'Bla mutation (Amp^r Cam^r)	This study
pPB4	pPB encoding a CpxPD$_{61}$V'-'Bla mutation (Amp^r Cam^r)	This study
pPB5	pPB encoding a CpxPM$_{59}$T'-'Bla mutation (Amp^r Cam^r)	This study
pPB6	pPB encoding a CpxPQ$_{128}$H'-'Bla mutation (Amp^r Cam^r)	This study
pPpiA	*ppiA* gene was amplified using PpiA*Kpn* and PpiA*Bam* primers and cloned into the KpnI and BamHI sites of p*trc*99A (Amp^r)	This study
ptrc99:Spy	*spy* open reading frame was amplified using the spy5'*Eco* and spy3'*Bam* primers and cloned into p*trc*99A using EcoRI and BamHI (Amp^r)	This study
ptrc99A	High-copy-number expression vector with a multiple cloning site following an IPTG-inducible *trc* promoter (Amp^r)	Pharmacia
pUC18	Cloning vector (Amp^r)	Invitrogen

TABLE 2. Primers used for PCR

Primer	Sequence
Bla3*Bam*	5′-CGG GAT CCT CAC GTT AAG GGA TTT TGG TC-3′
BlaCpxP	5′-CGG GAT CCA CGC TGG TGA AAG TAA AAG A-3′
CpxP*Bam*	5′-CGG GAT CCT GTG CCA GCA AAT AGA GCA G-3′
CpxPBla	5′-CGG GAT CCC TGG GAA CGT GAG TTG CTA C-3′
CpxP5*Kpn*	5′-GGG GTA CCT CGC GAC AGA AAG ATT TTG G-3′
CpxP*Kpn*	5′-GGG GTA CCG GCA AGG AAA ACA GGG TTT A-3′
CpxP5′*Eco*	5′-GGA ATT CCC TCT CTA TCG TTG AAT CGC G-3′
DegP*Bam*	5′-CGG GAT CCG CAC GGC TTA GCA TAA GGA A-3′
DegP*Kpn*	5′-GGG GTA CCC GAA TCT GAA GAA CAC AGC AA-3′
DsbA*Bam*	5′-CGG GAT CCT GAA TAC TCA CGG GCT TTA TG-3′
DsbA*Kpn*	5′-GGG GTA CCG TGG TTA ACC GGG GAA GAT T-3′
D-61-E1	5′-TCA GCA GAT GCG AGA ACT TAT GCA ACA GGC CC-3′
D-61-E2	5′-GGG CCT GTT GCA TAA GTT CTC GCA TCT GCT GA-3′
PpiA*Bam*	5′-CGG GAT CCG TTA CGC CGG GAG AGC AG-3′
PpiA*Kpn*	5′-GGG GTA CCT GAT CGT CAG GTT ACA TAT ATT TCA GA-3′
Q-55-P1	5′-AGT TTA ACC GAA CAT CCG CGT CAG CAG ATG CG-3′
Q-55-P2	5′-CGC ATC TGC TGA CGC GGA TGT TCG GTT AAA CT-3′
spy3′*Bam*	5′-CGG GAT CCC GCA GGG TAG TGG ACA AGA CCG-3′
spy5′*Eco*	5′-GGA ATT CCA ATA ACT GAA AGG AAG GAT ATA G-3′

FIG. 2. (A) Effect of overexpression of wild-type and mutant CpxP′-′Bla fusions on Cpx-mediated gene expression. β-Galactosidase produced from a *cpxP′-lacZ*+ fusion was measured as a reporter of Cpx-mediated gene expression in TR930 (lane 1), TR932 (lane 2), DB10 (lane 3), DB30 (lane 4), DB31 (lane 5), DB32 (lane 6), DB33 (lane 7), and DB40 (lane 8). VC, vector control; WT, wild-type CpxP′-′Bla protein. (B) Effect of overexpression of wild-type and mutant CpxP on *cpxP′-lacZ*+ activity. β-Galactosidase production was measured from strain TR50 carrying a chromosomal *cpxP′-lacZ*+ reporter and either p*trc*99A (lane 1), pCpxP (lane 2), pCpxPD$_{61}$E (lane 3), or pCpxPQ$_{55}$P (lane 4).

fusion from an exogenous promoter confers ampicillin resistance (Amp^r) and down-regulates Cpx-mediated gene expression three- to fivefold, the same as overexpression of the wild-type CpxP protein does (31, 32) (Fig. 2A, compare lanes 1 and 2). Thus, the CpxP′-′Bla protein possesses wild-type activity.

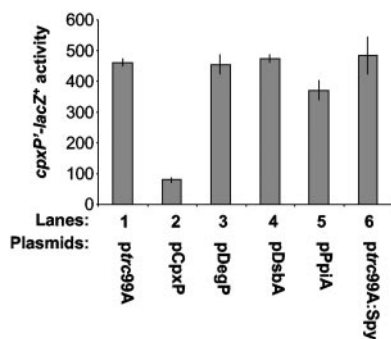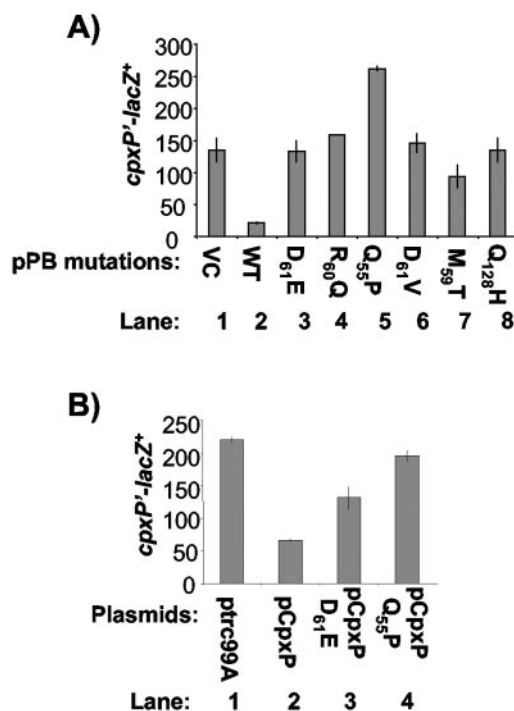FIG. 1. Inhibition of the Cpx response is specific to CpxP overexpression. β-Galactosidase levels were used to measure the effect of overproduction of specific Cpx-regulated genes on Cpx pathway activity, using strains carrying a *cpxP′-lacZ*+ fusion. The strains used were DB12 (lane 1), DB8 (lane 2), DB58 (lane 3), DB59 (lane 4), DB60 (lane 5), and DB61 (lane 6).

On lactose-MacConkey indicator medium, this inhibition phenotype is manifested as white or Lac$^-$ colonies. We used pPB in combination with a chromosomally encoded Cpx-regulated *cpxP′-lacZ*+ fusion to screen for Lac$^+$ and Amp^r phenotypes, thereby eliminating nonsense mutations that would destroy CpxP function by truncating the protein. pPB was subjected to random mutagenesis by passaging through the *E. coli* mutator strain XL1-Red (Stratagene). Over 10,000 Amp^r colonies, resulting from 20 mutated plasmid pools, were screened on lactose-MacConkey indicator plates, resulting in the isolation of 6 different *cpxP* mutations that yielded Lac$^+$ and Amp^r transformants upon plasmid isolation and retransformation into the reporter strain. Several of these mutations were isolated more than once, indicating that the screen was performed to saturation.

***cpxP* loss-of-function mutations localize to two distinct regions.** Both strands of the mutated pPB plasmids encoding potential loss-of-function CpxP′-′Bla mutants were sequenced to identify the precise mutations. Of the six loss-of-function mutations that we isolated, four of them were transversions and two were transitions in keeping with the broad mutator phenotype of the XL1-Red strain. Five of these mutations altered amino acids between residues 55 and 61 of the unprocessed CpxP and were localized to a single predicted α-helix in

the N-terminal region of the protein ($Q_{55}P$, $M_{59}T$, $R_{60}Q$, $D_{61}E$, $D_{61}V$), while the sixth mutation is located at position 128 in the last predicted α-helix in the C-terminal region of CpxP ($Q_{128}H$). Sequence analysis and recloning of the mutant *cpxP* genes showed that no mutations occurred in the *bla* gene, the *tac* promoter, or the plasmid backbone indicating that the observed affects on Cpx-mediated gene expression were due solely to the CpxP amino acid changes.

Loss-of-function CpxP mutants fail to inhibit the Cpx pathway. In order to determine the extent to which our mutants had lost the ability to inhibit the Cpx response, we transformed the vector control, wild-type and mutant pPB plasmids into a strain that contained a chromosomal $cpxP'$-$lacZ^+$ fusion, which allowed for detection of Cpx pathway activity. Activity of the Cpx pathway in the presence of the parent vector, pPB, carrying the wild-type CpxP′-′Bla was approximately three to five fold lower than in the presence of either the empty control vector or the six vectors carrying the mutated CpxP′-′Bla fusions (Fig. 2A, compare lane 2 to lanes 1 and 3 through 8). These results show that all of the mutations impair the ability of CpxP′-′Bla overexpression to inhibit $cpxP'$-$lacZ^+$ expression. To determine if these mutations affected activity of the wild-type CpxP protein in the same fashion and rule out the possibility that our mutations exerted their affects in a manner specific to the CpxP′-′Bla fusion protein, we transferred two of the mutations, $D_{61}E$ and $Q_{55}P$, to the previously characterized pCpxP vector, which inhibits the Cpx response via overexpression of the native CpxP protein (31, 32). Introduction of the $D_{61}E$ or $Q_{55}P$ mutations into pCpxP resulted in a diminished ability of these plasmids to inhibit $cpxP'$-$lacZ^+$ activity relative to the wild-type pCpxP control (Fig. 2B, compare lanes 3 and 4 to lane 2). Thus, the mutations we isolated exert their effects by altering the inhibitory capacity of the native CpxP protein.

Mutant CpxP′-′Bla proteins are expressed at varied levels. To examine the expression levels of the mutant compared to the wild-type CpxP′-′Bla proteins, whole-cell lysates were prepared and immunoblotting was carried out using a monoclonal antibody to β-lactamase. Of the six loss-of-function mutants, $R_{60}Q$, $D_{61}V$, and $D_{61}E$, produced similar or slightly elevated levels of protein of the same size as the wild-type CpxP′-′Bla protein (Fig. 3, compare lane1 with lanes 3, 5, and 9). The other three mutants, $Q_{55}P$, $M_{59}T$, and $Q_{128}H$, exhibited decreased protein expression compared to the wild-type CpxP′-′Bla fusion (Fig. 3, compare lane 1 to lanes 7, 11, and 13). Since sequence analysis and recloning of the mutant *cpxP* genes showed that no mutations in the *bla* gene, *tac* promoter, or plasmid backbone were responsible for the mutant phenotypes (data not shown), these data indicate that the decreased protein levels were due to effects on translation or protein stability.

Diminished mutant CpxP′-′Bla protein levels are restored by *degP* mutation. Since the levels of three of the six CpxP′-′Bla loss-of-function mutants were affected (Fig. 3), we wondered if CpxP might be controlled by proteolysis. To test this idea, we sought to determine if DegP, a Cpx-regulated protease, affected CpxP′-′Bla levels. The $cpxP'$-$'bla$ overexpression plasmid pPB and mutated plasmids pPB1 to pPB6 were transformed into a strain carrying a *degP*::Tn*10* mutation. Mutation of *degP* resulted in an increase in the level of the wild-type CpxP′-′Bla protein (Fig. 3, compare lanes 1 and 2). The
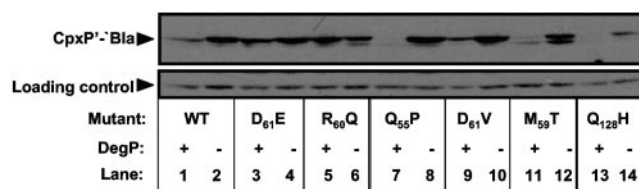

FIG. 3. Expression levels of wild-type and mutant CpxP′-′Bla fusion proteins in wild-type and *degP* null strain backgrounds. Western blots were performed on whole-cell lysates of wild-type (odd-numbered lanes) and *degP* null strains (even-numbered lanes) transformed with the CpxP′-′Bla encoding plasmid pPB (TR932 and DB4) (lanes 1 and 2) or the mutated pPB plasmids CpxPD$_{61}$E′-′Bla (DB10 and DB70) (lanes 3 and 4), CpxPR$_{60}$Q′-′Bla (DB30 and DB72) (lanes 5 and 6), CpxPQ$_{55}$P′-′Bla (DB31 and DB71) (lanes 7 and 8), CpxPD$_{61}$V′-′Bla (DB32 and DB73) (lanes 9 and 10), CpxPM$_{59}$T′-′Bla (DB33 and DB74) (lanes 11 and 12), and CpxPQ$_{128}$H′-′Bla (DB40 and DB75) (lanes 13 and 14) using a monoclonal β-lactamase antibody to label CpxP′-′Bla proteins. WT refers to the wild-type CpxP′-′Bla protein. The experiment was performed three times and a representative blot is shown.

levels of the three loss-of-function mutant proteins (CpxPQ$_{55}$P′-′Bla, CpxPM$_{59}$T′-′Bla, and CpxPQ$_{128}$H′-′Bla) shown to be present at lower levels than the wild-type control (Fig. 4, lanes 7, 11 and 13), were returned to close to wild-type levels in the *degP* null strain (Fig. 3, compare lane 2 to lanes 8, 12, and 14). In addition, the levels of the CpxPD$_{61}$E′-′Bla and CpxPD$_{61}$V′-′Bla proteins appeared to be slightly increased in the *degP* strain background (Fig. 4, compare lanes 3 and 4 and 9 and 10). The levels of a cross-reactive protein that served as a convenient loading control were unchanged in either background, as were levels of the periplasmic protein alkaline phosphatase (Fig. 3, bottom, and data not shown). The data suggest that the $Q_{55}P$, $M_{59}T$, and $Q_{128}H$ mutant proteins are present at reduced levels due to diminished stability and that DegP affects the stability of both the wild-type and mutant CpxP′-′Bla fusion proteins.

Cpx pathway activity and CpxP′-′Bla levels are affected by DegP in response to elevated pH. The above observations suggest that one reason some of the mutant CpxP′-′Bla fusion proteins have lost inhibitory activity is due to a loss of stability that is mediated by DegP-dependent proteolysis. Since we showed that our mutations affect the inhibitory activity of the native CpxP protein in the same fashion (Fig. 2), we speculated that DegP might influence Cpx signaling through proteolysis of CpxP under inducing conditions. To investigate whether DegP influences Cpx signaling, we examined the effects of mutating *degP* on Cpx pathway activity and CpxP′-′Bla levels in the presence and absence of inducing signals (Fig. 4). To induce the Cpx response, we used alkaline pH, a growth condition shown to induce the Cpx response in both *E. coli* and *Shigella flexneri* (9, 27).

We grew either wild-type or *degP* mutant strains of *E. coli* bearing a $cpxP'$-$lacZ^+$ reporter at pH 5.8, where the Cpx response has been shown to be uninduced, or pH 8.0, where the Cpx response is induced (9), and measured β-galactosidase activity. As observed previously, the Cpx response is strongly induced by growth at pH 8.0 relative to pH 5.8 (Fig. 4A, compare lanes 1 and 2). In a *degP* mutant strain, while the pathway was still induced, the level of $cpxP'$-$lacZ^+$ expression
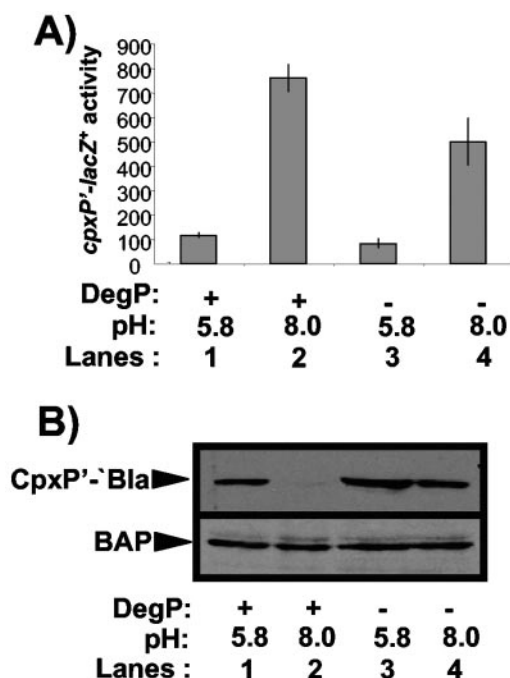
A)

```
E.coli            51  LTEHQRQQMRDLMQQARHE  69
Salmonella spp.   51  LTEHQRQQMRDLMQQARHE  69
S. flexneri        7  LTEHQRQQMRDLMQQARHE  25
Y. pestis         57  LTEQQRQQMRDLMRQSHQS  75
E. carotovora     55  LTEQQRQQMRDLMHQSRQD  73
P. luminescens    68  LTEQQRQQMRDLVGQKHQP  86
```

B)

```
E.coli           128  EQQAVLNEKHQQRMEQLRD-VAQWQ  150
Salmonella spp.  128  EQQAVLNEKHQQRMEQLRD-VAQWQ  150
S. flexneri       84  EQQAVLNEKHQQRMEQLRD-VTQWQ  106
Y. pestis        133  EQKEALNKKHQERIEKLQQKPA--A  155
E. carotovora    131  AQKEILELKHKQRMKEMQQQISMFN  155
P. luminescens   146  EQQAQLEQHYRQRMSQLPN        164
```
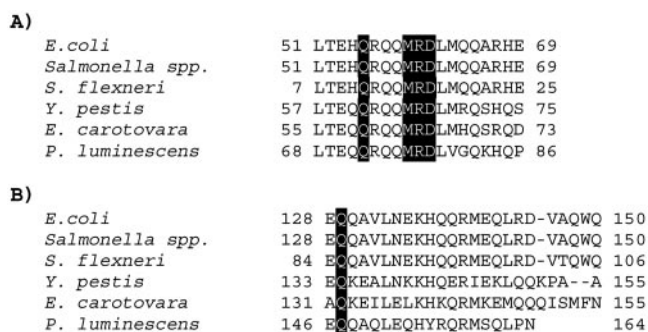
FIG. 5. Sequence alignment of selected regions of CpxP orthologues. The amino acid sequence of *E. coli* CpxP was aligned with those of *Salmonella* spp., *Shigella flexneri*, *Yersinia pestis*, *Erwinia carotovora*, and *Photorhabdus luminescens* using PepTool (Biotools Inc.). The locations of the six loss-of-function mutations are indicated by the dark boxes. A) The first predicted α-helix of CpxP, where five of the six mutations fall. B) The predicted C-terminal α-helix of CpxP, where the sixth mutation falls.

FIG. 4. (A) Induction of the Cpx pathway is reduced in a *degP* null strain. A chromosomal *cpxP'-lacZ*[+] fusion was used to determine the effect of pH on Cpx pathway activity in wild-type and *degP* null strains. β-Galactosidase levels were measured from strains grown in LB buffered with sodium phosphate buffer at a pH of 5.8 (odd-numbered lanes) or 8.0 (even-numbered lanes). The strains used in this experiment were TR50 (lanes 1 and 2) and TR757 (lanes 3 and 4). (B) CpxP'-'Bla levels are diminished in the presence of Cpx-inducing cues in a DegP-dependent fashion. Western blots were carried out on whole-cell lysates of wild-type (TR932) and *degP* null (DB4) strains overexpressing a CpxP'-'Bla fusion protein that had been grown in LB buffered with sodium phosphate buffer at a pH of 5.8 (lanes 1 and 3) or 8.0 (lanes 2 and 4). The experiment was performed three times and a representative blot is shown.

the native CpxP protein in the same fashion (Fig. 2) and affect either or both inhibitory function and stability (Fig. 2 and 3). The reduced levels of some mutant proteins can be restored by mutation of DegP and this led us to examine the effect of DegP on Cpx signal transduction and CpxP'-'Bla levels. Strikingly, we found that induction of the Cpx response by elevated pH is correlated with disappearance of the CpxP'-'Bla fusion protein and that mutation of *degP* abrogates both these effects (Fig. 4). Together, the data support a model in which DegP-mediated proteolysis of CpxP relieves inhibition of the Cpx response in the presence of inducing cues (Fig. 6).

Despite repeated attempts using fusion proteins and immu-

was diminished almost twofold (Fig. 4, compare lanes 1 and 2 to 3 and 4). When we examined the levels of CpxP'-'Bla present in wild-type strains at pHs of 5.8 and 8.0, we were unable to detect the fusion protein at a pH of 8.0 (Fig. 4B, compare lanes 1 and 2). Conversely, in the absence of DegP, the fusion protein was present at approximately equivalent levels at both pHs of 5.8 and 8.0 (Fig. 4B, compare lanes 3 and 4). The levels of a periplasmic control protein, alkaline phosphatase, were unaffected at either pH in wild-type or *degP* mutant strains (Fig. 4B, bottom). Thus, the ability to induce the Cpx response in a *degP* null mutant strain is compromised, and this correlates with increased levels of the CpxP'-'Bla fusion protein observed under the same conditions.

DISCUSSION

In this study, we have taken advantage of a CpxP'-'Bla fusion construct that mediates Cpx pathway inhibition in the same fashion as the native CpxP protein (Fig. 2) to identify the first essential inhibitory domain in CpxP. We screened over 10,000 mutagenized plasmids and isolated five mutations that localize to a single, highly conserved, predicted α-helix in the N terminus of CpxP (Fig. 5). The mutations alter the activity of
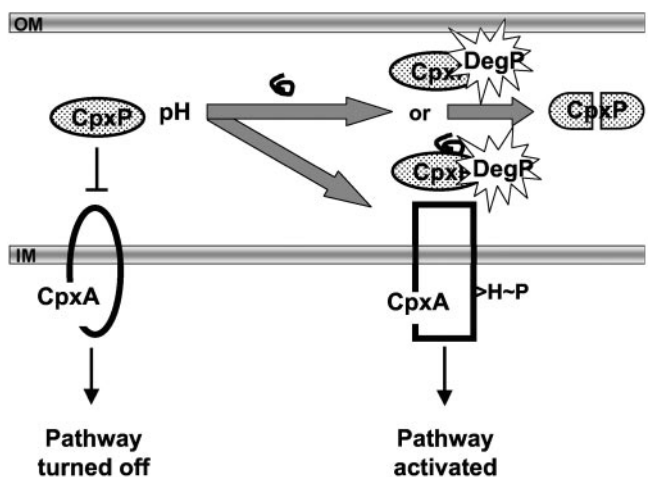

FIG. 6. Model for activation of the Cpx pathway. In the absence of envelope stress, CpxP (shaded oval) interacts with the sensing domain of CpxA (central region of the periplasmic domain of CpxA), maintaining the pathway in an off state. Inducing cues, such as elevated pH, lead to either a conformational change in CpxP or its association with misfolded envelope proteins (squiggly line) or both. CpxP or the complex of CpxP with misfolded protein is subsequently degraded by DegP (starburst) and can no longer inhibit CpxA. This leaves the CpxA sensing domain available for maximal induction by cues such as pH. OM, outer membrane; IM, inner membrane; H, histidine; P, phosphate.

nogenic epitopes as antigens, we have been unsuccessful in raising CpxP-specific antisera. Thus, we cannot be certain at this point that the chromosomally expressed CpxP is affected in a similar fashion by DegP in response to inducing cues. However, several lines of evidence suggest that this is the case. Firstly, the mutations we isolated in the CpxP′-′Bla construct affect the activity of the native, overexpressed CpxP in the same manner (Fig. 2). Second, the CpxP′-′Bla construct is dramatically destabilized by the Cpx-specific inducing cue of elevated pH (Fig. 4B) (9), and this destabilization is DegP dependent. Finally, mutation of *degP*, which would be expected to stabilize CpxP, leads to diminished Cpx pathway activity in the presence of inducing cues (Fig. 4A). Thus, although an analysis of the chromosomally encoded CpxP will be a necessary topic of future studies, these experiments provide strong support for a role of the N terminus of CpxP as well as DegP in a Cpx signaling cascade (Fig. 6).

Cpx envelope stress response is uniquely inhibited by CpxP overexpression. DiGiuseppe and Silhavy (14) have recently set forth the hypothesis that CpxP has dual functions as an inhibitory protein and a chaperone, suggesting that CpxP inhibition of the Cpx response upon overexpression may be a by-product of combating extracytoplasmic protein-mediated toxicity. If this is true, then overexpression of other protein folding and degrading factors that belong to the Cpx pathway should also cause a decrease in pathway activity. We overexpressed numerous Cpx-regulated genes, and only when CpxP was overexpressed was there a decrease in pathway activity (Fig. 1). This supports previous experiments (31, 32) and shows that simply overexpressing Cpx-regulated genes does not confer inhibition. These results do not allow us to support or refute the idea that CpxP has dual functions, however, they do allow us to speculate that CpxP has a unique function in inhibition of the Cpx response, which none of the other Cpx-regulated protein folding or degrading factors possess.

Predicted N-terminal α-helix plays an essential role in CpxP-mediated inhibition and stability. In this study we identified two classes of mutations that affect CpxP function and/or stability. Some mutations conferred a loss of inhibition but were otherwise present at comparable levels to the wild-type fusion protein (CpxPR$_{60}$Q′-′Bla, CpxPD$_{61}$E′-′Bla, and CpxPD$_{61}$V′-′Bla). Another class of mutations not only conferred loss of function but also clearly altered protein levels (CpxPQ$_{55}$P′-′Bla, CpxPM$_{59}$T′-′Bla, and CpxPQ$_{128}$H′-′Bla) (Fig. 3). Since most mutations fall in the N terminus, these observations suggest that the N terminus of CpxP contains a domain that is important both for the inhibition of CpxA via its sensing domain, as well as stabilization of CpxP.

Interestingly, five of the six *cpxP* mutations we identified localized to a span of seven amino acids in a predicted N-terminal α-helix. Since other CpxP homologues show some divergence in the N-terminal region, it has been suggested that the C terminus is responsible for inhibition and possibly interaction with the highly conserved CpxA (12). However, the sequence identities of the N-terminal domains in question compared to those of the entire *cpxP* open reading frame suggest that this predicted α-helix is highly conserved (Fig. 5). The identities of the predicted N-terminal α-helices relative to the entire *cpxP* open reading frame compared to those of *E. coli* are 100 compared to 88 for *Salmonella* spp., 100 compared

to 87.7 for *Shigella flexneri*, 65 compared to 53 for *Yersinia pestis*, 70 compared to 46 for *Erwinia carotovora*, and 60 compared to 44 for *Photorhabdus luminescens*. Even more strikingly, the five mutations that localize to this putative α-helix affect amino acids that are completely conserved between the seven species identified above (Fig. 5A). This is strong evidence that our mutations are in a critical area of the protein and that this N-terminal α-helix plays an important role in CpxP function.

The C-terminal mutation CpxPQ$_{128}$H′-′Bla also falls on a completely conserved amino acid (Fig. 5B), once again pointing to the importance of these amino acids in the function and stability of CpxP. Helical wheel analysis (Peptool, BioTools Inc.) shows that the N-terminal α-helix where five of our six loss-of-function mutations localized is polar except for CpxPL$_{51}$, CpxPL$_{62}$, and CpxPA$_{66}$. This suggests that this helix is most likely located on the outside of the protein and thus one possibility is that our mutations alter a possible protein-protein interaction, such as that which is proposed to occur with the sensing domain of CpxA. It has been suggested that CpxP forms a dimer (D. Issac and T. J. Silhavy, personal communication). Thus, another possibility is that this N-terminal α-helix plays a role in the formation of dimers, and that dimerization is essential for inhibition of the Cpx pathway. Experiments are in progress to differentiate among these hypotheses.

DegP influences both Cpx pathway activity and CpxP′-′Bla levels in response to inducing cues. Since some mutant CpxP′-′Bla proteins exhibited reduced protein levels (Fig. 3), we hypothesized that CpxP might be regulated by proteolysis. We reasoned that DegP might be involved in CpxP proteolysis since it is Cpx regulated (6) and has a broad substrate specificity (19, 21). We found that, in a *degP* knockout, we were able to recover full-length mutant CpxP′-′Bla proteins that were normally undetectable in a wild-type strain (Fig. 3), supporting the idea that CpxP is regulated by proteolysis and that the protease responsible for this degradation is DegP.

In order to determine if DegP-dependent proteolysis of CpxP plays a role in activation of the Cpx pathway, we analyzed Cpx pathway activity and CpxP′-′Bla levels in wild-type and *degP* null strains after exposure to the known Cpx-inducing signal of alkaline pH (9, 27). We found that in alkaline growth medium CpxP′-′Bla was undetectable, while at a pH of 5.8 CpxP′-′Bla seems to be stabilized (Fig. 4). CpxP′-′Bla instability at pH 8 was completely abolished in a *degP* null background (Fig. 4B), suggesting that DegP is responsible for CpxP′-′Bla degradation in response to elevated pH.

The levels of CpxP′-′Bla correlated inversely with induction of the Cpx response. In a wild-type strain, growth at pH 8.0 relative to pH 5.8 leads to a seven- to eightfold increase in *cpxP′-lacZ*$^+$ reporter activity and this increase was diminished by approximately twofold in the absence of DegP (Fig. 4A). Together, these data support a model for Cpx signal transduction in which CpxP is rapidly degraded by DegP in the presence of inducing cues, fostering increased pathway activation (Fig. 6). Interestingly, when DegP was overexpressed in the wild-type background we saw no evidence of increased pathway activity (Fig. 1). Thus, we hypothesize that inducing cues must be present in order for DegP-mediated proteolysis of CpxP to occur.

It is not surprising that pathway induction is not obliterated

in a *degP* null strain, since it has previously been shown that the Cpx response can still be induced in the presence of an over-expressed CpxP fusion protein, albeit to a lower level (14). Further, CpxP is not required for Cpx signaling (14, 31, 32). Thus, these results support previous reports that suggest an accessory role for CpxP in the signal transduction scheme. Interestingly, the RseB and TcpH regulators of *E. coli* and *Vibrio cholerae*, respectively, appear to play similar roles. The periplasmic RseB, although not essential for signaling, has been shown to protect the RseA anti-sigma factor from signal-independent proteolysis that would lead to inappropriate induction of the σ^E envelope stress response (16). Similarly, the periplasmic domain of TcpH appears to be involved in signaling by protecting the membrane-bound regulatory protein TcpP from untimely proteolysis (4). We propose that CpxP may serve a similar safeguarding function, protecting the sensing domain of CpxA from unwarranted activation or signaling noise in the absence of veritable envelope stresses.

At this time, we cannot say what event would trigger CpxP proteolysis by DegP in the presence of inducing cues. DegP possesses two PDZ domains (23) that are proposed to bind to substrates and direct them to the proteolytic central cavity of the hexameric protease, although the DegP substrate recognition motif remains undefined. Other PDZ-like domains recognize substrates by virtue of C-terminal recognition motifs baring hydrophobic determinants (39). Since the C terminus of CpxP is predominantly polar/charged, perhaps inducing cues lead to the exposure of an internal domain baring a hydrophobic recognition motif. Alternatively, CpxP may interact with denatured proteins generated by envelope stress, which then target the complex to DegP (Fig. 6).

Whatever the mechanism, the current work identifies the first conserved inhibitory domain in the novel regulator CpxP, implicates DegP-dependent proteolysis in the Cpx signaling cascade, and places CpxP among a new class of periplasmic regulatory proteins involved in shielding membrane-bound regulatory proteins from superfluous signaling events. Future studies will be aimed at working out the molecular details of these new facets of Cpx signal transduction.

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