

# Transduction of Envelope Stress in *Escherichia coli* by the Cpx Two-Component System

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**Disruption of normal protein trafficking in the *Escherichia coli* cell envelope (inner membrane, periplasm, outer membrane) can activate two parallel, but distinct, signal transduction pathways. This activation stimulates the expression of a number of genes whose products function to fold or degrade the mislocalized proteins. One of these signal transduction pathways is a two-component regulatory system comprised of the histidine kinase CpxA and the response regulator, CpxR. In this study we characterized gain-of-function Cpx\* mutants in order to learn more about Cpx signal transduction. Sequencing demonstrated that the *cpx\** mutations cluster in either the periplasmic, the transmembrane, or the H-box domain of CpxA. Intriguingly, most of the periplasmic *cpx\** gain-of-function mutations cluster in the central region of this domain, and one encodes a deletion of 32 amino acids. Strains harboring these mutations are rendered insensitive to a normally activating signal. In vivo and in vitro characterization of maltose-binding-protein fusions between the wild-type CpxA and a representative *cpx\** mutant, CpxA101, showed that the mutant CpxA is altered in phosphotransfer reactions with CpxR. Specifically, while both CpxA and CpxA101 function as autokinases and CpxR kinases, CpxA101 is devoid of a CpxR-P phosphatase activity normally present in the wild-type protein. Taken together, the data support a model for Cpx-mediated signal transduction in which the kinase/phosphatase ratio is elevated by stress. Further, the sequence and phenotypes of periplasmic *cpx\** mutations suggest that interactions with a periplasmic signaling molecule may normally dictate a decreased kinase/phosphatase ratio under nonstress conditions.**

A distinct set of protein folding factors and proteases are located in the cell envelope (inner membrane, periplasm, outer membrane) of the gram-negative bacterium *Escherichia coli*. These include the periplasmic protease DegP (37, 65), a number of enzymes involved in the catalysis of disulfide bond formation, DsbA to D (3–5, 29, 45, 48, 58), and three different peptidyl-prolyl isomerases, FkpA (21), PpiA (38), and SurA (32, 54). Several of these are directly involved in protein folding or degradation within the bacterial envelope. For example, DegP is required for survival above 40°C (35–37, 64, 65) and functions to degrade some abnormal or misassembled envelope proteins (12, 31, 63, 64). DsbA plays a critical role in catalyzing disulfide bond formation in a number of envelope proteins including alkaline phosphatase,  $\beta$ -lactamase, and OmpA (5, 29). SurA appears to play a role in an early folding step that occurs during the assembly of the trimeric outer membrane porin proteins (44, 54).

Two overlapping, but distinct, pathways modulate the expression of some of these factors in response to insults to the bacterial envelope (15, 16, 52).  $\sigma^E$  is an alternative sigma factor whose activity is induced by extremely high temperatures and increased expression, or mutations which cause misfolding, of outer membrane proteins (OMPs) (19, 36, 42, 44, 53, 54, 71). In response to such signals, RNA polymerase-containing  $\sigma^E$  mediates increased expression of several genes, including *degP* (19, 36, 42, 44, 53, 54, 71) and *fkpA* (15). Thus, it has been proposed that  $\sigma^E$  is the bacterial envelope counterpart for  $\sigma^{32}$ , the cytoplasmic heat shock sigma factor, functioning to monitor the status of extracytoplasmic protein folding events and induce the synthesis of corrective factors when needed (15, 42, 53).  $\sigma^E$  is linked to the inducing signal in the bacterial envelope

through an inner-membrane-localized anti- $\sigma$  factor, RseA (17, 46). However, the molecular mechanism used by this system to monitor and respond to envelope stresses remains unclear.

The Cpx pathway also plays a role in the regulation of protein trafficking factors in the bacterial envelope. The *cpx* locus encodes CpxR and CpxA, which comprise the response regulator (RR) and histidine kinase (HK), respectively, of a bacterial two-component regulatory system (18, 73). These systems function to sense and respond to a wide variety of environmental parameters through a conserved set of phosphotransfer reactions (20). Typically, a membrane-bound sensor protein, or HK, autophosphorylates at a conserved histidine residue in response to a specific cue. Transfer of this phosphate to a conserved aspartate in the cognate RR generally endows the RR with the ability to function as a transcriptional activator of downstream targets (20). Many HKs also possess an RR-P phosphatase activity which may ensure that the RR remains inactive in the absence of activating signals. Further, it has recently been demonstrated that aspartyl-phosphate phosphatases distinct from the HK can act directly on certain RRs to control the output of two-component systems, generally by downregulating the activity of the RR (51). Like many other HKs, CpxA is localized to the inner membrane by two transmembrane helices and contains both periplasmic and cytoplasmic domains (73). CpxR is predicted to encode an OmpR-like transcriptional activator (18). Both proteins display homology to the conserved phosphotransfer domains of other two-component systems.

Stresses to the bacterial envelope activate the Cpx pathway; however, these signals can be distinct from those that activate the  $\sigma^E$  pathway, implying that these two systems, although overlapping, carry out separate functions (16). Cpx-activating signals include overproduction of the novel outer membrane lipoprotein NlpE (63), elevated pH (14, 50), and an alteration in the lipid composition of the bacterial membrane (43). Ac-

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tivation of the Cpx signal transduction pathway results in elevated expression of DegP (16), DsbA (15), PpiA (52), and another periplasmic protein of unknown function, CpxP (14). The inducing signals are all predicted to disrupt the bacterial envelope and/or lead to aberrantly folded or targeted proteins in this compartment. Furthermore, since the downstream targets are involved in protein folding and proteolysis, it has been proposed that Cpx, like RseA/ $\sigma^E$ , is responsible for sensing and responding to perturbations of the bacterial envelope which could divert the normal flow of protein through this compartment (15, 16, 52). In support of this model, production of unfolded P pilus subunits, which aggregate and localize to the outer surface of the inner membrane, is a potent inducer of either the Cpx pathway or both the Cpx and  $\sigma^E$  pathways (27). Further, gain-of-function mutations mapping to the *cpx* locus (*cpx\**) are able to suppress the toxic effects of proteins that disrupt the bacterial envelope, and these effects are dependent on the downstream targets of Cpx (12). Moreover, *cpx\** mutations can suppress the temperature sensitivity of mutants lacking  $\sigma^E$  (11).

The molecular mechanism of signal transduction used by the Cpx two-component pathway is not clear. Biochemical data suggest that, like other RRs (30, 39, 40), CpxR can become phosphorylated by the low-molecular-weight phosphodonor acetyl phosphate and, further, that this form has a greater affinity for binding to the promoters of target genes (52). Similarly, it has been demonstrated that, in the absence of CpxA, CpxR cannot transcriptionally activate downstream target genes unless acetyl phosphate is present in the cell, suggesting that CpxR-P is responsible for transcriptional activation of target genes (16). This view is complicated by recently presented genetic evidence that suggests that two proteins with homology to eukaryotic serine-threonine phosphatases are involved in upregulation of the Cpx signal transduction pathway (47). Missiakas and Raina (47) have proposed that the effect of the phosphatases is direct and occurs through an alteration in the phosphotransfer reactions that occur between CpxA and CpxR that ultimately leads to lower levels of CpxR-P. Thus, it is unclear what the role of CpxR-P is in activation of the Cpx pathway.

It seems that phosphotransfer events between CpxA and CpxR must play an important role in Cpx signal transduction, as they do in other two-component regulatory systems (20). However, the role of CpxA in these events has not been addressed. If, as seems probable, CpxA is involved, then what is the nature of the signal(s) that is recognized, and how does it modulate the activities of the Cpx regulon? In this study, we have characterized several *cpx\** mutants in order to learn more about Cpx-mediated signal transduction. Our data suggest a model for how protein traffic in the bacterial envelope may be surveyed by CpxA in conjunction with a negatively acting periplasmic ligand.

#### MATERIALS AND METHODS

**Bacterial strains and bacteriophage.** Bacterial strains and bacteriophage used in this study are listed in Table 1. Strains were constructed by using standard microbiological techniques (60).

**Plasmids.** MBP fusions to CpxR and the cytoplasmic domains of CpxA and CpxA101 were constructed by using the pMal-C vector (New England Biolabs). The restriction-tagged PCR primers CpxRN (5'-CGG GAT CCA ATA AAA TCC TGT TAG TTG ATG-3') and CpxRC (5'-TGC ACT GCA GAT CAT GAA GCA GAA ACC ATC-3') were used to amplify the *cpxR* gene from the chromosome of MC4100. This PCR product was digested with *Bam*HI and *Pst*I and cloned into the *Bam*HI and *Pst*I sites of pMal-C to generate an in-frame fusion between a cytoplasmic derivative of MBP and the second amino acid of CpxR. The cytoplasmic domains of CpxA and CpxA101 (amino acids 218 to 458) were amplified from the chromosomes of MC4100 and TR20, respectively, by using the restriction-tagged PCR primers CpxABam (5'-CGG GAT CCG ATA

TABLE 1. Bacterial strains and bacteriophages

Bacterial strain or bacteriophage	Genotype	Reference
<b>Bacterial strains</b>		
PND325	MC4100 $\lambda$ RS88[ <i>degP-lacZ</i> ] <i>cpxR::spc</i>	16
CLC142	MC4100 <i>cpxA24 lamBA23D zjb::Tn10Kn</i>	12
CLC143	MC4100 <i>cpxA101 lamBA23D zjb::Tn10Kn</i>	12
CLC144	MC4100 <i>cpxA102 lamBA23D zjb::Tn10Kn</i>	12
CLC145	MC4100 <i>cpxA103 lamBA23D zjb::Tn10Kn</i>	12
CLC146	MC4100 <i>cpxA104 lamBA23D zjb::Tn10Kn</i>	12
CLC147	MC4100 <i>cpxA105 lamBA23D zjb::Tn10Kn</i>	12
CLC163	MC4100 <i>cpxA17 lamBA23D zjb::Tn10Kn</i>	12
CLC212	MC4100 <i>cpxA106 lamBA23D zjb::Tn10Kn</i>	12
CLC213	MC4100 <i>cpxA711 lamBA23D zjb::Tn10Kn</i>	12
CLC215	MC4100 <i>cpxA744 lamBA23D zjb::Tn10Kn</i>	12
CLC216	MC4100 <i>cpxA2 lamBA23D zjb::Tn10Kn</i>	12
JM109	F' <i>traD36 lacI<sup>q</sup> Δ(lacZ)M15 proA<sup>+</sup>B<sup>+</sup>/e14<sup>-</sup> (McrA<sup>-</sup>) Δ(lac-proAB) thi gyrA96 (Nal<sup>r</sup>) endA1 hsdR17(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) relA1 supE44 recA1</i>	75
JM109R-	JM109 <i>cpxR::spc</i>	This study
MC4100	F' <i>araD139 Δ(argF-lac) U169 rpsL150 (Str<sup>r</sup>) relA1 flbB5301 deoC1 ptsF25 rbsR</i>	6
PND325	MC4100 $\lambda$ RS88[ <i>degP-lacZ</i> ] <i>cpxR::spc</i>	16
TR20	MC4100 <i>cpxA24</i>	This study
TR33	MC4100 <i>cpxA24</i> $\lambda$ RS88[ <i>degP-lacZ</i> ]	This study
TR34	MC4100 <i>cpxA24</i> $\lambda$ RS88[ <i>cpxP-lacZ</i> ]	This study
TR45	MC4100 <i>cpxA104</i> $\lambda$ RS88[ <i>degP-lacZ</i> ]	This study
TR46	MC4100 <i>cpxA104</i> $\lambda$ RS88[ <i>cpxP-lacZ</i> ]	This study
TR47	MC4100 <i>cpxA101</i> $\lambda$ RS88[ <i>degP-lacZ</i> ]	This study
TR48	MC4100 <i>cpxA101</i> $\lambda$ RS88[ <i>cpxP-lacZ</i> ]	This study
TR49	MC4100 $\lambda$ RS88[ <i>degP-lacZ</i> ]	This study
TR50	MC4100 $\lambda$ RS88[ <i>cpxP-lacZ</i> ]	This study
<b>Bacteriophages</b>		
$\lambda$ RS88[ <i>degP-lacZ</i> ]		16
$\lambda$ RS88[ <i>cpxP-lacZ</i> ]		14

GGC AGC TTA ACC GCG CGC-3') and CpxAPst (5'-TGC ACT GCA GCG AAG TTT AAC TCC GCT TAT ACA G-3'). The PCR products were digested with *Eco*RI, which cuts at an internal site corresponding to amino acid 218 of CpxA, and *Pst*I and cloned into the same restriction sites in pMal-C to generate fusions between a cytoplasmic derivative of MBP and amino acid 218 of CpxA (pMCA) or CpxA101 (pMCA101). Expression of MBP-CpxR, MBP-CpxA, and MBP-CpxA101 was driven by the *tac* promoter upstream of *malE* on pMal-C. The fusion joints of pMCR, pMCA, and pMCA101 were sequenced to confirm that all constructs encoded the correct in-frame product. The entire cytoplasmic domains of CpxA and CpxA101 in pMCA and pMCA101 were sequenced to confirm the presence of the *cpxA101* mutation and the absence of other PCR-generated mutations.

pND10 contains a clone of the wild-type *cpxR* gene plus upstream sequences and has been described previously (16). pLD404 is a pBR322 derivative containing a clone of the *nlpE* gene (63).

All molecular biological techniques were performed as described in reference 57.

**$\beta$ -Galactosidase assays.** Single colonies of each bacterial strain were inoculated into overnight cultures of Luria broth containing 150  $\mu$ g of ampicillin (Sigma) per ml and grown overnight at 30°C. The next day, strains were subcultured 1:40 to the same media and grown at 30°C to late log phase.  $\beta$ -Galactosidase was measured by a microtiter plate assay (62). All assays were performed in triplicate.

**Protein purification.** The MBP-CpxR fusion protein was purified from JM109(pMCR). We reasoned that overproduction of CpxA or CpxA101 (or the cytoplasmic domain derivatives) could be deleterious to the cell due to hyperactivation of the Cpx pathway. Therefore, MBP-CpxA and MBP-CpxA101 fusion proteins were purified from a *cpxR*-null derivative of JM109, JM109R<sup>-</sup>, transformed with either pMCA or pMCA101. MBP fusion proteins were purified by amylose affinity chromatography according to the specifications of the manufacturer (New England Biolabs). Briefly, a single colony of the fusion-containing strain was inoculated into 10 ml of Luria broth containing 150  $\mu$ g of ampicillin per ml and 0.2% glucose and grown overnight at 30°C. The next day, all of this culture was used to inoculate a secondary 1-liter culture of the same medium.

Cells were grown to an  $A_{600}$  of approximately 0.5, at which time IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) was added to 0.3 mM to induce expression of the fusion protein. Cells were grown an additional 3 h and harvested by centrifugation at  $4,000 \times g$  for 20 min. The pellet was resuspended in 50 ml of column buffer (20 mM Tris [pH 7.4], 400 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and subjected to one cycle of freeze-thaw. Cells were disrupted by sonication, and then debris and membranes were removed by centrifugation at  $9,000 \times g$  for 30 min. The soluble fraction was diluted to 2.5 mg of protein per ml with column buffer and loaded on an amylose column that had been pre-equilibrated with column buffer. The column was washed with 12 column volumes of buffer, and MBP fusion protein was eluted with column buffer containing 10 mM maltose. MBP-CpxA and MBP-CpxA101 were desalted by using an Econo-Pac 10 DG column (Bio-Rad) and replaced in a final buffer of 20 mM Tris (pH 7.4)–100 mM NaCl–5 mM  $\beta$ -mercaptoethanol ( $\beta$ ME). They were used at a final concentration of approximately 0.15 mg/ml for autokinase and kinase reactions and at a final concentration of approximately 15  $\mu$ g/ml for phosphatase assays. MBP-CpxR was also desalted and replaced in 50 mM sodium phosphate (pH 7.1)–150 mM NaCl–2 mM  $\beta$ ME. MBP-CpxR was used at the final concentrations indicated.

Purification of EnvZ115 and OmpR has been described previously (25, 26).

**Mobility shift assay.** The *degP* promoter was obtained by PCR amplification from the chromosome of MC4100 using the primers Htra5 (5'-GCG TGG GAT GAA TAC CGA CGT CTG ATG G-3') and DegPPro3 (5'-CGG TGC AAG GCT TGG CAT CTG CTG GGC-3'). This fragment was end labeled in a 50- $\mu$ l reaction mixture containing approximately 50 pmol of 5' termini, 70 mM Tris (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol (DTT), 50 pmol of [ $\gamma$ -<sup>32</sup>P]ATP, and 20 U of T4 polynucleotide kinase (New England Biolabs). The reaction mixture was incubated at 37°C for 30 min, at which time the enzyme was inactivated by heating to 65°C for 20 min. Labeled DNA was purified by using a Genelute purification kit according to the manufacturer's (Supelco) instructions. In each assay, 1.5 pmol of the purified, end-labeled PCR product was used. The *ompF* promoter was PCR amplified from the chromosome of MC4100 by using the primers KEG1 (5'-CTA ATT TAG CGA ATT CAA GAG CC) and KEG15 (5'-GTC TGG ATC CAT CTT TCC ATT CAA ACT AAC G-3'). This DNA fragment was used as nonspecific competitor DNA in some reactions at a final concentration of 5 pmol. CpxR fusion protein (15 or 150 pmol) or MBP (125 pmol) was incubated in the presence or absence of acetyl phosphate (20 mM) at 37°C for 30 min in a 15- $\mu$ l reaction volume containing 10 mM Tris (pH 7.4), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50  $\mu$ g of bovine serum albumin per ml, 1 mM DTT, 20 mM potassium glutamate, and 10 mM MgSO<sub>4</sub>. End-labeled PCR product (1.5 pmol) containing the *degP* promoter, and in some cases 5 pmol of competitor *ompF* promoter DNA was added, and the reaction mixture was incubated another 30 min at 37°C. Reactions were stopped by the addition of 3  $\mu$ l of loading dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF [Sigma]), and products were electrophoresed on 5% nondenaturing acrylamide gels in TAE running buffer (0.04 M Tris-acetate, 0.001 M EDTA). Gels were dried and exposed to X-ray film (Kodak).

**Sequencing *cpx\** mutations from the bacterial chromosome.** *cpx\** mutations were sequenced directly from the bacterial chromosome by using the technique previously described by Russo et al. (55). Briefly, the entire *cpxA* gene was PCR amplified from the chromosomes of CLC142, CLC143, CLC144, CLC145, CLC146, CLC147, CLC163, CLC212, CLC213, CLC215, and CLC216 by using the primers *cpxA5'* (5'-TAA GCC AGG AAG TGT TGG GC-3') and *cpxA3'* (5'-CCC GGA GTG TAG GCC TGA TA-3'). PCR products were gel purified and sequenced by using Sequenase (United States Biochemical) and *cpxA*-specific sequencing primers.

**Autokinase assay.** MBP-CpxA or MBP-CpxA101 (1.8  $\mu$ M) was incubated at room temperature in the presence of 0.1 M Tris (pH 8.0), 50 mM KCl, 5 mM CaCl<sub>2</sub>, 5% glycerol, 1 mM MnCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 0.5  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP. Aliquots were removed to an equal volume of 2 $\times$  sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye (125 mM Tris [pH 6.8], 20% glycerol, 10%  $\beta$ ME, 6% SDS, 0.2% bromophenol blue) after the indicated periods, heated to 55°C for 5 min, and electrophoresed on SDS–10% polyacrylamide gels. Gels were dried and exposed to X-ray film.

**Kinase assay.** MBP-CpxA or MBP-CpxA101 were radiolabeled with [ $\gamma$ -<sup>32</sup>P]ATP for 20 min at room temperature as above. MBP-CpxR was added to the reaction mixture at a final concentration of 1.75  $\mu$ M. Aliquots were removed to an equal volume of 2 $\times$  SDS-PAGE loading buffer after the indicated periods, heated to 55°C, and electrophoresed on SDS–10% polyacrylamide gels. Gels were dried and exposed to X-ray film.

**Phosphatase assay.** EnvZ115 (95  $\mu$ M) was phosphorylated by incubation in the presence of 0.1 M Tris (pH 8.0), 50 mM KCl, 5 mM CaCl<sub>2</sub>, 10% glycerol, and 0.02  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP for 10 min at room temperature. Labeled EnvZ115-P was pelleted by centrifugation at 14,000 rpm in a microcentrifuge for 15 min and resuspended in 1 ml of assay buffer. After pelleting again at 14,000 rpm in a microcentrifuge for 15 min, this step was repeated two more times for a total of three washes to remove residual [ $\gamma$ -<sup>32</sup>P]ATP. The pellet, containing EnvZ115-P, was resuspended to the original concentration in assay buffer. To obtain labeled MBP-CpxR-P, the purified protein was mixed with the EnvZ115-P at a final concentration of 13  $\mu$ M and incubated for 1 h at room temperature. The majority of the EnvZ115-P was removed by ultracentrifugation at 100,000  $\times$  rpm for 20 min. The remaining supernatant was used as a source of MBP-CpxR-P for the

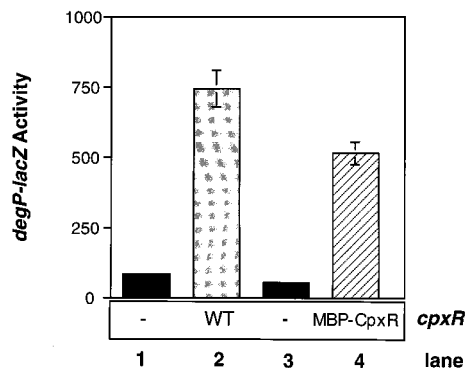


FIG. 1. An MBP-CpxR fusion protein is active in vivo. Activity from the *degP* promoter was measured by assaying  $\beta$ -galactosidase production of PND325 (MC4100  $\lambda$ RS88[*degP-lacZ*] *cpxR::spc*) (lane 1), PND325(pND10) (lane 2), PND325(pMal-C) (lane 3), and PND325(pMCR) (lane 4). pND10 encodes the wild-type (WT) *cpxR*, pMal-C is a vector control, and pMCR contains an in-frame fusion between a cytoplasmic derivative of MBP and the second amino acid of CpxR.

ensuing phosphatase assays. MBP-CpxA or MBP-CpxA101 was added to a final concentration of 0.07  $\mu$ M. In addition, in some assays, MgCl<sub>2</sub> was included at a concentration of 5 mM and ATP was added to a final concentration of 3 mM. Aliquots were removed to an equal volume of 2 $\times$  SDS-PAGE loading buffer at the indicated times, heated to 55°C, and electrophoresed on SDS–10% polyacrylamide gels. Gels were dried and exposed to X-ray film.

## RESULTS

**An MBP-CpxR fusion has in vivo and in vitro activities.** To facilitate the analysis of the biochemical activities of CpxA and CpxR, we constructed fusions to a cytoplasmic derivative of MBP. pMCR encodes an in-frame fusion between MBP and the second amino acid of CpxR (see Materials and Methods for construction details). To determine if pMCR encoded a functional protein, we assayed the ability of this plasmid to complement expression of *degP* in a *cpxRA* mutant background. pMCR, together with the vector control pMal-C and a clone of the wild-type *cpxR*, pND10, was transformed into a strain carrying a *degP-lacZ* reporter and a polar, null allele of *cpxR* (16). In the absence of CpxR and CpxA, there is low-level expression from the *degP* promoter (Fig. 1, lanes 1 and 3), consistent with the previous finding that Cpx-independent transcription of the *degP* promoter can be initiated by RNAP $\sigma^E$  (16). A multicopy plasmid carrying the wild-type *cpxR* gene elevates expression of the *degP* promoter more than sevenfold (Fig. 1, lane 2). Similarly, pMCR, encoding the MBP-CpxR fusion, enhanced *degP* transcription approximately fivefold (Fig. 1, lane 4). As expected (16), expression of *degP* in strains lacking CpxA was partially dependent on the presence of acetyl phosphate (data not shown). The parent vector used to construct pMCR had no effect on *degP* expression (Fig. 1, lane 3). We conclude that pMCR encodes a fusion protein which functions like the wild-type CpxR to activate expression from the *degP* promoter.

To investigate the biochemical activities of CpxR, the MBP-CpxR fusion protein was purified by amylose affinity chromatography. CpxR binds to a specific consensus DNA binding site (52). Further, this activity is enhanced in the presence of acetyl phosphate, which can be used as a phospho-donor to phosphorylate some RRs in the absence of the cognate HK (30, 39, 40). To ensure that the purified MBP-CpxR was active, it was used in mobility shift assays with the radiolabeled *degP* promoter region (see Materials and Methods for details) (Fig. 2). Pure MBP-CpxR was able to bind the *degP* promoter, as evi-

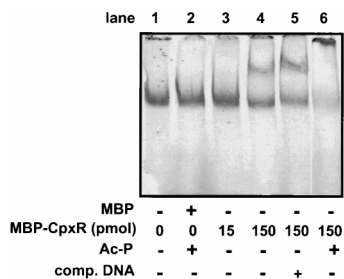


FIG. 2. MBP-CpxR is a DNA-binding protein whose activity is enhanced in the presence of acetyl phosphate. Mobility shift assays were performed with pure MBP-CpxR and a  $^{33}\text{P}$ -end-labeled *degP* promoter fragment in the presence and absence of competitor DNA or acetyl phosphate (Ac-P). Each lane contains 1.5 pmol of the  $^{33}\text{P}$ -end-labeled *degP* promoter fragment (lane 1) plus 115 pmol of MBP and 20 mM acetyl phosphate (lane 2), 15 pmol of pure MBP-CpxR (lane 3), 150 pmol of pure MBP-CpxR (lane 4), 150 pmol of pure MBP-CpxR and 5 pmol of *ompF* promoter competitor DNA (lane 5), or 150 pmol of pure MBP-CpxR and 20 mM acetyl phosphate.

denced by a shift in the migration of the DNA after electrophoresis (Fig. 2, lane 4). This activity was specific, as the same shift was observed in the presence of an excess of *ompF* promoter competitor DNA (Fig. 2, lane 5). No difference in the mobility of the *degP* promoter was observed upon incubation with comparable amounts of MBP (Fig. 2, lane 2), demonstrating that this effect was due to the CpxR moiety of the protein. Finally, when the assay was carried out in the presence of acetyl phosphate, both the amount of *degP* promoter shifted and the degree of the band shift were markedly enhanced. Acetyl phosphate had no effect on a similar reaction containing equal amounts of MBP in place of MBP-CpxR (lane 2). Cumulatively, these data suggest that (i) CpxR is a DNA-binding protein with affinity for specific sequences upstream of the *degP* promoter, (ii) the MBP-CpxR fusion protein can be phosphorylated by acetyl phosphate, and (iii) the DNA binding activity of the fusion protein is enhanced by phosphorylation.

**Cpx\* gain-of-function mutants are clustered in CpxA.** Our lab previously isolated several gain-of-function *cpx\** mutants (12). These mutants confer a 3- to 10-fold elevation in expression of the downstream targets of the Cpx regulon (16; also, unpublished observations). To learn more about Cpx-mediated signal transduction, these mutant genes, and one independently isolated *cpx\** allele, *cpxA2* (41), were PCR amplified and sequenced. *Cpx\** mutations are clustered in or near three regions of CpxA: the periplasmic domain, the second transmembrane helix, and the region adjacent to the predicted conserved site of autophosphorylation, H249 (Fig. 3). *cpxA2*(G97S), *cpxA102*(E92K), and *cpxA104*(R33C) contain missense mutations in the periplasmic domain (Fig. 3). Interestingly, the *cpxA24*( $\Delta$ 93-124) mutation is a deletion which encompasses 32 amino acids in the central region of the periplasmic loop (Fig. 3). A second cluster of mutations occurs in or very near the second transmembrane helix of CpxA: *cpxA17*(A188E), *cpxA711*(A184T), and *cpxA744*(P178S) (Fig. 3). The mutations in *cpxA103*(R164P) and *cpxA106*(R192L) occur on the periplasmic (PP) and cytoplasmic sides of transmembrane domain 2 (TM2), respectively (Fig. 3). Finally, two independent *cpx\** alleles (*cpxA101* and *cpxA105*) have exactly the same change and result in the alteration of T253 to a proline residue. T253 occurs near the putative site of autophosphorylation, within the region of CpxA having homology with the conserved H box of HKs (20).

**Fusion proteins containing the cytoplasmic domains of CpxA and CpxA101 confer opposite phenotypes in vivo.** To

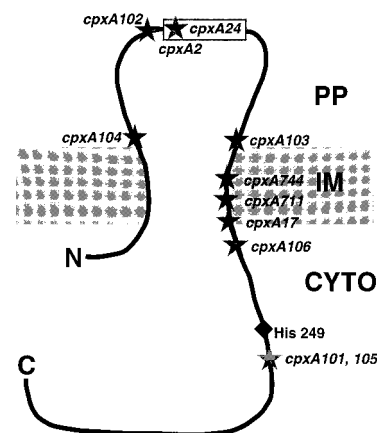


FIG. 3. Gain-of-function *cpx\** mutations are clustered in *cpxA*. *cpx\** mutations were sequenced from a PCR fragment encoding the complete *cpxA* gene derived from strains CLC142 (*cpxA24*), CLC143 (*cpxA101*), CLC144 (*cpxA102*), CLC145 (*cpxA103*), CLC146 (*cpxA104*), CLC147 (*cpxA105*), CLC163 (*cpxA17*), CLC212 (*cpxA106*), CLC213 (*cpxA711*), CLC215 (*cpxA744*), and CLC216 (*cpxA2*). The genetic background in all the above strains is MC4100 *lamBA23D zjb::Tn10Kn* (12). Stars denote the locations of missense mutations, the rectangle indicates a 96-bp deletion, and the diamond at H249 represents the putative conserved site of autophosphorylation. PP, periplasm; IM, inner membrane; CYTO, cytoplasm. N and C, amino and carboxyl termini, respectively, of CpxA.

study the biochemical activities of CpxA and identify those that might be altered in the *cpx\** mutants, we constructed, purified, and analyzed fusions between a cytoplasmic derivative of MBP and the cytoplasmic domains of CpxA and CpxA101. pMCA encodes an in-frame fusion between MBP and amino acid 218 of CpxA. pMCA101 encodes the same fusion, but with the C terminus of CpxA101(T253P). We reasoned that if these fusion proteins, which lack the periplasmic domain and transmembrane helices of CpxA, possessed any *in vivo* activities, they should be reflected in an alteration in Cpx-mediated gene expression. To test this prediction, we transformed pMCA and the control vector, pMal-C, into *cpxA*-null and wild-type backgrounds containing either a *degP-lacZ* or a *cpxP-lacZ* reporter construct and measured  $\beta$ -galactosidase activity. In the absence of wild-type CpxA, we found that pMCA caused a slight reduction in expression of both *degP* and *cpxP* (data not shown). This effect was much more obvious in the wild-type background, in which the MBP-CpxA fusion conferred a greater than threefold reduction in *cpxP* transcription (Fig. 4; compare lane 1 with 2 and lane 4 with 5). In contrast, the construct encoding the MBP-CpxA101 protein caused slightly elevated expression from both promoters in the wild-type background (Fig. 4; compare lane 1 with 3 and lane 4 with 6). These observations suggest that both fusion constructs have activity *in vivo* and that the activities of MBP-CpxA and MBP-CpxA101 are opposite of each other. Further, if CpxR-P is the competent form for transcriptional activation (the active form) (16, 52) (Fig. 2), then these data imply that the wild-type fusion acts to decrease levels of CpxR-P while the MBP-CpxA101 protein leads to increased amounts of CpxR-P.

**MBP-CpxA and MBP-CpxA101 have autokinase and CpxR kinase activities.** Many HKs possess three biochemical activities that are critical for two-component signal transduction: autokinase, RR kinase, and RR-P phosphatase (20). To assay the biochemical activities of CpxA and compare them to those of CpxA101, we purified the MBP-CpxA and MBP-CpxA101 fusion proteins using amylose affinity chromatography (Fig. 5B). When these proteins were incubated in the presence of [ $\gamma$ -

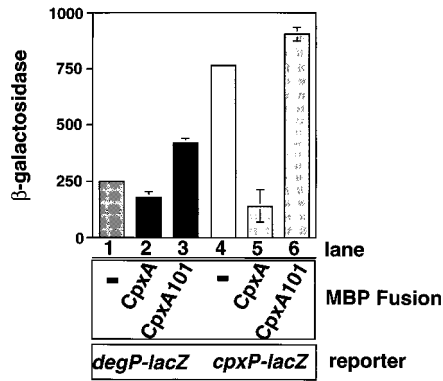


FIG. 4. MBP fusions to the cytoplasmic domains of CpxA and CpxA101 exhibit opposite phenotypes in vivo. Transcription of the *degP* and *cpxP* promoters was measured by assaying  $\beta$ -galactosidase production of TR49 (MC4100  $\lambda$ RS88[*degP-lacZ*]) (lanes 1 to 3) and TR50 (MC4100  $\lambda$ RS88[*cpxP-lacZ*]) (lanes 4 to 6) transformed with pMal-C (lanes 1 and 4), pMCA101 (lanes 2 and 5), or pMCA101 (lanes 3 and 6). pMal-C is the vector control, pMCA encodes a fusion between a cytoplasmic derivative of MBP and the cytoplasmic domain of CpxA, and pMCA101 contains the same fusion but with the T253P mutation found in CpxA101.

$^{33}\text{P}$ ]ATP, radiolabeled MBP-CpxA-P and MBP-CpxA101-P accumulated over time, although MBP-CpxA101-P appeared to accumulate somewhat slower (Fig. 5A). Thus, both proteins can catalyze autophosphorylation. Pure MBP alone did not become phosphorylated in the presence of  $[\gamma\text{-}^{33}\text{P}]$ ATP (data not shown). We conclude that *cpxA101*(T253P) decreases but does not abolish the autokinase activity.

Upon autophosphorylation, the next step in two-component signal transduction is phosphotransfer to the RR effector molecule (20). Thus, the HKs also function as RR kinases (20). We measured the CpxR kinase activity of CpxA and CpxA101 by incubating MBP-CpxA- $^{33}\text{P}$  or MBP-CpxA101- $^{33}\text{P}$  (Fig. 6, lane 1) with purified MBP-CpxR for increasing periods (Fig. 6, lanes 2 to 7). A phosphorylated species corresponding to the molecular weight (MW) of MBP-CpxR accumulated with increasing reaction time in the presence of radiolabeled MBP-CpxA or MBP-CpxA101, although this reaction appeared to be diminished in the presence of the mutant MBP-CpxA101 (Fig.

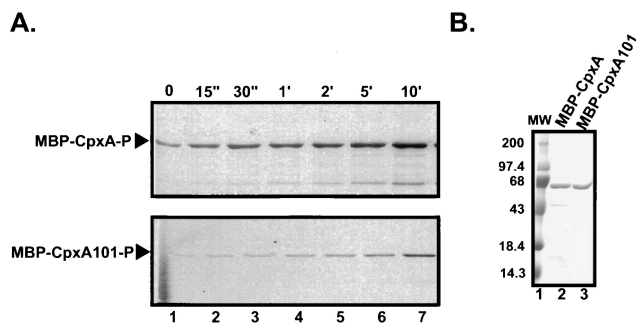


FIG. 5. MBP-CpxA and MBP-CpxA101 exhibit autokinase activity. (A) Equal amounts (final concentration, 1.8  $\mu\text{M}$ ) of pure MBP-CpxA (upper panel) or MBP-CpxA101 (lower panel) were incubated in the presence of 0.5  $\mu\text{M}$   $[\gamma\text{-}^{33}\text{P}]$ ATP for the indicated periods prior to SDS-PAGE and autoradiography (lanes 1 to 7). A slightly longer exposure of the MBP-CpxA101 gel is shown. (B) Equal volumes of pure preparations of MBP-CpxA (lane 2) or MBP-CpxA101 (lane 3) were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue to ensure that protein concentrations were identical. Western blot analysis demonstrated that the low-MW bands in lane 2 are breakdown products of the MBP-CpxA fusion protein (data not shown).

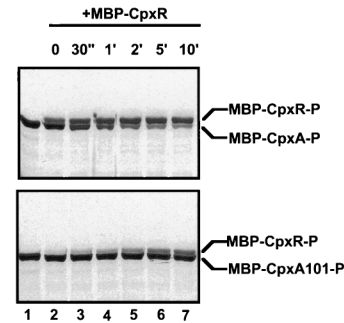


FIG. 6. MBP-CpxA and MBP-CpxA101 exhibit kinase activity. MBP-CpxA- $^{33}\text{P}$  (upper panel, lane 1) or MBP-CpxA101- $^{33}\text{P}$  (lower panel, lane 1) was incubated in the presence of 1.75  $\mu\text{M}$  MBP-CpxR plus  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  for the indicated periods prior to SDS-PAGE and autoradiography (lanes 2 to 7). A slightly longer exposure of the MBP-CpxA101 gel is shown. ', minutes; ", seconds.

6). No MBP-CpxR- $^{33}\text{P}$  was seen when the reaction was carried out in the absence of MBP-CpxA or MBP-CpxA101 or in the presence of MBP (data not shown). Thus, both MBP-CpxA-P and MBP-CpxA101-P can catalyze phosphotransfer to MBP-CpxR. We conclude that, although *cpxA101*(T253P) diminishes the kinase activity of CpxA, it does not abolish it.

**MBP-CpxA is an MBP-CpxR-P phosphatase.** To test if CpxA is a CpxR-P phosphatase, purified MBP-CpxA was incubated in the presence of MBP-CpxR-P for increasing periods. To obtain radiolabeled MBP-CpxR, we took advantage of the fact that, in the absence of the cognate HK, an RR can become phosphorylated by an alternate HK, albeit much less efficiently (24). We used a truncated version of the HK EnvZ, EnvZ115, to phosphorylate MBP-CpxR (25). The advantage of using EnvZ115 is that this protein, although functionally competent to communicate with its cognate RR through phosphotransfer (Fig. 7A, lane 2) (23–25), forms insoluble aggregates which can be removed from the reaction mixture by centrifugation. Incubation of EnvZ115 in the presence of  $[\gamma\text{-}^{33}\text{P}]$ ATP resulted in rapid accumulation of phospho-EnvZ115 (Fig. 7A, lane 1). Addition of the cognate RR OmpR to this reaction mixture demonstrated that EnvZ115-P is an efficient OmpR kinase (Fig. 7A, lane 2), as previously demonstrated (23). Further, upon prolonged incubation with MBP-CpxR, EnvZ115-

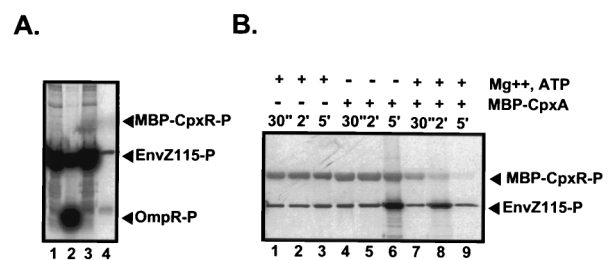


FIG. 7. MBP-CpxA is an MBP-CpxR-P phosphatase. Pure MBP-CpxA was incubated with MBP-CpxR- $^{33}\text{P}$  for the indicated periods in the presence and absence of  $\text{Mg}^{2+}$  and ATP (B). (A) MBP-CpxR- $^{33}\text{P}$  was obtained by labeling with EnvZ115- $^{33}\text{P}$ . EnvZ115 (95  $\mu\text{M}$ ) was phosphorylated by incubation with  $[\gamma\text{-}^{33}\text{P}]$ ATP (lane 1). After several washes to remove the remaining  $[\gamma\text{-}^{33}\text{P}]$ ATP, phosphotransfer to either OmpR (lane 2) or MBP-CpxR (lane 3) was achieved through the addition of the RR to a final concentration of 13  $\mu\text{M}$ . The majority of the EnvZ115- $^{33}\text{P}$  was removed by ultracentrifugation, and the supernatant (lane 4), containing MBP-CpxR- $^{33}\text{P}$ , was used as a substrate to measure the phosphatase activity of MBP-CpxA or MBP-CpxA101. (B) MBP-CpxR- $^{33}\text{P}$  was incubated with (lanes 4 to 9) or without (lanes 1 to 3) MBP-CpxA in the presence (lanes 1 to 3 and 7 to 9) or absence (lanes 4 to 6) of 5 mM  $\text{MgCl}_2$  and 3 mM ATP for the indicated periods. ', minutes; ", seconds.

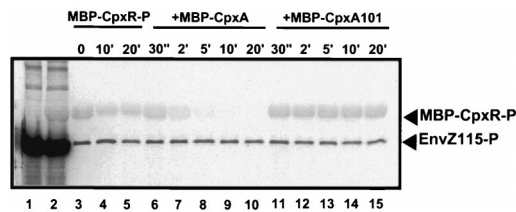


FIG. 8. MBP-CpxA101 lacks MBP-CpxR-P phosphatase activity. MBP-CpxR-<sup>33</sup>P was incubated in the presence of MBP-CpxA or MBP-CpxA101. MBP-CpxR was phosphorylated with EnvZ115-<sup>33</sup>P. To obtain EnvZ115-<sup>33</sup>P, EnvZ115 (95  $\mu$ M) was incubated with [ $\gamma$ -<sup>33</sup>P]ATP (lane 1). After several washes to remove remaining [ $\gamma$ -<sup>33</sup>P]ATP, MBP-CpxR (13  $\mu$ M) was added to this reaction mixture and incubated for 1 h (lane 2). The majority of the EnvZ115-<sup>33</sup>P was removed by ultracentrifugation. The supernatant, containing MBP-CpxR-<sup>33</sup>P was used as a substrate to assay the phosphatase activity of MBP-CpxA and MBP-CpxA101. MBP-CpxR-<sup>33</sup>P was incubated in the presence of 5 mM MgCl<sub>2</sub> and 3 mM ATP either alone (lanes 3 to 5), with 0.07  $\mu$ M pure MBP-CpxA (lanes 6 to 10), or with 0.07  $\mu$ M pure MBP-CpxA101 for the indicated periods prior to SDS-PAGE and autoradiography. ', minutes; ", seconds.

<sup>33</sup>P was able to phosphorylate this RR as well (Fig. 7A, lane 3). After high-speed ultracentrifugation, the majority of the remaining EnvZ115-P was pelleted, leaving MBP-CpxR-P in the supernatant (Fig. 7A, lane 4). The supernatant was used as a source of MBP-CpxR-P for subsequent MBP-CpxA phosphatase assays (Fig. 7B). When MBP-CpxR-P was incubated in the presence of either Mg<sup>2+</sup> and ATP or pure MBP-CpxA, the phosphoprotein was stable over the time of the assay (Fig. 7B, lanes 1 to 3 and 4 to 6). In contrast, in the presence of Mg<sup>2+</sup>, ATP, and MBP-CpxA, MBP-CpxR-P was rapidly dephosphorylated (Fig. 7B, lanes 7 to 9). These data show that MBP-CpxA is an MBP-CpxR-P phosphatase and, like other HKs (20), this activity is dependent on Mg<sup>2+</sup> and/or ATP. By extension, we conclude that CpxA possesses CpxR-P phosphatase activity.

**MBP-CpxA101 lacks MBP-CpxR-P phosphatase activity.** To determine if CpxA\* mutants have altered phosphatase activity, we tested the ability of MBP-CpxA101 to dephosphorylate MBP-CpxR-P in the same assay described above. The truncated EnvZ115 protein was phosphorylated by incubation with [ $\gamma$ -<sup>33</sup>P]ATP (Fig. 8, lane 1) and then used to label MBP-CpxR (Fig. 8, lane 2). After high-speed centrifugation to remove the majority of EnvZ115-P, the supernatant, containing MBP-CpxR-P (Fig. 8, lane 3), was incubated alone (Fig. 8, lanes 3 to 5), with pure MBP-CpxA (Fig. 8, lanes 6 to 10) or MBP-CpxA101 (Fig. 8, lanes 11 to 15) in the presence of Mg<sup>2+</sup> and ATP. As previously observed, MBP-CpxA functioned as an efficient MBP-CpxR-P phosphatase, removing the entire label after a very short incubation time (Fig. 8, lanes 6 to 10). Conversely, MBP-CpxR-P levels were unaltered in the presence of the MBP-CpxA101 protein over the period assayed (Fig. 8, lanes 11 to 15), demonstrating that MBP-CpxA101 is devoid of phosphatase activity. We conclude that *cpxA101* (T253P) abolishes the phosphatase activity of CpxA.

**Gain-of-function mutations in the periplasmic domain of CpxA confer a signal-blind phenotype.** Our data and that of others suggest that it is ultimately alterations in the HK kinase/phosphatase activity ratio which mediate signal transduction (56). Presumably, these alterations are brought about by signal-induced conformational changes in the sensor. Very little is understood about the precise molecular mechanism by which this is accomplished; however, the periplasmic domains of these molecules have been implicated (7, 28, 59, 68). The location of several *cpx\** mutations near the center of the periplasmic domain of CpxA (Fig. 3) suggests that this region

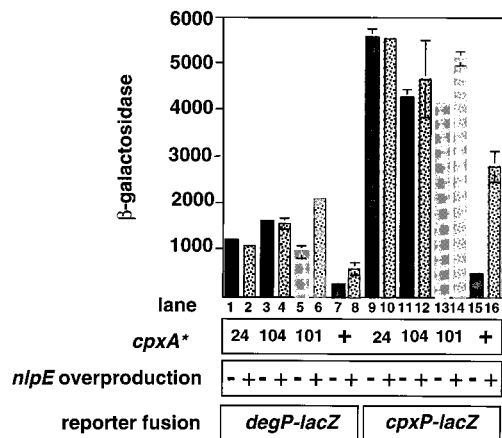


FIG. 9. Periplasmic *cpxA\** mutations confer a signal-blind phenotype.  $\beta$ -Galactosidase assays were used to measure the effect of NlpE overproduction on expression from *degP-lacZ* (lanes 1 to 8) and *cpxP-lacZ* (lanes 9 to 16) reporter fusions in the presence of the *cpxA24* (lanes 1, 2, 9, and 10), *cpxA104* (lanes 3, 4, 11, and 12), *cpxA101* (lanes 5, 6, 13, and 14), and wild-type *cpxA* (lanes 7, 8, 15, and 16) alleles. Lane 1, TR33(pBR322); lane 2, TR33(pLD404); lane 3, TR45(pBR322); lane 4, TR45(pLD404); lane 5, TR47(pBR322); lane 6, TR47(pLD404); lane 7, TR49(pBR322); lane 8, TR49(pLD404); lane 9, TR34(pBR322); lane 10, TR34(pLD404); lane 11, TR46(pBR322); lane 12, TR46(pLD404); lane 13, TR48(pBR322); lane 14, TR48(pLD404); lane 15, TR50(pBR322); lane 16, TR50(pLD404).

might be involved in sensing, and further that these *cpx\** mutants are constitutively active because of an inability to correctly sense the appropriate signals. To test this prediction, we measured the ability of strains containing the wild type, a cytoplasmic *cpx\** mutation (*cpxA101*), or one of two periplasmic *cpx\** mutations (*cpxA24* or *cpxA104*) to respond to a signal that normally activates the Cpx pathway by assaying transcription from the *degP* and *cpxP* promoters in the absence and presence of NlpE overproduction (Fig. 9). As previously demonstrated, overproduction of NlpE resulted in induction of both *degP* and *cpxP* expression in a wild-type background (15a, 16) (Fig. 9). A strain containing a *cpx\** mutation in the cytoplasmic domain exhibited higher basal-level expression than the wild type, but the mutant HK still responded to NlpE overexpression, showing that this molecule is still able to sense and respond to activating signals (Fig. 9, *cpxA101*). In contrast, two different strains harboring *cpx\** mutations in the periplasmic domain were no longer able to mediate elevated transcription of *degP* and *cpxP* in response to the activating signal of *nlpE* overproduction (Fig. 9, *cpxA24* and *cpxA104*). Thus, activating mutations in the periplasmic domain of CpxA confer a signal-blind phenotype.

## DISCUSSION

The CpxA HK is capable of both increasing and decreasing levels of CpxR-P to control the output of the Cpx regulon (Fig. 5 to 7). Like other HKs, CpxA is an efficient autokinase when incubated in the presence of ATP (Fig. 5). This autophosphorylation event presumably takes place at the His residue corresponding to the conserved site of autophosphorylation, H249. CpxA-P, in turn, phosphorylates CpxR (Fig. 6), demonstrating that CpxA is a CpxR kinase. This reaction is greatly stimulated by the presence of Mg<sup>2+</sup> and/or Mn<sup>2+</sup> ions (data not shown), similar to the reactions catalyzed by other HKs (20). Homology predicts that phosphorylation of CpxR would occur on D51. Like many other HKs, CpxA can also catalyze the removal of phosphate from CpxR-P (Fig. 8). Addition of MBP-CpxA to

radiolabeled MBP-CpxR-P results in rapid dephosphorylation that, akin to other HKs that function as phosphatases, is dependent on  $Mg^{2+}$  and ATP (24). Thus, CpxA has three enzymatic activities: autokinase, kinase, and phosphatase and therefore the ability to both increase and decrease levels of CpxR-P and, ultimately, the Cpx regulon.

Previous studies suggested that the phosphorylated form of CpxR is the species responsible for transcriptional activation of the Cpx downstream targets *degP*, *cpxP*, *dsbA*, and *ppiA*. Danese et al. (16) demonstrated that in the absence of CpxA, CpxR-mediated transcriptional activation could occur in vivo only when the phosphodonor acetyl phosphate was present in the cell. Similarly, Pogliano and coworkers (52) showed that preincubation of purified CpxR with acetyl phosphate increased its affinity for specific DNA binding sites upstream of the *degP*, *dsbA*, and *ppiA* genes. These observations suggest that in the absence of its cognate HK, CpxR, like other RRs (30, 39, 40), can become phosphorylated in the presence of acetyl phosphate and that the phosphorylated species is competent for DNA binding and transcriptional activation.

Our data support and confirm the notion that CpxR-P is the active form of this protein. A plasmid encoding a MBP-CpxR fusion protein was able to complement *degP-lacZ* transcription in a *cpxRA* mutant background to levels comparable to those of a plasmid encoding the wild-type CpxR (Fig. 1). This complementation was partially ablated by a deletion of the genes responsible for the synthesis of acetyl phosphate (data not shown), which suggests that in the absence of CpxA, the MBP-CpxR fusion protein, like CpxR, is dependent upon alternative phospho-donors such as acetyl phosphate to mediate transcriptional activation. Further, when mobility shift experiments were performed with pure MBP-CpxR and the *degP* promoter region, the presence of acetyl phosphate resulted in an increase in both the amount of DNA shifted and the degree of the shift (Fig. 2). Most convincingly, an activated allele of CpxA that leads to increased expression of the regulon is defective in removal of phosphate from CpxR-P (Fig. 8). Taken together, these data strongly argue that phosphorylated CpxR is the form competent for DNA binding and transcriptional activation.

These observations suggest that the activating effects on the Cpx pathway of the recently identified PrpA and PrpB phosphatases do not occur, as suggested (47), through direct effects on the phosphotransfer events between CpxA and CpxR which result in lower levels of CpxR-P. Missiakas and Raina argue that perhaps the Prp phosphatases fine-tune Cpx-mediated transcription by dephosphorylating CpxR-P to levels that would prevent binding of CpxR-P to "low affinity repression sites which may block the transcriptional process." Our data suggest that in *Cpx\** mutants, levels of CpxR-P accumulate to high levels due to the absence of CpxA phosphatase activity (Fig. 8) and, further, that CpxR-P possesses higher affinity for the promoters of Cpx-regulated genes (Fig. 2). Further, we have seen no repressive effect on any Cpx-regulated gene identified to date in any *cpx\** mutant background. These observations and others (16, 52) suggest that the effects of these phosphatases on Cpx signal transduction are indirect and are not mediated through reductions in the amount of CpxR-P in the cell. Since these phosphatases appear to have many effects on the phosphorylation status of numerous proteins (47), it is not possible to say at this time how their effects on Cpx signal transduction are manifested.

The fact that the *degP* promoter fragment is supershifted in the presence of acetyl phosphate suggests that phosphorylation may enhance the cooperativity with which DNA binding occurs. In support of this idea, DNA footprinting studies show an

extended CpxR binding region in the presence of acetyl phosphate (52). At the level of sequence homology, CpxR is most closely related to the RR OmpR. OmpR also exhibits an enhanced DNA footprint upon phosphorylation (1), and it has recently been shown that phosphorylation acts to stimulate cooperative binding (22). Although this hypothesis awaits further experimentation, it is clear that phosphorylation of CpxR renders the molecule competent for transcriptional activation and that enhanced DNA binding ability is a part of this mechanism.

To understand how CpxA modulates levels of CpxR-P, we studied gain-of-function *cpx\** mutants that constitutively activate expression of Cpx regulon members (12, 16). All *cpx\** mutations we studied clustered within three regions of CpxA: the periplasmic, transmembrane, and H-box domains. Intriguingly, three of these mutations were located near the center of the periplasmic domain. One of these is a 96-bp deletion, while the other two point mutations are either within or very near this deletion (Fig. 3). These observations suggest two things. First, the periplasmic locale of these mutations suggests that this region of CpxA may be involved in sensing and responding to signals. Second, the simplest explanation for the activated nature of these alleles is that they must somehow act to relieve a negative effect that is normally mediated through the wild-type periplasmic domain. In other sensor kinases, the periplasmic domain is implicated or shown to be directly involved in sensing relevant signals (7, 28, 59). For example, genetic analysis suggests that the periplasmic domain of the plant pathogen *Agrobacterium tumefaciens* HK VirA binds plant wound sugars complexed with the periplasmic sugar binding molecule ChvE to activate the Vir regulon and cause concomitant expression of tumor-inducing genes. The TorA HK is proposed to interact with the alternate electron acceptor TMAO to activate expression of the TMAO reductase anaerobic respiratory system. Finally, mutations in the periplasmic domain of the NarX sensor-transmitter, which controls expression of anaerobic electron transport and fermentation genes, prevent response to nitrate, the normal activating signal. We propose that the central region of the periplasmic domain of CpxA is directly involved in responding to signals of envelope stress. A prediction of this hypothesis is that periplasmic mutants will no longer have the capacity to respond to activating signals. Indeed, while a strain containing a *cpx\** mutation in the cytoplasmic domain retained the ability to upregulate the Cpx regulon in response to a normally activating signal, those harboring one of two different *cpx\** periplasmic mutations were rendered signal blind (Fig. 9). Thus, these mutations affect a domain of CpxA that is critical for responding to envelope stress.

The activated nature of these mutations suggests that they relieve a negative effect, which maintains the HK in a down-regulated state normally. Similar "locked on" mutations have been isolated in putative binding sites for effector molecules in the periplasmic domains of the HKs VirA, TorA, NarX, and EnvZ (7, 28, 59, 68). Such phenotypes could potentially occur through the acquisition of an activated conformational state. We favor a model in which a negative effector molecule interacts with the periplasmic domain of CpxA under unstressed conditions to maintain the HK in an off state and the regulon at a basal level of expression (Fig. 10). Envelope stresses would result in loss of interaction with the effector and activation of the regulon. The nature of the activating signals and the downstream targets of the Cpx two-component system suggest that CpxA monitors protein trafficking events in the periplasm. Thus, we propose that the negative-effector molecule is a periplasmic protein normally involved in folding and/or sorting envelope proteins. Accordingly, under unstressed conditions,

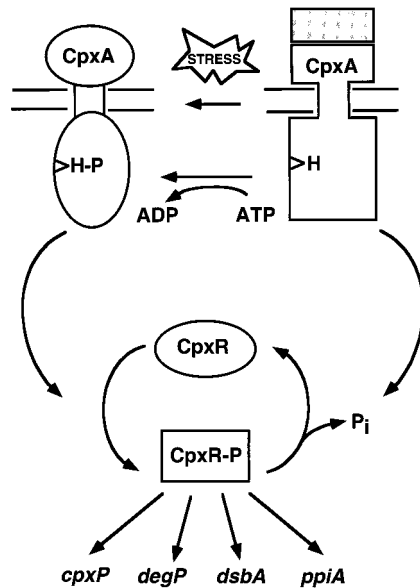


FIG. 10. A model for Cpx-mediated signal transduction. Under normal physiological conditions, the CpxA HK is maintained predominantly as a CpxR-P phosphatase via interaction with a putative periplasmic ligand, resulting in a low kinase/phosphatase ratio and depressed production of the downstream targets. During times of envelope stress, when there is an increased demand for envelope protein folding and degrading enzymes, the periplasmic ligand is titrated away from CpxA, triggering a conformational change which leads to an increase in the kinase/phosphatase ratio through stimulation of the autokinase and CpxR kinase activities and concomitant elevated expression of downstream targets. See the text for more details.

such a molecule would be abundant and could maintain CpxA and the Cpx regulon in a downregulated state. Conversely, under conditions where protein trafficking is perturbed in the envelope and there is an increased demand for this and other folding/sorting factors, it would be titrated away from CpxA, resulting in activation of the HK and upregulated expression of the regulon. This model provides a satisfying explanation for the activated nature of the clustered periplasmic *cpx\** alleles—in particular, the *cpxA24*( $\Delta$ 93-124) mutation; these mutant receptors can no longer recognize the negative effector.

Similar models have been proposed to describe the regulation of other stress responses. Biochemical and genetic evidence support a model in which the protein folding status of the bacterial cytoplasm is monitored through interactions between  $\sigma^{32}$  and the free pool of chaperones, which can in turn affect RpoH activity through alterations in synthesis, stability, or activity (33, 34, 66, 67).  $\sigma^E$  has been shown to interact with a membrane-bound anti-sigma factor, RseA, that is hypothesized to release  $\sigma^E$  in response to misfolded proteins in the bacterial periplasm (17, 46). Interestingly, the unfolded protein response in the endoplasmic reticulum (ER) of yeast also involves a membrane-bound kinase, Ire1p, which is involved in activating the expression of protein folding factors in the ER in response to unfolded proteins in this compartment (13, 49). It has been proposed that Ire1p may sense unfolded proteins through free levels of the ER chaperone KAR2 (13, 49).

How is such a signal transduced to the cytoplasm? In the structurally related chemoreceptors of *E. coli*, binding of attractant or repellent to the periplasmic face causes movement of TM2, which is thought to be the critical event in propagating the signal to the cytoplasmic domain of the molecule (8, 9). Interestingly, when the periplasmic and transmembrane domains of the aspartate chemoreceptor are fused to the enzymatic

region of the EnvZ HK, the hybrid protein functions to regulate porin expression in response to levels of aspartate, suggesting that the HK may utilize a similar signal transduction mechanism (70). Five of the 11 activating *cpx\** mutations characterized in this study occur in or very near TM2 (Fig. 3). Thus, we suggest that, as with the chemoreceptors, TM2 is involved in propagating a conformational change in response to effector binding and/or release that ultimately modifies the enzymatic activities of the cytoplasmic domain of CpxA.

That the periplasmic and TM domains are important for maintaining the proper signaling conformation of the cytoplasmic domain is evidenced by the observation that replacement of these domains with a soluble protein, MBP, drastically alters the activity of the wild-type CpxA (Fig. 4). A soluble MBP-CpxA fusion caused as much as a threefold decrease in Cpx-mediated gene expression in a *cpxRA*<sup>+</sup> background (Fig. 4). Since CpxR-P is the active form, we conclude that this fusion protein mediates a reduction in CpxR-P levels. This could potentially occur through one of two mechanisms. First, it is possible that the MBP-CpxA fusion protein forms inactive multimers with the wild-type CpxA, thus decreasing the amount of the HK available to phosphorylate CpxR. It has been proposed that the EnvZ HK functions as a dimer (74), and thus this remains a formal possibility. However, since the pure MBP-CpxA fusion protein demonstrates both autokinase and kinase activities in vitro (Fig. 5 and 6), we favor the possibility that this protein depresses CpxR-P levels through an elevated phosphatase activity. In fact, the pure MBP-CpxA rapidly and efficiently dephosphorylates CpxR-P in vitro (Fig. 7 and 8). Accordingly, it appears that the PP and TM domains are required to maintain normal, basal functioning of the CpxA HK in the absence of envelope perturbations and by extension that these domains play a critical role in transducing signals concerning the extracytoplasmic compartments to modulate the enzymatic activities of the HK.

This idea is supported by the observation that gain-of-function mutations in the cytoplasmic domain of CpxA are able to subvert the effects of losing the normal signaling domains of the protein. Mutations in the conserved H-box of the HK EnvZ can lock this protein in an on state (2, 61). Similarly, the presence of the *cpxA101* H-box mutation in the MBP-CpxA fusion confers an activated phenotype on this protein (Fig. 4). In vivo this is reflected by the ability of this fusion protein to confer an increase in Cpx-mediated gene expression in a *cpxRA*<sup>+</sup> background (Fig. 4). Thus, the presence of the *cpxA101* mutation overcomes the defective signaling status conferred on the wild-type CpxA protein by loss of the PP and TM domains, implying that this mutation locks the HK in an on state.

To determine what this state is, we assayed the purified MBP-CpxA101 fusion protein for the autokinase, kinase, and phosphatase activities demonstrated for the wild-type protein (Fig. 5 to 7). MBP-CpxA101 retains the ability to autophosphorylate in the presence of ATP and to transfer this phosphate to CpxR (Fig. 5 to 6), demonstrating that, as expected, the autokinase and kinase activities of the mutant, although reduced, are intact. Conversely, we find that the MBP-CpxA101 fusion protein is completely devoid of phosphatase activity (Fig. 8). Thus, the *cpxA101* mutation confers an activated phenotype by increasing the kinase/phosphatase ratio. Since the effects of this mutation mimic the activated state of the Cpx regulon, we conclude that modulation of the Cpx regulon occurs through alterations in the CpxA kinase/phosphatase activity ratio (Fig. 10). Mutations in other HKs which are thought to confer a loss of phosphatase activity result in similar activated phenotypes in vivo (2, 10, 68, 69, 72),



suggesting that this is a common mechanism used to control the output of these transducers. Although it is not possible for us to say at this time what the precise effects of mutations in the PP and TM domains of CpxA are on the enzymatic activity of the HK, studies with the closely related protein EnvZ have demonstrated that similar mutations do indeed affect the porin regulon through alteration of the kinase/phosphatase activity ratio (56, 68, 69). Therefore, we think it highly likely that the PP and TM mutations in CpxA confer an increase in the kinase/phosphatase ratio similar to that of *cpxA101*.

In sum, our results provide the framework for a working model of Cpx-mediated signal transduction (Fig. 10). The CpxA HK controls the levels of CpxR-P through its autokinase, kinase, and phosphatase activities (Fig. 5 to 7). Phosphorylation of CpxR confers increased affinity for the promoters of Cpx regulon members (Fig. 2), leading to transcriptional activation and elevated expression. The output of the regulon is controlled through modulations in the CpxA kinase/phosphatase activity ratio in response to the status of protein trafficking in the bacterial envelope. We propose that envelope perturbations are sensed as disruptions in protein trafficking in the bacterial envelope through the use of a negative-effector molecule involved in protein folding and/or sorting that interacts with CpxA (Fig. 10). In "relaxed" times, the demand for such an effector molecule would be low and it would be available to bind to the periplasmic domain of CpxA and maintain the sensor at a low kinase/phosphatase ratio, or off state (Fig. 10). Envelope perturbations, such as elevated pH, overproduction of NlpE, etc., would necessitate increased demand for the effector molecule, releasing CpxA and causing conformational changes which would be propagated via TM2 to the enzymatic domain in the cytoplasm to mediate an elevated kinase/phosphatase ratio.

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