Signal Detection and Target Gene Induction by the CpxRA Two-Component System

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The Cpx pathway is a two-component signal transduction system that senses a variety of envelope stresses, including misfolded proteins, and responds by upregulating periplasmic folding and trafficking factors. CpxA resides in the inner membrane and has both kinase and phosphatase activities. CpxR, the response regulator, mediates a response by activating transcription of stress-combative genes. Signal transduction is subject to feedback inhibition via regulon member CpxP and autoamplification. Recently, it was shown that the Cpx pathway is also upregulated when cells adhere to hydrophobic surfaces and that this response is dependent on the outer membrane lipoprotein NlpE. Here we show that while NlpE is required for induction of the Cpx pathway by adhesion, induction by envelope stress and during growth is NlpE independent. We show that while all of the envelope stresses tested induce the Cpx pathway in a manner that is dependent on the periplasmic domain of CpxA, induction during growth is independent of CpxA. Therefore, we propose that the Cpx pathway can sense inducing cues that enter the signaling pathway at three distinct points. Although CpxP is not required for induction of the Cpx pathway, we show that its activity as a negative regulator of CpxA is inactivated by envelope stress. Moreover, the cpxP promoter is more inducible than any other regulon member tested. Consistent with these results, we suggest that CpxP performs a second function, most likely that of a chaperone. Finally, we show that two Cpx-regulated genes are differentially upregulated in response to different envelope stresses, suggesting the existence of three stress-responsive systems.

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TABLE 1. Bacterial strains and plasmids used in this study

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chemiluminescence Western blotting reagents were purchased from Amersham. Binding protein (MBP)-CpxR antiserum (34) was used at a 1:5,000 dilution. Maltose-

Western blot analysis. Following induction of the Cpx pathway by alkaline pH (see above), the OD600 was determined. The reciprocal of the OD600 was used to determine the sample volume, normalizing the number of cells per milliliter. According to this calculation, the appropriate culture volume was harvested by centrifugation, resuspended in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and lysed by boiling for 5 min. Samples (15 μl) were electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel, transferred to nitrocellulose, and subjected to Western blot analysis. Maltose-binding protein (MBP)-CpxR antiserum (34) was used at a 1:5,000 dilution. Enhanced-chemiluminescence Western blotting reagents were purchased from Amersham Life Sciences.

RESULTS

Inputs. Components of the Cpx pathway reside in each compartment of the bacterial cell. NlpE resides in the outer membrane. CpxP, a negative regulator of the pathway, resides in the periplasm (5). CpxA and CpxR reside in the inner membrane and cytoplasm, respectively (15, 44). Many different envelope stresses, which could affect any of the four bacterial compartments, are known to upregulate the Cpx pathway. Given that members of the signaling cascade exist in all compartments and that stresses that upregulate the pathway may affect each compartment, we hypothesized that potential signals could enter the pathway at different points in the signaling pathway. For example, a stress originating in the outer membrane could enter the pathway at NlpE. A stress originating in the periplasm could enter the pathway downstream of NlpE, at CpxP or at CpxA. Inner membrane stresses could signal through the transmembrane domains of CpxA, while stresses that affect the cytoplasm could signal to the pathway by affecting the phosphotransfer between CpxA and CpxR or by affecting CpxR directly.

Accumulation of misfolded pilus subunits PapG and PapE in the periplasm is known to upregulate the Cpx pathway (20). However, the signals generated by other envelope stresses are less obvious. Accumulation of lipid II ECA intermediate, most likely in the inner membrane, upregulates the Cpx pathway and causes sensitivity to bile salts, suggesting an outer membrane defect (4). Decreased levels of phosphatidylethanolamine upregulate the Cpx pathway in an NlpE-independent manner, causing both inner and outer membrane defects (25). Alkaline pH upregulates the Cpx regulon, but the effects of increased pH on the envelope are unclear (5). EDTA induces the Cpx pathway (data not shown), most likely by disrupting lipoplysaccharide (LPS) in the outer membrane (28). It is possible that each of these envelope stresses generates a unique signal that is sensed distinctly by the Cpx pathway or all of the stresses cause the same general signal.

(i) Envelope stress activates the Cpx pathway in an NlpE-independent manner. Since adhesion to hydrophobic surfaces activates the Cpx pathway in an NlpE-dependent manner, one possibility is that adhesion damages the outer membrane and NlpE thus functions as an outer membrane “damage sensor.” It is also possible that adhesion sends a signal to the Cpx pathway that is distinct from that of envelope stress and the role of NlpE in signal transduction is specific to adhesion. In order to elucidate the role of NlpE in signal transduction, wild-type and mutant nlpE strains were tested for Cpx induction in response to a variety of envelope stresses. Using a cpxA::lacZ fusion strain as a reporter, we found that PapG expression in the absence of the PapD chaperone induced the Cpx pathway in an NlpE-independent manner (Fig. 1). The same results were observed for PapE expression, induction by alkaline pH, accumulation of the ECA intermediate lipid II, and exposure to EDTA (data not shown), suggesting that envelope stress is sensed by Cpx in an NlpE-independent manner. We suggest that NlpE does not function to sense damage but rather that NlpE functions specifically in adhesion.

(ii) The periplasmic domain of CpxA is required for induction of the Cpx pathway by envelope stress. The cpxA* mutation, cpxA4, is an in-frame deletion in the periplasmic domain of CpxA. cpxA4 activates the Cpx pathway but, unlike other cpxA* alleles, is not further induced by stress, suggesting that this mutation renders CpxA signal blind (34). By using this signal-blind cpxA* allele, the periplasmic domain of CpxA was shown to be required for induction of the Cpx pathway by NlpE overexpression and for repression of the Cpx pathway by overexpression of CpxP (34). We expected that if all of the stresses present the same signal to the Cpx pathway, then all of
the stresses should be sensed in the same way and require the periplasmic sensing domain of CpxA; cpxA24 mutant strains should be blind to envelope stress signals.

In order to determine if induction requires the periplasmic domain of CpxA, the cpxP-lacZ fusion was analyzed by using β-galactosidase assays of wild-type and cpxA24 strains in the presence or absence of PapG expression. As shown in Fig. 1, an intact periplasmic domain of CpxA is required for induction of the Cpx pathway by PapG overexpression. The cpxA24 mutant strain was blind to all of the stresses tested, including alkaline pH, PapE overexpression, NlpE overexpression, exposure to EDTA, and lipid II accumulation (data not shown). These data indicate that an intact periplasmic domain of CpxA is required for induction of the Cpx pathway by envelope stress.

(iii) The periplasmic domain of CpxA is not required for induction of the Cpx pathway during growth. During the course of these studies, we observed that the Cpx pathway was induced during growth in LB broth and that the induction was not entirely due to pH changes throughout growth. While it was previously reported by De Wulf and Lin that Cpx is induced upon entry into stationary phase in an RpoS-dependent manner in strains derived from MG1655 (13), we found that the induction occurs in an RpoS-independent manner (32). However, upon exposure to alkaline pH, the cpxP null strain was induced to levels comparable to those of the wild-type strain (Fig. 3). The same results were obtained for the other stresses tested, including PapG and PapE expression, exposure to EDTA, and lipid II accumulation (data not shown).

The Cpx pathway is induced by growth phase (compare lanes 1 and 2 in panels A and B) in a manner that is dependent on CpxR (lanes 3 and 4 in panel A). Induction during growth also occurs in the cpxA24 mutant background (compare lanes 5 and 6 to 1 and 2 in panel B). WT, wild type.

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(iv) Induction by envelope stress is CpxP independent. We hypothesized that if CpxP were required for induction of the Cpx pathway during envelope stress, cpxP null strains would be blind to signals. In order to better determine the role of CpxP in signal transduction, β-galactosidase assays were performed on wild-type and cpxP null strains containing the cpxP-lacZ fusion in response to a variety of envelope stresses. The induced level of transcription from the cpxP promoter in the cpxP null strain was twofold higher than the uninduced level of transcription from cpxP in the wild type, consistent with prior experiments showing that CpxP is a negative regulator under noninducing conditions (32). However, upon exposure to alkaline pH, the cpxP null strain was induced to levels comparable to those of the wild-type strain (Fig. 3). The same results were obtained for the other stresses tested, including PapG and PapE expression, exposure to EDTA, and lipid II accumulation (data not shown).
CpxP is a member of the OmpR family of winged helix-turn-helix transcription factors. Consequently, OmpR and CpxR are highly homologous response regulators. On the basis of analogy to OmpR, where Asp at position 55 is the putative site of 5 and 6), suggesting that alkaline pH does not compromise the stability of the MBP-CpxP fusion proteins. Similar experiments were performed by using PapE expression as the inducing signal and yielded comparable results (data not shown). These data suggest that under conditions that induce the Cpx pathway, MBP-CpxP is not sufficient to prevent induction and therefore does not act as a negative regulator. MBP-CpxP is inactivated by envelope stress. Moreover, since all envelope stresses upregulate CpxP, then all envelope stresses must also inactivate CpxP.

Outputs. (i) CpxP is the most highly induced target gene. Previously, it was observed that the cpxP and degP promoters have different binding affinities for CpxR–P (T. L. Raivio, personal communication), which implies different sensitivities of these promoters to levels of CpxR–P. In an effort to further characterize differences in CpxR–P affinity at Cpx-regulated target genes, induction of Cpx-regulated lacZ transcriptional fusions was analyzed in response to a variety of envelope stresses. When conditions known to induce the Cpx pathway, such as cpxA* alleles, alkaline pH, and NlpE overexpression, were used to induce target gene expression, the fold induction of the cpxP–lacZ expression was greater than the fold induction of the other fusions tested. For example, upon induction by pH 8.5, the cpxP–lacZ fusion was induced approximately 15-fold in a Cpx-dependent manner while other fusions were induced 2- or 3-fold (Fig. 5). In addition, Western blot analysis showed that CpxP protein levels were induced more strongly than CpxR and DegP upon induction by alkaline pH, consistent with fusion analysis (data not shown). These data suggest that cpxP is the most inducible member of the Cpx regulon.

(ii) The CpxP promoter has high affinity for CpxR–P. CpxR is a member of the OmpR family of winged helix-turn-helix transcription factors. Consequently, OmpR and CpxR are highly homologous response regulators. On the basis of analogy to OmpR, where Asp at position 55 is the putative site of
phosphorylation and Val at position 203 is in the putative site of phosphorylation (Asp at position 51 in CpxR) and in the recognition helix (Met at position 199 in CpxR) in CpxR. The cpxRD51A and cpxRM199A mutations were recombined into the chromosome at the cpxR locus by allelic exchange (see Materials and Methods). CpxRD51A and CpxRM199A mutant proteins are stable on the basis of Western blot analysis but are present at levels lower than that of wild-type CpxR (data not shown). All strains were grown as described in Materials and Methods.

Strains containing either the cpxRD51A mutation or the cpxRM199A mutation and Cpx-regulated lacZ transcriptional fusions were analyzed by using β-galactosidase assays to determine if the CpxR mutant proteins can activate transcription. Strains containing the cpxRD51A mutation behaved like cpxR null strains with all of the Cpx-regulated lacZ fusions tested (data not shown), which, on the basis of analogy with OmpR studies, suggests that phosphorylation at the Asp residue at position 51 is required for transcriptional activation by CpxR. Interestingly, strains containing the cpxRM199A mutation behave like cpxR null strains with respect to all of the Cpx-regulated lacZ fusions tested except for the cpxP-lacZ fusion, where it retains ~10% of wild-type activity (data not shown). On the basis of analogy with OmpR studies, this suggests that the CpxRM199A mutant protein retains some ability to bind DNA. Moreover, only the cpxP promoter is inducible by alkaline pH in a cpxRM199A-containing strain (compare Fig. 6A and B). Because the mutant protein is leaky and retains some wild-type activity, this suggests that the cpxP promoter has greater affinity for CpxR–P than the other promoters tested.

(iii) The spy and degP promoters are “differentially upregulated.” Analysis of Cpx-regulated lacZ transcriptional fusions in the presence and absence of CpxR showed that transcription of cpxP is almost entirely dependent on CpxR–P, while the other regulon members tested showed between 50 and 70% of the wild-type level of transcription in cpxR null strains. Transcription in the absence of CpxR could suggest either that the cpxR, ppiA, yihE-dsbA, spy, and degP promoters have high levels of basal transcription or that these target genes are also regulated by other signal transduction pathways. We reasoned that genes regulated solely by Cpx would be induced to a comparable degree regardless of the envelope stress tested. On the other hand, target genes that are upregulated to different degrees in response to different stresses must have multiple regulatory inputs. We refer to this idea as differential upregulation.

The Cpx and σE pathways are overlapping, but functionally distinct, envelope stress response pathways, and degP transcription is regulated by both (6, 30). Therefore, we would expect that stresses that induce both pathways would upregulate the degP promoter to a greater degree than stresses that induce the Cpx pathway only. That is, the degP promoter should exhibit differential upregulation. Indeed, the degP promoter was strongly upregulated by both PapG expression (8-fold) and lipid II accumulation (14- to 17-fold) but only moderately induced by the other stresses tested, such as increased pH (2-fold [Fig. 5]). PapG expression and lipid II accumulation have been shown to upregulate both the Cpx and σE pathways (4, 20); pH induces only the Cpx pathway.

We found that the spy promoter was strongly upregulated by expression of both PapG (~14-fold) and PapE (~8-fold) but only moderately induced by the other stresses tested, such as lipid II accumulation (2-fold) and alkaline pH (2-fold [Fig. 5]). Because the induction profile of spy varies depending on the stress tested, we suggest that, like degP, spy is differentially upregulated. Moreover, the profile of spy differential upregulation is distinct from that of degP and it has been shown that spy is not regulated by the σE pathway (31). Thus, spy is likely to be coregulated by a third stress-responsive system.

DISCUSSION

The Cpx pathway can sense inducing cues that enter the signaling pathway at three distinct points. A large number of seemingly distinct stresses upregulate the Cpx pathway. Because components of the Cpx pathway reside in all four cellular compartments, numerous entry points into the signaling pathway could exist (Fig. 7). However, in this report, we have shown that a variety of envelope stresses induce the Cpx pathway in a manner that is dependent on the periplasmic domain of CpxA but not NlpE. That is, a variety of stresses upregulate the Cpx pathway by entering the signaling cascade at a common point, downstream of NlpE, at CpxA.

It is possible that CpxA senses each stress differently or that all of the envelope stresses tested present the same signal to the periplasmic domain of CpxA. Given that the envelope stresses known to induce Cpx have seemingly disparate origins and effects, what does CpxA sense?

Expression of misfolded PapG and PapE pilin subunits have previously been shown to upregulate the Cpx pathway (20),...
both adhesion and envelope stress. Growth phase induction is clearly distinct from not signal blind. Although the precise nature of this induction does not require CpxP since the than 3-fold (29). The induction of Cpx over the growth curve 8- to 15-fold, adhesion induces /H11011 ever, unlike envelope stresses, which induce disparate stresses lead to a common periplasmic inducing signal, misfolded envelope proteins, sensed by the Cpx pathway. While all of the stresses tested in this study lead to a common periplasmic signal, adhesion and growth phase induction are distinct from envelope stress. Adhesion enters the Cpx pathway upstream of NlpE. Like envelope stress, adhesion does not require CpxP for induction of the Cpx pathway. However, unlike envelope stresses, which induce cpxP transcription ~8- to 15-fold, adhesion induces cpxP transcription no more than 3-fold (29). The induction of Cpx over the growth curve does not require CpxA since the cpxA242 allele is inducible and not signal blind. Although the precise nature of this induction is unknown, growth phase induction is clearly distinct from both adhesion and envelope stress. CpxP may act as a periplasmic chaperone. Our findings that CpxP is greatly induced under conditions of envelope stress and that the activity of CpxP as a negative regulator is inactivated under these conditions strongly suggests that CpxP not only functions as a negative regulator but also performs a second function in the cell. We propose that CpxP may function as a chaperone.

Negative autoregulation by chaperones is a common theme in stress-responsive systems. The heat shock response, which is positively regulated by the alternative sigma factor σE, is negatively autoregulated by regulon members DnaJ, DnaK, and GrpE (41, 42, 43). DnaK, DnaJ, and GrpE function as a "chaperone machine" (17), sequestering σE from core RNA polymerase following clearance of stress (22).

The σE stress response, which is thought to monitor outer membrane β-barrel proteins (19), is negatively autoregulated by regulon members RseA and RseB (11, 27). RseA acts as an anti-sigma factor by complexing with σE and sequestering it from core RNA polymerase. RseB is a periplasmic protein that negatively regulates the pathway by increasing the stability of the σE · RseA complex (2). In strains lacking RseB, the σE regulon is induced approximately twofold, is not completely derepressed (11), and still shows wild-type induction. In this respect, RseB is similar to CpxP. Moreover, RseB has been shown to bind aggregated periplasmic proteins, so it is thought that upon binding misfolded proteins, RseB can no longer interact with the σE · RseA complex, leading to the release and activation of σE (2). Analogously, it is possible that CpxP functions as the primary chaperone for the Cpx pathway and is thus greatly induced by misfolded periplasmic proteins. Misfolded proteins could titrate CpxP from CpxA, allowing for activation of the Cpx pathway.

Another possibility, which is not mutually exclusive with CpxP acting as a chaperone, is that CpxP is involved in shutting off the stress response pathway following the removal of stress. Perhaps the high induction of CpxP results in a periplasmic pool of CpxP that allows for rapid shutoff of the Cpx pathway following stress clearance. Experiments to further distinguish between these possibilities are under way.

CpxP is not the only negative regulator of the Cpx pathway. In this report, we showed that overexpression of MBP-CpxP (both periplasmic and tethered) is not sufficient to prevent induction of the Cpx pathway under stress conditions. Moreover, induction of the Cpx pathway is comparable in the presence or absence of CpxP. Therefore, CpxP is inactivated during envelope stress, potentially by the signal itself (misfolded envelope proteins).

It was previously shown that spheroplasting induces the Cpx pathway (31). While overexpression of periplasmic MBP-CpxP was insufficient to prevent induction of the Cpx pathway during spheroplasting, tethered MBP-CpxPA24D prevented induction of the pathway (31). Because spheroplasting does not inactivate tethered MBP-CpxPA24D, we suggest that spheroplasting is not an envelope stress and consequently does not result in a stress signal. In genetic studies presented here, we showed that losing CpxP is not sufficient for derepression of the Cpx pathway. This suggests that CpxP cannot be the only negative regulator of the pathway. We propose that induction by spheroplasting results from the loss of CpxP and another, unidentified, periplasmic regulator(s) of the Cpx pathway. This implies that both CpxP and the negative regulator(s) potentially function as sensors for the Cpx pathway.

Moreover, the negative regulators can function independently. For example, during spheroplasting, tethered MBP-CpxP is sufficient for repression (31). Yet, in the absence of CpxP, the Cpx pathway is not fully derepressed, suggesting that
another negative regulator(s) keeps the pathway off in the absence of CpxP.

Given that deletions in the periplasmic sensing domain of CpxA activate the Cpx pathway (34), it is also possible that the periplasmic domain of CpxA is the second negative regulator. This would require that spheroplasting affects the integrity of the periplasmic domain of CpxA and that the presence of the tethered MBP-CpxP protein protects this domain.

There are at least three systems that sense and respond to envelope stress. We have shown that CpxP is solely controlled by the Cpx pathway, while other target genes are subject to multiple regulatory inputs. It was previously shown that degP is regulated by both the σE and Cpx pathways. We found that spy is differentially upregulated by expression of both PapE and PapG, suggesting that spy transcription is induced by yet another stress response pathway that overlaps both the Cpx and σE pathways and responds to misfolded periplasmic proteins. It has been suggested that spy transcription is only partially dependent on the Cpx pathway (31), and it was previously shown that spy is induced by the oxidant indole (16). It seems, therefore, that spy transcription is dependent on another stress response pathway that is induced by indole, PapE and PapG expression.

ppiA, cpxRA, and yihE-dshA were only moderately induced by all of the stresses tested; these data are consistent with two possibilities. It is possible that these promoters, like cpxP, are solely regulated by Cpx; the fold induction of transcription at these promoters is constant regardless of the stress tested. This suggests that these promoters have a high level of CpxR-independent basal transcription. Another possibility is that these promoters are regulated by other, as yet unknown, regulatory pathways, the inducing cues of which are also unknown.

This method of transcriptional analysis can be used to better define overlap between regulatory pathways at both the input (identification of inducing cues) and output levels. Such experiments could assign inducing cues to multiple regulons. For example, NlpE overexpression and cpxRA* alleles are known to specifically upregulate the Cpx pathway. Because NlpE overexpression, cpxRA* alleles, and alkaline pH lead to the same profile of target gene upregulation and because alkaline pH does not induce degP or spy transcription differentially, this suggests that alkaline pH does not induce the σB or the other regulatory pathway that induces spy. This method can also be used to place target genes into multiple regulatory pathways, as in the case of spy transcription, and can uncover the existence of regulatory pathways but cannot provide clues as to identity.

Following the submission of the manuscript, Raffa et al. (30a) showed that spy is coregulated by a second stress-responsive system, BaeSR, in response to misfolded PapG, consistent with our predictions.

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REFERENCES


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