The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP

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DegP is a heat-shock inducible periplasmic protease in *Escherichia coli*. Unlike the cytoplasmic heat shock proteins, DegP is not transcriptionally regulated by the classical heat shock regulon coordinated by σ^32_. Rather, the degP gene is transcriptionally regulated by an alternate heat shock σ factor, σ^E_. Previous studies have demonstrated a signal transduction pathway that monitors the amount of outer-membrane proteins in the bacterial envelope and modulates degP levels in response to this extracytoplasmic parameter. To analyze the transcriptional regulation of degP, we examined mutations that altered transcription of a degP-lacZ operon fusion. Gain-of-function mutations in cpxA, which specifies a two-component sensor protein, stimulate transcription from degP. Defined null mutations in cpxA or the gene encoding its cognate response regulator, cpxR, decrease transcription from degP. These null mutations also prevent transcriptional induction of degP in response to overexpression of a gene specifying an envelope lipoprotein. Cpx-mediated transcription of degP is partially dependent on the activity of Eσ^E, suggesting that the Cpx pathway functions in concert with Eσ^E and perhaps other RNA polymerases to drive transcription of degP.

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and, hence, DegP synthesis. Conversely, mutations that decrease the number of proteins found in the outer membrane concomitantly decrease EoE activity. Thus, oE responds to a general extracytoplasmic parameter, just as o32 responds to general conditions known to damage cytoplasmic proteins [Bukau 1993].

If oE does serve a complementary function to that of o32, it is likely that EoE directs transcription of various envelope stress proteins in response to alterations in the physiology of the bacterial envelope. To examine this intercompartmental communication, we analyzed mutations that affected transcription of degP. Using a degP-lacZ operon fusion we have identified a two-component regulatory system [Stock et al. 1990] that is involved in transcriptional regulation of degP.

Results

The degP-lacZ operon fusion accurately reflects degP transcription

A degP-lacZ operon fusion was constructed to study the transcriptional regulation of the degP locus in E. coli. The fusion was recombined onto a λ phage and was placed in single copy at the attB locus on the E. coli chromosome [see Materials and methods for details]. Like the wild-type degP locus, transcription from this fusion is altered by genetic backgrounds that alter the activity of EoE, the RNA polymerase responsible for transcriptional initiation at the degP locus [Erickson and Gross 1989; Wang and Kaguni 1989]. EoE activity is modulated in response to the synthesis of outer-membrane proteins. For example, high-level synthesis of outer-membrane proteins such as OmpF or OmpC increases EoE activity. Conversely, mutations that decrease the number of proteins found in the outer membrane decrease EoE activity [Mecsas et al. 1993]. Figure 1 shows that high-level synthesis of the outer-membrane proteins OmpF or OmpC stimulates transcription of the degP-lacZ fusion. In contrast, the ompR::Tnl0 mutation, which decreases EoE activity by eliminating expression of ompF and ompC [Slauch et al. 1988], decreases transcription from the degP-lacZ fusion (Fig. 1). Thus, the degP-lacZ fusion is an accurate reporter of transcription at the degP locus.

Mutations in cpxA stimulate transcription of degP

To identify genes involved in transcriptional regulation of degP, we sought mutations that altered transcription of the degP-lacZ fusion. Our first candidate for such a gene arose from concurrent studies of two novel envelope proteins in our laboratory. The LamB–LacZ–PhoA fusion protein [Snyder and Silhavy 1995] and the signal-sequence processing-defective maltoporin, LamBA23D [Carlson and Silhavy 1993], are both targeted to the bacterial envelope and both damage the envelope when their genes are highly expressed. Such high-level synthesis of LamB–LacZ–PhoA causes cell lysis because of an undefined perturbation in the bacterial envelope [Snyder and Silhavy 1995]. LamBA23D contains a mutation that renders signal-sequence cleavage of this protein inefficient.

High-level synthesis of this protein confers hypersensitivity to detergents, suggesting that LamBA23D perturbs the integrity of the outer membrane [Carlson and Silhavy 1993].

Independent attempts to identify extragenic suppressor mutations of the lethality conferred by either LamB-LacZ-PhoA or LamBA23D yielded gain-of-function mutations in a previously identified locus, cpxA [W.B. Snyder, C.L. Cosma, and T.I. Silhavy, unpubl.]. cpxA specifies an inner-membrane sensor homolog in the two-component family of regulatory proteins [Weber and Silverman 1988]. cpxR, the gene specifying the putative cognate response regulator of CpxA, lies immediately upstream of the cpxA locus and shares homology with other two-component response regulators [Dong et al. 1993].

Based on homology between cpxA and this family of regulatory proteins, it seemed likely that the cpxA suppressor mutations played a regulatory function in relieving the toxicity conferred by LamB–LacZ–PhoA and LamBA23D. Biochemical analysis has shown that the cpxA suppressors enhance the rate of proteolysis of...
LamB–LacZ–PhoA (W.B. Snyder and T.J. Silhavy, unpubl.). This result prompted us to examine the effects of the cpxA suppressor mutations on transcription from our degP–lacZ fusion. Each cpxA suppressor allele was transduced into the degP–lacZ fusion strain and the resulting β-galactosidase activity was determined. Figure 2 shows that the cpxA suppressor alleles increase transcription from the degP–lacZ fusion from 3- to 10-fold.

It is interesting to note that the cpxA24, cpxA17, and cpxA41 alleles were originally characterized as stronger suppressors of the lethality conferred by LamB–LacZ–PhoA than cpxA723, cpxA741, and cpxA744 (W.B. Snyder and T.J. Silhavy, unpubl.). Figure 2 shows that cpxA24, cpxA17, and cpxA41 also increase transcription from degP to a higher level than cpxA723, cpxA741, and cpxA744. Thus, the cpxA alleles that stimulate transcription of degP most strongly are also the strongest cpxA suppressor alleles.

Analysis of cpxA and cpxR null mutations

Previous work has demonstrated that degP transcription is modulated in response to the amount of outer-membrane proteins localized to the bacterial envelope (Mecas et al. 1993). This effect is mediated by modulating the activity of ErR, and presently the mechanism of this modulation is unknown. Because CpxA is an inner-membrane sensor homolog and because the cpxA suppressors stimulate degP transcription, it seemed possible that CpxA and CpxR were responsible for sensing the extra-cytoplasmic parameter that influences ErR activity. If the Cpx proteins were the sole sensory circuit for modulating ErR activity, cpx null strains should no longer alter ErR activity in response to fluctuations in the number of outer-membrane proteins found in the bacterial envelope.

cpxA and cpxR were each inactivated by insertion of antibiotic-resistance cassettes within their respective open reading frames (see Materials and methods). The insertion within cpxR is polar and strains containing this insertion are cpxR– and cpxA– (see Materials and methods for details). When introduced into the degP–lacZ fusion strain, the cpxA and cpxR null alleles both conferred a decrease in transcription from this fusion (e.g., Fig. 3a, cf. lanes 1, 3, and 5). However, the cpx null mutations were not epistatic to transcriptional induction of the degP–lacZ fusion by any factors known to alter ErR activity. Specifically, overexpression of ompF or ompC still caused the same magnitude increase in transcription from the degP–lacZ fusion with or without functional Cpx proteins [Fig. 3a,b]. Also, the ompR::Tn10 mutation decreases degP transcription in the cpxA– and cpxA– R– strains [Fig. 3c]. Thus, cpxA is not required for modulating ErR activity under these circumstances.

Acetyl-phosphate can stimulate degP transcription through CpxR

Most two-component sensors are responsible for phosphorylating and dephosphorylating their cognate response regulators to elicit appropriate internal responses to a particular external input. The phosphorylated species of the response regulator is typically the species that actively elicits the internal responses (Stock et al. 1990, Parkinson 1993). Many response regulators can also be phosphorylated by the low-molecular-weight compound, acetyl-phosphate [Ac-P] (Lukat et al. 1992, McCleary et al. 1993). In wild-type cells, the effect of phosphorylation of response-regulators by Ac-P is negligible. However, when a given sensor molecule is lost to mutation, the effect of phosphorylation of the cognate response regulator by Ac–P can become significant. With the sensor molecule rendered nonfunctional, there is no phosphatase activity to counteract the phosphorylation of the response regulator by Ac–P (McCleary et al. 1993). Because Ac–P can be synthesized from acetyl-CoA and P₃ (McCleary et al. 1993), the intracellular concentration of Ac–P rises with the use of the glycolytic pathway. Hence, carbon sources utilized in glycolysis increase the intracellular concentration of Ac–P and this ultimately leads to an increase in the phosphorylation of certain "orphaned" response regulators.

Our analysis of the cpxA and cpxR null mutations indicates that CpxR can be phosphorylated by Ac–P under certain conditions. When grown in Luria broth, the cpxA– strain displays a decrease in degP transcription compared with an isogenic cpxA+ strain [Fig. 3a–c, cf. lanes 1 and 3]. However, when cpxA+ and cpxA– strains are grown in the presence of carbon sources such as D-glucose, maltose, or lactose, the cpxA– strain displays

**Figure 2.** cpxA suppressor mutations activate transcription of degP–lacZ. β-Galactosidase activities of PND2000 [MC4100, λRS8(λdegP–lacZ)] [lane 1] and PND2000 containing the various mutant cpxA alleles indicated [lanes 2–9] were assayed. The mutant cpxA alleles stimulated transcription of degP–lacZ ~3- to 10-fold over that of wild type. Strains were grown in Luria broth as described in Materials and methods.
Figure 3. The Cpx proteins do not modulate degP-lacZ transcription in response to outer-membrane protein levels. (a) (Lanes 1,3,5) β-Galactosidase levels of strains transformed with pLG339 (control for pPR272); (lanes 2,4,6) β-galactosidase levels of strains transformed with pPR272 (overexpresses ompF). (b) (Lanes 1,3,5) β-galactosidase levels of strains transformed with pRAM1005 (control for pRAM1006); (lanes 2,4,6) β-galactosidase levels of strains transformed with pRAM1006 (overexpresses ompC). (c) The Cpx proteins do not mediate the decrease in transcription from degP-lacZ conferred by the ompR::Tn10 mutation. (Lanes 1,3,5) ompR+; (lanes 2,4,6) ompR::Tn10. In a–c, lanes 1 and 2 display β-galactosidase levels of PND2000 [MC4100, AR588[degP-lacZ], cpxA+, cpxR+]; lanes 3 and 4 display β-galactosidase levels of PND242 [PND2000, cpxA::cam]; lanes 5 and 6 display β-galactosidase levels of PND325 [PND2000, cpxR::Tn10]. All strains were grown in Luria broth and the appropriate antibiotic for plasmid selection when necessary as described in Materials and methods.

A dramatic increase in degP transcription (Fig. 4, cf. lanes 1 and 2, data not shown). In contrast, the cpxR−A− double mutant does not alter degP transcription when grown in these same carbon sources (Fig. 4, cf. lanes 2 and 3), indicating that the transcriptional induction of degP observed in the absence of CpxA proceeds through CpxR via another factor. This other factor is Ac−P. Deletion of pta and ackA, the two genes responsible for Ac−P synthesis (McCleary et al. 1993), is epistatic to the effect of glucose on degP transcription in a cpxA− background (Fig. 4, cf. lanes 2 and 4). These results further imply that CpxR-phosphate (CpxR-P) can direct transcriptional induction of degP and also provide indirect evidence that CpxA can dephosphorylate CpxR-P.

Figure 4. Ac−P mediates transcriptional induction of degP−lacZ by phosphorylating CpxR in the absence of CpxA. (Lane 1) PND495 [PND2000, zei::Tn10]; (lane 2) PND422 [PND2000, cpxA::cam, zei::Tn10]; (lane 3) PND496 [PND2000, cpxR::Ω, zei::Tn10]; (lane 4) PND421 [PND2000, cpxA::cam, zei::Tn10 Δ[pta, ackA, hisQ, hisP]]. Strains were grown in M63 minimal media with 0.4% glucose as a carbon source (see Materials and methods for details).

The Cpx pathway mediates transcriptional induction of degP in response to high-level synthesis of an envelope lipoprotein

The observed induction of degP transcription by Ac−P in a cpxA− background is clearly not a reflection of wild-type regulation. From the data presented thus far, several models can explain the Cpx-mediated transcriptional regulation of degP. For example, it is possible that the Cpx proteins are normally involved in transcriptional induction of degP in response to an unknown extracytoplasmic parameter. If so, the observed induction of degP synthesis by Ac−P or cpxA mutations has simply highlighted a normal function of the Cpx pathway by perturbing this pathway. It is also possible that the Cpx proteins normally regulate the synthesis of an outer-membrane protein which, when overproduced by activation of the Cpx pathway, increases E~r E activity, ultimately stimulating transcription of degP. Alternatively, the induction of degP transcription by Ac−P in the cpxA null strain or by the cpxA mutations could reflect situations whereby a hyperactivated Cpx pathway would al-

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After the transcription of genes outside of its normal regulon. Evidence presented below favors the first model.

In an independent attempt to identify high-copy suppressors of the lethality conferred by LamB-LacZ-PhoA, a new envelope lipoprotein, NlpE, was identified (W.B. Snyder, L.J.B. Davis, and T.J. Silhavy, unpubl.). High-level synthesis of NlpE increases transcription of degP, and (2) the observed transcriptional induction of degP itself is unaffected by the cpx null mutations [data not shown]. The Cpx-dependent transcriptional induction of degP highlights two points: (1) The wild-type Cpx proteins can activate degP transcription in response to an extracytoplasmic stimulus, and (2) the observed transcriptional induction of degP by Ac~P and the cpxA suppressor mutations likely reflects a normal function of the Cpx pathway even though the observed induction occurs by perturbing this pathway.

The Cpx pathway is not a general monitor of lipoprotein synthesis

Because overproduction of NlpE activates degP transcription via the Cpx pathway, it seemed possible that the Cpx proteins were responsible for modulating degP transcription in response to the high-level synthesis of lipoproteins in general. In this case, the Cpx pathway would alter degP transcription in response to the level of lipoproteins, just as degP transcription is modulated in response to the levels of outer-membrane proteins (Mecsa et al. 1993). However, high-level synthesis of a variety of lipoproteins, including OsmB (Jung et al. 1989), Lpp (Nakamura et al. 1982), Pal (Chen and Henning 1987), lipoprotein-28 (Yu et al. 1986), NlpD (Ichikawa et al. 1994), and TraT (Ogata et al. 1982), does not induce transcription of degP-lacZ [data not shown]. Thus, the Cpx pathway does not appear to monitor lipoprotein synthesis in general, implying that the enhanced transcription observed by overexpression of nlpE is related to the actual function of NlpE.

The Cpx pathway activates transcription from degP but not transcription from a minimal rpoH 

Figure 5. Overexpression of nlpE induces transcription of degP-lacZ through the Cpx signal transduction pathway. (Lanes 1,3,5) β-Galactosidase activity of strains transformed with pBAD18 [control for pND18]; (lanes 2,4,6) β-galactosidase activity of strains transformed with pND18 [overexpresses nlpE]. (Lanes 1,2) PND497 [PND2000, zej::Tn10 Δpta, ackA, hisQ, hisP, ara- ]; (lanes 3,4) PND498 [PND497, cpxA::cam]; (lanes 5,6) PND499 [PND497, cpxR::Sii]. All strains were grown in Luria broth containing 0.4% L-arabinose and 50 μg/ml of ampicillin [see Materials and methods for details]. The experiments in Figure 5 were performed with strains deleted for pta and ackA. Because NlpE synthesis is driven from the araB promoter (Guzman et al. 1992) in this experiment, full transcriptional induction requires growth in arabinose. Hence, Ac~P synthesis must be eliminated to prevent hyperphosphorylation of CpxR in the cpxR-A- background.

Presently, there are only two promoters in E. coli that are known to be utilized by EσE: the degP promoter and the P3 promoter of rpoH (Lipinska et al. 1988; Erickson and Gross 1989; Wang and Kaguni 1989).Using a rpoH- lacZ operon fusion, Mecsa et al. (1993) have shown that the minimal P3 promoter is regulated by EσE in a fashion analogous to that seen with the degP promoter.

We wished to determine whether activation of the Cpx pathway directly altered EσE levels. If this were true, activation of the Cpx pathway would affect transcription of the rpoH-rpoH-lacZ fusion in a fashion similar to that observed with degP. However, activation of the Cpx pathway had no effect on transcription from the rpoH-rpoH-lacZ operon fusion. Figure 6a shows that in a cpxA- background, carbon sources such as glucose do not affect transcription from the rpoH-rpoH promoter (cf. lanes 1 and 2). This is in contrast to the 10-fold stimulation of degP transcription observed under the same conditions [see Fig. 4]. Also, whereas overexpression of ompX or ompC stimulates transcription from both rpoH-rpoH and degP [Fig. 6b, lanes 2,4,6; Mecsa et al. 1993], overexpression of nlpE has no effect on rpoH-rpoH transcription [Fig. 6b, lane 6]. These results reveal a new layer in the regulation of degP transcription. The Cpx pathway does not directly alter σE levels. Rather, this pathway affects degP transcription by working in parallel with EσE.

Activation of degP transcription by the Cpx pathway is dependent on EσE activity

The fact that activation of the Cpx pathway stimulated transcription of degP and not of rpoH-rpoH-lacZ suggested
that the Cpx pathway functions specifically at the degP promoter, perhaps working in concert with ErE to activate degP transcription. If this were true, activation of degP transcription by the Cpx pathway would be dependent on a functional copy of the rpoE gene, which encodes ErE (Raina et al. 1995; Rouviere et al. 1995). Figure 7 indicates that activation of degP transcription by the Cpx pathway is partially dependent on the activity of ErE. Inactivation of rpoE decreases the transcriptional induction of degP by overexpression of nlpE (Fig. 7, cf. lanes 2 and 4). The results illustrated in Figure 7 suggest that the Cpx pathway can function in concert with ErE to induce degP transcription. However, the rpoE null mutation is not completely epistatic to the transcriptional induction of degP by activation of the Cpx pathway. This indicates that the Cpx pathway can function in concert with at least one other RNA polymerase to drive degP transcription.

It should be noted that the experiments performed to generate the data illustrated in Figure 7 utilized the degP-lacZ fusion in the MC1061 (Silhavy et al. 1984) strain background. This was necessitated because of the instability of the rpoE:cam mutation in the MC4100 background [P.N. Danese, unpubl.]. Importantly, this change in strain background is not problematic, as it does not affect the observed transcriptional regulation of degP in response to activation of the Cpx pathway (cf. lanes 1 and 2 in Fig. 7 with lanes 1 and 2 in Fig. 5).

Because the transcriptional induction of degP observed by activation of the Cpx pathway was not entirely dependent on ErE, we were interested in determining the transcriptional initiation sites for the Cpx-mediated induction. S1 nuclease mapping of the transcription start sites of degP was performed with RNA prepared from rpoE+ strains that contained either an nlpE-overexpressing plasmid [pND18] or a control plasmid [pBAD18]. Figure 8 shows the transcripts induced by overexpression of nlpE. These transcripts begin between nucleotides 147 and 165 of the published degP sequence (Lipinska et al. 1988). Figure 8a shows that transcription initiated at position 159, which is attributed to ErE (Lipinska et al. 1988; Erickson and Gross 1989), can be induced by the Cpx pathway. This is consistent with the results presented in Figure 7, indicating that the Cpx pathway can function in concert with ErE to activate degP transcription. Figure 8a also shows the induction of other transcripts that initiate near position 159. Presently, we do not know which (if any) of these other transcripts is also a product of ErE-directed transcription. However, we note that activation of the Cpx pathway generates novel protected fragments initiating at positions 147 and 148 which are not present in the uninduced strain (Fig. 8a, cf. lanes 1 and 2). These fragments may represent the source of the residual transcriptional induction of degP by the Cpx pathway in the absence of ErE.

Discussion

The molecular nature of the pleiotropic cpxA mutations

cpxA was first identified by mutations that prevented efficient transfer of F plasmids from donor to recipient strains (conjugative plasmid expression) (McEwen and Silverman 1980a). Subsequent analyses have shown that these alleles, as well as the cpxA suppressor mutations identified in our laboratory (see Figure 2), are highly pleiotropic. For example, these cpxA mutations confer
Cpx regulates \( \text{degP} \) transcription

The E. coli osmosensor EnvZ can be altered by mutation to a phosphatase + kinase + species, and this mutant EnvZ hyperphosphorylates its cognate response regulator, OmpR, ultimately altering transcription from its normal downstream targets, \( \text{ompF} \) and \( \text{ompC} \), as well as other loci (Slauch et al. 1988).

This model also clarifies another observation in the cpx literature. Many of the previous phenotypic characterizations of the pleiotropic cpxA mutations noted that a large chromosomal deletion encompassing cpxA and spanning from \( \text{rha} \) to \( \text{pfkA} \) reverted the pleiotropic phenotypes (Dong et al. 1993). However, the precise meaning of the results obtained with this deletion is confounded by its sheer size. The deletion removes several genes and at least 10 kb of DNA (Miller 1992). From our analysis, it is clear that deletion of both cpxA and cpxR prevents activation of the Cpx pathway altogether.

**The function of the Cpx proteins**

Whereas there are a variety of phenotypes conferred by many (but not necessarily all) of the following phenotypes: decreased stability of inner- and outer-membrane proteins, including Lpp and OmpF; low-level resistance to aminoglycosides; impairment of ion-driven transport systems such as those for lactose and proline; inability to grow on nonfermentable carbon sources such as succinate; the ability to utilize L-serine as a carbon source; and isoleucine/valine auxotrophy (McEwen and Silverman 1980b, 1982; Rainwater and Silverman 1990; Dong et al. 1993).

Our analysis indicates that the pleiotropic cpxA mutations are not null mutations. Whereas the pleiotropic cpxA suppressors increase \( \text{degP} \) transcription, a defined cpxA null mutation actually decreases \( \text{degP} \) transcription in the absence of cross-phosphorylation by Ac~P. Moreover, the cpxA null also unveils the basis for the observed pleiotropy of the cpxA missense mutations. These mutant CpxA proteins phosphorylate CpxR in an unregulated manner and ultimately cause CpxR-P to accumulate to high levels. Under these conditions, transcription from genes normally regulated by CpxR is altered and the regulation of genes not normally found in the cpx regulon is affected as well. Thus, the pleiotropic mutations probably disrupt the phosphatase activity or enhance the kinase activity of CpxA. This is not unpre-

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**Figure 7.** Cpx-mediated stimulation of \( \text{degP} \) transcription is partially dependent on the activity of Er5. (Lanes 1,3) \( \beta \)-Galactosidase activity of strains transformed with pBAD18 [control for pND18]; [lanes 2,4] \( \beta \)-galactosidase activity of strains transformed with pND18 [overexpresses nlpE]. [Lanes 1,2] PND819 [MC1061 ARS88[\text{degP}--\text{lacZ}]]; [lanes 3,4] PND818 [MC1061, ARS88[\text{degP}--\text{lacZ}] rpoE::cam]. All strains were grown at 30°C in Luria broth containing 0.4% L-arabinose and 50 µg/ml of ampicillin (see Materials and methods for details). Although the standard deviation for the \( \beta \)-galactosidase activity shown in lane 4 is relatively large, Student’s t-test for comparison of two means indicates that the difference between the values shown in lanes 3 and 4 is statistically significant at a confidence level >0.99 [Harris 1987].

**Figure 8.** Activation of the Cpx pathway stimulates \( \text{degP} \) transcription at the \( \sigma^5 \) promoter and nearby sites. (a) Lanes 1 and 2 show the \( \text{degP} \) transcription start sites for strain PND2000 ara + transformed with either a control plasmid, pBAD18 (lane 1), or the nlpE-overexpressing plasmid, pND18 (lane 2). The undigested probe (340 nucleotides in length) is shown. The DNA fragments corresponding to transcripts that initiate at positions 147, 148, 156, 159, and 165 of the \( \text{degP} \) sequence (Lipinska et al. 1988) are indicated at right. RNA was prepared from strains grown at 30°C in Luria broth containing 0.4% L-arabinose and 50 µg/ml of ampicillin. (see Materials and methods for details). (b) The transcription start sites are designated with asterisks (*).

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the pleiotropic cpxA mutations (see above), it is unclear whether these phenotypes reflect a normal function of the Cpx proteins or whether they are attributable to aberrant properties of the hyperactivated Cpx pathway. For example, Silverman has examined the effects of cpxA mutations on F plasmid transmission. These studies have shown that the pleiotropic cpxA mutations decrease the steady-state levels of the cytoplasmic TraJ protein (Silverman et al. 1993), providing an explanation for the inefficient transfer. However, deletion of the cpx genes reverts the F plasmid transfer phenotypes to a wild-type state (Silverman et al. 1993), suggesting that the effects observed on F plasmid transfer may reflect aberrant properties of the hyperactivated Cpx pathway.

In contrast, the data presented here suggest that the Cpx proteins are normally involved in regulating degP transcription. First, the cpxR-A- double mutant decreases degP transcription twofold, indicating that the Cpx proteins normally contribute to degP transcription in a wild-type cell. Second, activation of the Cpx pathway (either by Ac-P or by mutation of cpxA) increases degP transcription 3- to 10-fold. Third, an extracytoplasmic stimulus (nlpE overexpression) activates degP transcription through the wild-type Cpx proteins, arguing that transcriptional induction of degP is a normal function of the Cpx pathway.

Although the precise molecular basis for many of the other phenotypes conferred by the pleiotropic cpxA mutations remains to be determined, at least a subset of these phenotypes may be attributable to the activation of degP transcription. For example, Silverman and colleagues noted that both inner- and outer-membrane proteins, including Lpp and OmpF, were destabilized in a cpxA2 background (McEwen and Silverman 1982; McEwen et al. 1983). It seems likely that this phenotype is attributable to activation of degP transcription. Other phenotypes such as low-level resistance to aminoglycosides and impairment of ion-driven transport systems also originate from alterations in the physiology of the bacterial envelope (Rainwater and Silverman 1990). It is possible that these phenotypes are also attributable to the destabilization of certain envelope proteins by increased levels of DegP.

**The Cpx regulon**

The Cpx pathway stimulates transcription at sites upstream of the degP open reading frame, including the site utilized by EoE (Figs. 7 and 8). The simplest explanation for this result is that CpxR binds to a site upstream of the degP open reading frame and works in concert with EoE and perhaps other RNA polymerases to drive degP transcription (Fig. 9). CpxR shares homology with other two-component DNA-binding proteins, including OmpR and ArcA (Dong et al. 1993). There are >100 nucleotides upstream of the -35 site of degP in our degP-lacZ operon fusion which could support binding of CpxR. However, direct biochemical analysis is required to test this model.

Whereas activation of the Cpx pathway does not increase transcription from rpoHv3-lacZ, it is still possible that the Cpx proteins might influence transcription from rpoHP3 at its wild-type chromosomal locus. In the rpoHz3-lacZ fusion, only the minimal promoter containing the -10 and -35 sites of rpoHP3 is used to drive transcription (Mecsas et al. 1993). There are no upstream sequences present in this fusion that could potentially support binding of transcription factors. Thus, we do not know whether the Cpx regulon stimulates transcription at other σE promoters or whether the Cpx and σE regulons simply intersect at degP. There is reason to believe that degP is only one of a group of genes regulated by the

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**Figure 9.** A model for the action of the Cpx signal transduction pathway on degP transcription. The CpxA inner-membrane sensor responds to the level of the NlpE lipoprotein present in the bacterial envelope. CpxA communicates this information to its cognate response regulator, CpxR, which in turn activates degP transcription by working in concert with EoE and perhaps other RNA polymerases. The activity of EoE is modulated by an unknown signal transduction system in response to the expression level of outer-membrane proteins.
Cpx pathway. Although the toxicity conferred by induction of either LamB–LacZ–PhoA or LamBA23D is suppressed by activation of the Cpx pathway, removal of a functional degP gene is only partially epistatic to the suppression, indicating that other gene products participate in the observed suppression (W.B. Snyder, C.L. Cosma, and T.J. Silhavy, unpubl.).

What does CpxA sense?

The Cpx proteins regulate degP transcription in response to the high-level synthesis of the envelope lipoprotein NlpE. However, increased levels of lipoproteins in general do not induce transcription of degP–lacZ, implying that the enhanced transcription of degP caused by overexpression of nlpE is probably related to the actual function of NlpE. Presently, this function is unknown, but we suggest that NlpE may be regulated in response to some physiological parameter associated with the outer membrane. Alteration of the levels and/or activity of NlpE would be communicated to the Cpx pathway to appropriately alter envelope physiology. We note that NlpE contains a serine protease inhibitor motif [W.B. Snyder, unpubl.]. If NlpE is a protease inhibitor, CpxA may sense the need for increased expression of degP and other gene products when the function of another envelope protease is inhibited.

Materials and methods

Media, reagents, and enzymes

Media were prepared as described by Silhavy et al. [1984]. Liquid cultures were grown either in Luria broth or M63 minimal medium supplemented with thiamine (50 μg/ml) and 0.4% of the indicated carbon source. The final concentration of antibiotics used in the growth medium was as follows: 50 μg/ml of ampicillin, 50 μg/ml of kanamycin, 20 μg/ml of tetracycline, 50 μg/ml of spectinomycin, 20 μg/ml of chloramphenicol. Standard microbiological techniques were used for strain construction and bacterial growth [Silhavy et al. 1984].

Strains

PND2000 [MC4100 ARS88[degP–lacZ]] is the parent of all strains containing the degP–lacZ operon fusion except for strains used to generate data illustrated in Figure 7. Strains described in Figure 7 contain the degP–lacZ fusion in strain MC1061. The use of the MC1061 background for experiments described in Figure 7 was necessitated because of the instability of the rpoE::cam mutation in the MC4100 background. Lysozyme resistant ARS88[degP–lacZ] was performed as described by Simons et al. [1987]. PND381 [MC4100 ARS45[spoH::degP–lacZ]] is the parent of all strains containing the rpoH::degP–lacZ operon fusion. PND381 was created by P1 transduction using CAG16037 (Mecsas et al. 1993) as a donor strain. All fusions were shown to be located in single copy at the λatt locus by P1 transduction. cxPA suppressor mutations were moved by P1 transduction, selecting for growth on minimal media with a metF− recipient strain. The presence of the cxPA suppressor mutations was verified by rescuing each allele by P1 transduction and demonstrating the suppressor function of each allele in strains carrying either lamB–lacZ–phoA or lamBA23D. The pta–ackA deletion was moved by a linked transposon [zes::Tn10], and the presence of the deletion was scored by assaying growth on minimal acetate media [Wanner and Wilmes-Resienberg 1992]. The cxpA–cam, cpxR::fl, ompR::Tnl0, and rpoE::cam mutations were moved by P1 transduction selecting for resistance to the appropriate antibiotic [cam] chloramphenicol, [fl] spectinomycin, [Tn10] tetracycline. The rpoE::cam insertion [Rouviere et al. 1995] was assayed further for the confirmation of temperature-sensitive growth at 42°C.

Plasmids and phage

ARS88 has been described elsewhere (Simons et al. 1987). The following kanamycin-resistant plasmids were used in this study; pPR272 (overexpressing ompF, Misra and Reeves 1987), pLG338 (parent of pPR272 and pPR2C, Stoker et al. 1982), pGP1-2 [T7 RNA polymerase for overexpression of nlpD, Ichikawa et al. 1994]; pPR2C [overexpresses the gene for peptidoglycan-associated lipoprotein (pall); Chen and Henning 1987]. The following ampicillin-resistant plasmids were used in this study: pRAM1005 [parent plasmid for pRAM1006, Misra and Benson 1988]; pRAM1006 [overexpresses ompC; Misra and Benson 1988]; plE100 (overexpresses ompX, Mecsas et al. 1993); pBR322 [parent of pE100]; pK110 [overexpresses nlpD, Ichikawa et al. 1994]; pCC242 [overexpresses osmB, Jung et al. 1989]; pPW3 [overexpresses traT, Ogata et al. 1982]; pFY108 [overexpresses the gene for lipoprotein-28, Yu et al. 1986]; pKEN125 [overexpresses lpp, Nakamura et al. 1982]; pND18 [overexpresses nlpE, this study]; pBAD18 [parent for pND18, Guzman et al. 1992]. The expression levels of the various lipoproteins are at least 10-fold greater than with plasmid-borne genes, spanning a range that exceeds (Lpp), is comparable to (NlpD, Lpp-28), or is less than (TraT, Pal, OsmB) the level of NlpE when it is overproduced (Nakamura et al. 1982, Ogata et al. 1982; Yu et al. 1986; Henning et al. 1987; Jung et al. 1989; Ichikawa et al. 1994).

pND18 was constructed as follows: nlpE was amplified from the chromosome of MC4100 by the polymerase chain reaction (PCR) using the following primers: NlpE5 [5′-TCAAGCGTCGACGCGCGGCAAAGTG-3′] and NlpE3 [5′-GATGCGGCGTAAAAGCTTTATCCGGCC-3′]. The amplified NlpE5, nlpE3, respectively, to facilitate subcloning. The amplified DNA was subcloned into the araB promoter (Guzman et al. 1992).

Construction of the degP–lacZ fusion

The degP–lacZ fusion was created by amplifying the chromosomal degP locus of MC4100 by PCR using the following primers: Htra5 [5′-GGCTGGGATGATTTCTCGAGCTGAGGG-3′] and Htrami3 [5′-CCATGTTACCCGGCGATACCAAC-3′]. EcoRI and SmaI restriction endonuclease sites were incorporated into NlpE and NlpE3, respectively, to facilitate subcloning. The amplified DNA was subcloned into the EcoRI and SmaI sites of pRS451 (Simons et al. 1987), creating pSINATRA131. The nucleotide sequence of the degP insert of pSINATRA131 was confirmed by dideoxy nucleotide sequencing. The cloned degP insert includes nucleotides from position –122 with respect to the degP transcription start site to position +845 with respect to this same site. The degP–lacZ fusion of pSINATRA131 was then recombinated onto phage ARS88 [Simons et al. 1987], and recombinants
were used to lysogenize MC4100 as described [Simons et al. 1987].

**Construction of the cpxA and cpxR null mutations**

The chromosomal cpxA locus was amplified by PCR using the following primers: Cpxkpn (5'-GTGAACGGTCAGGTT-TACCCCTG-3'), Cpxa3 (5'-CCGGAGTATTCGGCTGATAA-G-3'). KpnI and SphI restriction endonuclease sites were incorporated into Cpxkpn and Cpxa3, respectively, to facilitate subcloning. The amplified cpxA DNA was then subcloned into the KpnI and SphI sites of pAMPTs [G. Phillips, unpublished plasmid], creating pND8. The pAMPTs vector is temperature sensitive for replication. Strains grown in media selecting for this plasmid at the restrictive temperature (42°C) must integrate the plasmid into the chromosome to maintain viability. This temperature-sensitive replication provides a means for replacing chromosomal DNA with homologous plasmid-encoded DNA [Hamilton et al. 1989].

pND8 contains a unique EcoRI restriction site within the open reading frame of cpxA. This EcoRI site lies upstream of the codon for the conserved histidine residue of two-component sensors [Weber and Silverman 1988; Stock et al. 1990]. The chloramphenicol antibiotic resistance cassette described by Fellay et al. [1987] was inserted within the cpxA open reading frame at the unique EcoRI site, creating pND9. This insertion/disruption of cpxA was then recombined onto the chromosome of MC4100 as described in Hamilton et al. [1989].

The cpxR locus was inactivated in a similar fashion to cpxA. Using primers Cpxr5 (5'-GATATCCACCAGCGGATCCAC-CTG-3′) and Cpxr3 (5'-CGTGGGCGATCTCCTGGC-CAGGCTCCG-3′), cpxR was amplified from the MC4100 chromosome by PCR. To facilitate the subcloning of cpxR, BamHI restriction endonuclease sites were incorporated into primers Cpxr5 and Cpxr3. The amplified cpxR DNA was subcloned into the BamHI site of pAMPTs, creating pND10. pND10 contains a unique XhoI restriction site 20 codons downstream of the 5' end of the cpxR open reading frame. pND10 was digested with XhoI, the 3' overhangs were filled in with Klenow fragment [Sambrook et al. 1989], and the resulting fragment was ligated to the spectinomycin-resistance cassette described by Fellay et al. [1987]. This subcloning created pND11. This cpxR disruption was also recombined onto the chromosome as described [Hamilton et al. 1989]. The chromosomal disruptions of cpxR and cpxA were confirmed by Southern hybridization. The insertion within the cpxR open reading frame is polar and strains containing this insertion are cpxR+ and cpxA+. When a cpxA null strain is complemented with plasmid pND10 (containing cpxR), the strain behaves as a cpxA- strain in that degP-lacZ transcription is increased dramatically in the presence of carbon sources such as D-glucose (see Fig. 4 and Results). Acetyl-phosphate can stimulate degP transcription through CpxR.

**β-Galactosidase assays**

Cells were grown at 37°C overnight in Luria broth or M63 minimal medium supplemented with 0.4% of a given carbon source. Cells were then subcultured [1:40] into 2 ml of the same medium and grown to mid-log phase at 30°C or 37°C. β-Galactosidase activities were determined utilizing a microtiter plate assay [Slauch and Silhavy 1991] and are expressed as [U/ A₆₀₀]×10³, where units [U] equal micromoles of product formed per minute. A minimum of four independent assays were performed on each strain, and the results were averaged to obtain the indicated activities. Error bars indicate the standard deviation. The absence of error bars indicates that the standard deviation fell below the resolution limit of the graphing program.

**Preparation of E. coli RNA, S1 nuclease protection assays, and DNA sequencing**

RNA was prepared from strains grown at 30°C in Luria broth as described by Barry et al. [1980]. A 340-nucleotide-long fragment, spanning from position 1 to 340 of the published degP sequence (Lipinska et al. 1988), was used to create a radioactive probe for S1 experiments. The probe was phosphorylated with [γ-³²P]ATP in the forward reaction as described [Sambrook et al. 1989]. Total RNA (60 μg) was used in each S1 assay, and the assays were performed as described in Sambrook et al. [1989]. The DNA sequence of degP was determined as described previously [Russo et al. 1993]. The degP sequencing reactions and S1 nuclease samples were electrophoresed on 8% polyacrylamide sequencing gels and analyzed using the PhosphorImager ImageQuant [Molecular Dynamics] analysis program.

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