The $\sigma^E$ and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*

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In *Escherichia coli*, the heat shock-inducible $\sigma$-factor $\sigma^E$ and the Cpx two-component signal transduction system are both attuned to extracytoplasmic stimuli. For example, $\sigma^E$ activity rises in response to the overproduction of various outer-membrane proteins. Similarly, the activity of the Cpx signal transduction pathway, which consists of an inner-membrane sensor (CpxA) and a cognate response regulator (CpxR), is stimulated by overproduction of the outer-membrane lipoprotein, NlpE. In response to these extracytoplasmic stimuli, $\sigma^E$ and CpxA/CpxR stimulate the transcription of *degP*, which encodes a periplasmic protease. This suggests that CpxA/CpxR and $\sigma^E$ both mediate protein turnover within the bacterial envelope. Here, we show that CpxA/CpxR and $\sigma^E$ also control the synthesis of periplasmic enzymes that can facilitate protein-folding reactions. Specifically, $\sigma^E$ controls transcription of *fkpA*, which specifies a periplasmic peptidyl-prolyl cis/trans isomerase. Similarly, the Cpx system controls transcription of the *dsbA* locus, which encodes a periplasmic enzyme required for efficient disulfide bond formation in several extracytoplasmic proteins. Taken together, these results indicate that $\sigma^E$ and CpxA/CpxR are involved in regulating both protein-turnover and protein-folding activities within the bacterial envelope.

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conferred by the synthesis of certain mutant envelope proteins (Cosma et al. 1995; Snyder et al. 1995). The Cpx system performs this stress-combative function, in part, by stimulating the synthesis of the periplasmic protease, DegP. However, the Cpx system can partially combat these extracytoplasmic protein stresses even in the absence of DegP (Cosma et al. 1995; Snyder et al. 1995). Therefore, the Cpx pathway must control at least one other factor that can combat envelope protein toxicities.

The results described above indicate that although σE and the Cpx proteins are influenced by and affect extracytoplasmic events, the precise functions of the σE and Cpx systems are not firmly established. To further our understanding of the roles of both σE and Cpx, we sought to identify and characterize new members of each of these regulons.

We have found that the Cpx and σE systems each control the synthesis of periplasmic enzymes that can aid in protein folding. Because the Cpx and σE systems also control the synthesis of the periplasmic protease, DegP, the results described in this study suggest that the primary functions of these two regulons may be to mediate protein folding and protein turnover within the bacterial envelope.

Results

Activation of the σE and Cpx regulons alters the profile of periplasmic proteins

Because the σE and Cpx systems exert their effects on the bacterial envelope, it seemed reasonable to assume that some members of their respective regulons would be found in the periplasm of E. coli. Accordingly, we screened for periplasmic proteins whose synthesis could be activated by σE or by the Cpx signal transduction system.

Figure 1a shows a Coomassie-stained, steady-state profile of periplasmic proteins from strain CLC198 [MC4100, degP::Tn10] transformed with either [1] a control plasmid [pBR322, lane 1] or [2] a plasmid that overproduces σE (pND12, lane 2). The most striking difference between these two lanes is the increased intensity of a band that migrates in the 32-kD size range [Fig. 1a, cf. lanes 1 and 2]. Note that CLC198 contains a degP::Tn10 mutation, which was utilized to mitigate any effects that increased proteolysis would have on the profile of periplasmic proteins during these experiments.

In a similar fashion, Figure 1b shows the Coomassie-stained, steady-state profile of periplasmic proteins from strains SP779 [MC4100, ΔRS88(degE::lacZ), ara74::cam, zab::Tn10] and SP781 [pND18, cpxR::spc] transformed with either [1] a control plasmid [pBAD18, lanes 1,3] or [2] a plasmid that overproduces the outer-membrane lipoprotein, NlpE [pND18, lanes 2,4]. Because overproduction of NlpE activates the Cpx signal transduction system (Danese et al. 1995; Snyder et al. 1995), the strain used to prepare the periplasmic extract shown in lane 2 of Figure 1b possesses an activated Cpx signal transduction system. Lanes 3 and 4 of Figure 1b show extracts from transformant derivatives of SP781, which contain a cpxR null mutation. Thus, these lanes serve as controls to help in determining whether any changes between the protein profiles of lanes 1 and 2 are actually dependent on the Cpx pathway. Figure 1b shows a band of ~23-kD in size whose intensity rises from lane 1 to lane 2. No change in the intensity of this band is observed when comparing lanes 3 and 4, indicating that the increased intensity of this band is dependent on CpxR.

Amino-terminal sequencing of the 32- and 23-kD bands

The 32- and 23-kD bands shown in Figure 1 were promising candidates for proteins that could be regulated by σE and Cpx, respectively. Accordingly, the identity of the first 11 amino acid residues from the amino terminus of
increased levels of (rE concomitantly increase functional signal sequence. Note that the sequence determined for DsbA also corresponds to the signal-sequence processed form of the protein. For example, Mecsas and coworkers [1993] demonstrated that overproduction of the outer-membrane protein, OmpX, stimulates σE activity approximately fourfold. We were therefore interested in determining whether fkpA transcription would also be induced by overproduction of OmpX. We measured the amount of fkpA-lacZ transcription generated from SP887 [MC4100, ARS888(fkpA–lacZ)] that contained either (1) a control plasmid, pBR322 [Fig. 2b, lane 1] or (2) pJE100, a plasmid that overproduces OmpX [Mecsas et al. 1993]. Comparison of lanes 1 and 2 of Figure 2b shows that overproduction of OmpX stimulates fkpA–lacZ transcription approximately twofold when compared with a control strain. Thus, overproduction of OmpX, which stimulates σE activity, also stimulates fkpA transcription, albeit to a lesser extent than is observed with degP transcription.

There are several possible explanations for the increased amount of FkpA found in periplasmic extracts of strains overproducing σE. However, because σE is involved in transcriptional initiation, the simplest model posits that increased levels of σE concomitantly increase fkpA transcription.

Accordingly, we constructed an fkpA–lacZ operon fusion to determine whether the σE-overproducing plasmid affected fkpA transcription. This 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]. β-Galactosidase activities were determined from derivatives of SP887 [MC4100, ARS888(fkpA–lacZ)] that were transformed with either (1) the pBR322 control plasmid [Fig. 2a, lane 1] or (2) pND12, which overproduces σE [Fig. 2a, lane 2]. Figure 2a illustrates that the σE-overproducing plasmid stimulates fkpA transcription approximately sevenfold when compared with its control strain. Thus, σE can stimulate fkpA transcription.

Figure 2. Overproduction of σE stimulates transcription of fkpA–lacZ. (a) β-Galactosidase activities of SP887 [MC4100, ARS888(fkpA–lacZ)] transformed with pBR322 [control for pND12] [lane 1] or pND12 (overexpresses tpoE) [lane 2] were assayed. The tpoE overexpressing strain stimulates transcription of fkpA–lacZ approximately twofold over that of the control strain. (b) Overproduction of the outer-membrane protein, OmpX, stimulates fkpA–lacZ transcription. β-Galactosidase activities of SP887 [MC4100, ARS888(fkpA–lacZ)] transformed with either pBR322 (control for pJE100) [lane 1] or pJE100 (overproduces OmpX) [lane 2] were determined. The OmpX overproducing plasmid increases fkpA–lacZ transcription approximately twofold when compared with the control strain. (c) The surA° degP° double mutant stimulates fkpA–lacZ transcription. β-Galactosidase activities of SP887 [lane 1], SP940 [SP887, degP::Tn10] [lane 2], SP921 [SP887, surA::kan] [lane 3] and SP942 [SP921, degP::Tn10] [lane 4] were determined. The degP::Tn10 mutation alone does not stimulate fkpA transcription [cf. lanes 1 and 2]. In contrast, the surA null mutation alone stimulates fkpA transcription 1.4-fold, whereas the surA° degP° double mutant stimulates fkpA–lacZ transcription 4-fold when compared with the control strain. Strains were grown in Luria broth with ampicillin when needed, and all procedures were performed as described in Materials and Methods.

σE and Cpx control synthesis of protein-folding enzymes

Transcriptional regulation of fkpA

There are several possible explanations for the increased amount of FkpA found in periplasmic extracts of strains overproducing σE. However, because σE is involved in transcriptional initiation, the simplest model posits that increased levels of σE concomitantly increase fkpA transcription.

Accordingly, we constructed an fkpA–lacZ operon fusion to determine whether the σE-overproducing plasmid affected fkpA transcription. This 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]. β-Galactosidase activities were determined from derivatives of SP887 [MC4100, ARS888(fkpA–lacZ)] that were transformed with either (1) the pBR322 control plasmid [Fig. 2a, lane 1] or (2) pND12, which overproduces σE [Fig. 2a, lane 2]. Figure 2a illustrates that the σE-overproducing plasmid stimulates fkpA transcription approximately sevenfold when compared with its control strain. Thus, σE can stimulate fkpA transcription.

fkpA transcription is affected by fluctuations in σE activity

To determine the extent of σE’s influence on fkpA transcription, we quantified the amount of fkpA transcription that is generated during situations in which σE activity is altered by extracytoplasmic events.

For example, Mecsas and coworkers [1993] demonstrated that overproduction of the outer-membrane protein, OmpX, stimulates σE activity approximately fourfold. We were therefore interested in determining whether fkpA transcription would also be induced by overproduction of OmpX. We measured the amount of fkpA–lacZ transcription generated from SP887 [MC4100, ARS888(fkpA–lacZ)] that contained either (1) a control plasmid, pBR322 [Fig. 2b, lane 1] or (2) pJE100, a plasmid that overproduces OmpX [Mecsas et al. 1993]. Comparison of lanes 1 and 2 of Figure 2b shows that overproduction of OmpX stimulates fkpA–lacZ transcription approximately twofold when compared with a control strain. Thus, overproduction of OmpX, which stimulates σE activity, also stimulates fkpA transcription, albeit to a lesser extent than is observed with degP transcription.

The surA null mutation impairs the assembly of the porins LamB, OmpF, and OmpC, and as a result of this
assembly defect, the mutation also increases $\sigma^E$ activity approximately fivefold [Lazar and Kolter 1996; Missiakas et al. 1996; data not shown]. $\text{surA}$ specifies a periplasmic peptidyl-prolyl cis/trans isomerase, and it is believed that the SurA protein directly catalyzes a step(s) in the folding of the porins mentioned above [Lazar and Kolter 1995; Missiakas et al. 1996].

The porin assembly defect conferred by the $\text{surA}$ null mutation is aggravated by the absence of the DegP protease. Although the $\text{degP}$ null mutation does not stimulate $\sigma^E$ activity on its own, when it is introduced into a $\text{surA}$ null strain, $\sigma^E$ activity rises 10-fold [data not shown].

We were interested in determining the extent of $\text{fkpA}$ transcriptional induction under these circumstances as well. Specifically, we quantified the transcription generated from the $\text{fkpA}-\text{lacZ}$ fusion in either (1) a wild-type background [Fig. 2c, lane 1]; (2) a $\text{degP}^+$ background [Fig. 2c, lane 2]; (3) a $\text{surA}^-$ background [Fig. 2c, lane 3], or (4) a $\text{surA}^-\text{degP}^-$ double-mutant background [Fig. 2c, lane 4]. As expected, the $\text{degP}$ null mutation has no effect on $\text{fkpA}$ transcription. In contrast, the $\text{surA}$ null increases $\text{fkpA}$ transcription 1.4-fold when compared with the wild-type strain [Fig. 2c, cf. lanes 1 and 3]. The $\text{surA}^-\text{degP}^-$ double mutant displays the largest induction of $\text{fkpA}$ transcription at fourfold over that of the wild-type strain [Fig. 2c, cf. lanes 1 and 4]. Again, these results indicate that extracytoplasmic defects that increase $\sigma^E$ activity also stimulate $\text{fkpA}$ transcription.

A $\sigma^E$ promoter stimulates $\text{fkpA}$ transcription

The transcriptional induction of $\text{fkpA}$ by overproduction of $\sigma^E$ as well as by stimuli that activate $\sigma^E$ suggests that $\text{fkpA}$ transcription is controlled, at least in part, by a $\sigma^E$ promoter. An analysis of the noncoding upstream sequence of $\text{fkpA}$ highlights a putative $\sigma^E$ promoter [Fig. 3a]. Nucleotides 342–347 of the published $\text{fkpA}$ sequence [Horne and Young 1995] contain 4 of the 6 consensus nucleotides for a $\sigma^E$-10 promoter site (aaAACAA). This site is followed by a consensus 16-nucleotide-long spacer region that contains a string of 5 adenine nucleotides that is also characteristic of $\sigma^E$ promoters [Lipinska et al. 1988; Erickson and Gross 1989; Raina et al. 1995; Rouviere et al. 1995]. This spacer region is followed by a 30-nucleotide-long spacer region that contains a string of 5 adenine nucleotides that is also characteristic of $\sigma^E$ promoters [Lipinska et al. 1988; Erickson and Gross 1989; Raina et al. 1995; Rouviere et al. 1995].

In light of this putative $\sigma^E$ promoter, we wanted to determine the start site of the $\text{fkpA}$ transcripts that are induced upon overproduction of $\sigma^E$. To this end, we performed S1 nuclease protection assays using RNA prepared from a strain that was transformed with either (1) pBR322 [control for pNND12] or (2) pNND12 [overproduces $\sigma^E$]. Figure 3b shows that the transcripts induced by overproduction of $\sigma^E$ begin at nucleotides 370, 371, and 372 of the published $\text{fkpA}$ sequence, which correspond to positions +4, +5, and +6 with respect to the $\sigma^E$-10 consensus promoter site [see Fig. 3a] [Horne and Young 1995].

Although this initiation site is relatively close to the $\sigma^E$-10 site [the strongest $\sigma^E$ transcripts begin at positions +8 and +9 [Raina et al. 1995; Rouviere et al. 1995]],
other E. coli promoters also initiate transcription at these early sites (Harley and Reynolds 1987). Moreover, the results presented in Figure 3b may help to explain the attenuated induction of fkpA transcription that is observed upon activation of $\sigma^E$.

Recall that situations in which $\sigma^E$ induces activity 4-fold will stimulate fkpA transcription only by 1.4- to 2-fold (Fig. 2b). There are at least three possible explanations for this observation. First, the position of the transcriptional initiation sites relative to the $\sigma^E$ –10 site may diminish the ability of RNA polymerases containing $\sigma^E$ to transcribe this promoter. Second, the $\sigma^E$ –35 site of fkpA does not possess 100% identity with the consensus –35 site (see Figs. 3a and 4). When $\sigma^E$ is not activated (i.e., nonstressing conditions), transcriptional initiation at sites 370–372. Thus, the total amount of transcriptional induction that is observed with the fkpA–lacZ fusion is attenuated because $\sigma^E$ overproduction stimulates transcriptional initiation at nucleotides 370–372, at the expense of transcription initiation at nucleotide 401.

We have also noted that the rpoE null mutation [which eliminates $\sigma^E$ synthesis] does not reduce transcription of fkpA–lacZ (data not shown). However, this is not surprising in light of the results presented in Figure 3b. When $\sigma^E$ is not activated (i.e., nonstressing conditions), the majority of transcription generated from fkpA initiates at position 401. It is only upon activation of $\sigma^E$ that we observe transcriptional initiation at sites 370–372.

Taken together, the results of Figure 3b show that fkpA possesses a $\sigma^E$-activatable promoter that has the standard features recognized by $\sigma^E$. Figure 4 shows this fkpA promoter aligned with the three $\sigma^E$ promoters of E. coli characterized previously.

Transcriptional regulation of dsbA

In a similar fashion to $\sigma^E$'s regulation of FkpA, there are several possible explanations for the increased amount of DsbA found in periplasmic extracts of strains possessing an activated Cpx signal transduction system. However, because the Cpx pathway controls the transcription of $\text{degP}$ (Danese et al. 1995; Raina et al. 1995), and because CpxR is homologous to the OmpR subfamily of two-component transcription factors (Dong et al. 1993), the simplest interpretation of the results presented in Figure 1b is that activation of the Cpx pathway stimulates dsbA transcription.

To test the possibility that the Cpx pathway could activate transcription from the dsbA locus, we created an $\text{orfA}$–dsbA–lacZ operon fusion. This 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).

The incorporation of $\text{orfA}$ into the operon fusion was necessitated because the dsbA gene is situated in an operon with an upstream gene, $\text{orfA}$, of unknown function [Fig. 5a]. Transcription of dsbA is directed from two promoters, each of which contributes to approximately one-half of DsbA synthesis. The first promoter is situated within the $\text{orfA}$ [pdsbA] coding sequence, whereas the second is positioned upstream of $\text{orfA}$ [porfA–dsbA]. This latter promoter cotranscribes both orfA and dsbA [Fig. 5a] (Belin and Boquet 1994).

To ensure that all of the transcripts used to synthesize DsbA were represented in the operon fusion, we fused the promoter region of orfA, the entire orfA coding sequence [which contains the first dsbA promoter], and the first 86 nucleotides of the dsbA coding sequence to the lac operon, creating porfA–dsbA–lacZYA [Fig. 5a].

We first wanted to determine whether dsbA transcription could be stimulated by the $\text{cpxA24}$ allele, which possesses a mutation that hyperactivates the Cpx pathway and stimulates $\text{degP}$ transcription approximately eightfold (Danese et al. 1995). Accordingly, we introduced the $\text{cpxA24}$ mutation into strain SP969 [MC4100, ΔR588[orfA–dsbA–lacZ]], generating strain SP971 (SP969, cpxA24). Comparison of lanes 1 and 2 in Figure 5b shows that the $\text{cpxA24}$ mutation stimulates porfA–dsbA–lacZ transcription sixfold, indicating that activation of the Cpx pathway stimulates dsbA transcription.

The transcriptional induction of dsbA is also observed when the outer membrane lipoprotein, NlpE, is overproduced. Overproduction of NlpE has previously been shown to activate the wild-type Cpx pathway and stimulate degP transcription (Danese et al. 1995; Snyder et al. 1995). Figure 5c shows the results of β-galactosidase assays performed on strains SP994 [MC4100, ΔR588[orfA–dsbA–lacZ]], ara74:cam. zab::Tn10], SP995 [SP994, cpxA::cam], and SP996 [SP994, cpxR::spc] transformed with either (1) pBAD18 [control for pND18] or (2) pND18 (overproduces NlpE). Comparison of lanes 1 and 2 in Figure 5c shows that NlpE overproduction stimulates

\begin{figure}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Alignment of $\sigma^E$-activatable promoters. The -35, -10, and poly[A] regions are boxed. The transcription start sites are underlined. See text for details.}
\end{figure}
Figure 5. Activation of the Cpx pathway stimulates transcription of the porfA–dsbA–lacZ operon fusion. (a) The orfA–dsbA operon and the porfA–dsbA–lacZ operon fusion. The orfA and dsbA open reading frames are shown as shaded rectangles. The two promoters that transcribe dsbA (porfA–dsbA and pdsbA) are depicted as arrows emanating from their respective initiation sites. The genomic DNA used to create the porfA–dsbA–lacZ operon fusion is shown as a thin line fused to the lacZ operon above the orfA and dsbA coding sequences. A 3000-nucleotide-long scale is shown below the operon for reference. (b) A hyperactive cpxA allele stimulates transcription of porfA–dsbA–lacZ. β-Galactosidase activities from SP969 (MC4100, KRS88[porfA–dsbA–lacZ]) and SP971 (SP969, cpxA24) were determined. The cpxA24 mutation stimulates dsbA–lacZ transcription approximately sixfold. (c) Overproduction of NlpE stimulates porfA–dsbA–lacZ transcription by the Cpx pathway. Lanes 1, 3, and 5 show β-galactosidase activity of strains transformed with pBAD18 (control for pND18). Lanes 2, 4, and 6 show the β-galactosidase activity of strains transformed with pND18 (overexpresses nlpE). [Lanes 1, 2] SP994 [MC4100, KRS88[porfA–dsbA–lacZ], ara74::cam, zab::Tnl0]. [Lanes 3, 4] SP995 (SP994, cpxA::carn). [Lanes 5, 6] SP996 (SP994, cpxR::spc). All strains were grown in Luria broth (containing 0.4% L-arabinose and ampicillin when needed).

porfA–dsbA–lacZ transcription 5.3-fold. Only a minor stimulatory effect is observed in the cpxA− and cpxR− cpxA− backgrounds (Fig. 5c, cf. lanes 1 and 2 with lanes 3 and 4; 5 and 6), indicating that overproduction of NlpE stimulates dsbA transcription by activating the wild-type CpxA protein.

Activation of the Cpx pathway stimulates dsbA transcription from the promoter upstream of the orfA locus

Because dsbA transcription originates from two promoters, one within the orfA coding sequence and one that also cotranscribes orfA [Fig. 5a], we were interested in determining which of these two promoters was utilized by the activated Cpx pathway. To address this issue, we used S1 nuclease protection assays to quantify the amount of transcription generated from the porfA–dsbA promoter and the pdsbA promoter using RNA prepared from strains that contain either an nlpE overexpressing plasmid (pND18) or a control plasmid (pBAD18). Recall that overproduction of NlpE activates dsbA transcription in a CpxA-dependent fashion [Fig. 5c].

The pdsbA promoter is unaffected by overproduction of NlpE (data not shown). In contrast, Figure 6a shows that transcription from the porfA–dsbA promoter is stimulated by overproduction of NlpE. Also, the primary transcriptional initiation site induced by overproduction of NlpE corresponds to the +1 site described for porfA–dsbA by Belin and Boquet (1994) [see Fig. 6a,b]. Therefore, overproduction of NlpE increases the synthesis of DsbA by stimulating transcription from the porfA–dsbA promoter.

σE and Cpx do not coregulate transcription of fkpA and dsbA

Previous studies have indicated that the Cpx signal transduction system and σE jointly control the transcription of the degP locus [Danese et al. 1995; Raina et al. 1995]. Accordingly, we were interested in determining whether the transcription of any of the other members of the Cpx and σE regulons were also regulated jointly. To address this issue, we determined whether the overproduction of any of the other members of the Cpx and σE regulons were also regulated jointly. To address this issue, we determined whether the overproduction of σE could activate dsbA transcription, and we also determined whether activation of the Cpx pathway (by overproduction of NlpE) could activate fkpA transcription. In the former case, overproduction of σE does not activate dsbA transcription, and in the latter case, overproduction of NlpE does not stimulate fkpA transcription (data not shown). These results indicate that

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The results presented here provide the first evidence that the cr E and Cpx regulons not only contain proteases like DegP, indicating that there are other stress-combative members of the Cpx regulon. DsbA is a promising candidate for such a factor. However, Snyder and Silhavy (1995) have demonstrated that the dsbA null mutation does not impede the ability of the Cpx pathway to ameliorate the periplasmic toxicity associated with the LamB–LacZ–PhoA fusion protein. Although this does not preclude the possibility that the Cpx pathway can utilize DsbA in other stress-combative situations, the result does imply that there is still at least one other unidentified factor utilized by the Cpx regulon to combat extracytoplasmic protein-mediated toxicities.

Second, from the results presented, it is not clear

although Cpx and αE intersect in their regulation of degP, their regulons do not completely overlap.

Discussion

The control of protein-folding agents within the bacterial envelope

The results presented here provide the first evidence that the αE and Cpx regulons not only contain proteases like DegP, but that they also contain periplasmic enzymes that can engage in protein-folding activities. This result implies that there is a second envelope-protein stress-

combative tool at the disposal of E. coli—specifically, protein-folding agents. Thus, much like α32 controls the synthesis of a host of cytoplasmic proteases and molecular chaperones (Gross 1996), the αE and Cpx regulons may perform a complementary function, mediating protein folding and protein turnover within the bacterial envelope.

FkpA and the αE regulon

Missiakas et al. (1996) have recently provided evidence that high-level synthesis of FkpA can suppress extracytoplasmic stresses, such as the accumulation of unfolded periplasmic and outer-membrane proteins. These authors have also demonstrated that FkpA is a peptidyl-prolyl cis/trans isomerase, suggesting that the function of this protein is to facilitate the folding of other extracytoplasmic proteins.

The results presented here provide a satisfying complementary analysis to that performed by Missiakas et al. (1996). Specifically, we have shown that both the overproduction of αE and the creation of extracytoplasmic stresses that stimulate αE activity will increase the synthesis of FkpA. This increased synthesis is mediated by a αE-activatable promoter that shares the common features of the other known αE-regulated promoters of E. coli (Fig. 4). Taken together, these results suggest that fkpA is the newest member of the αE regulon.

Cpx and DsbA

In addition, the results presented here suggest that the Cpx pathway is also involved in mediating protein-folding functions within the bacterial envelope. Specifically, DsbA synthesis is increased by activation of the Cpx pathway. Interestingly, this study indicates that the Cpx pathway also stimulates transcription of orfA, the gene upstream of dsbA (Fig. 5a). However, the function of this gene is presently unknown.

The Cpx-mediated stimulation of DsbA synthesis is intriguing for several reasons: First, we have shown previously that activation of the Cpx pathway can combat extracytoplasmic protein-mediated toxicities (Cosma et al. 1995; Snyder et al. 1995). The activated Cpx system performs this function, in part, by stimulating the synthesis of DegP. However, the activated Cpx system can still partially suppress these stresses even in the absence of DegP, indicating that there are other stress-combative members of the Cpx regulon. DsbA is a promising candidate for such a factor. However, Snyder and Silhavy (1995) have demonstrated that the dsbA null mutation does not impede the ability of the Cpx pathway to ameliorate the periplasmic toxicity associated with the LamB–LacZ–PhoA fusion protein. Although this does not preclude the possibility that the Cpx pathway can utilize DsbA in other stress-combative situations, the result does imply that there is still at least one other unidentified factor utilized by the Cpx regulon to combat extracytoplasmic protein-mediated toxicities.
whether transcription of any of the other Dsb proteins (DsbB, DsbC, and DsbD) can also be stimulated by the Cpx pathway (Bardwell et al. 1993; Dailey and Berg 1993; Missiakas et al. 1993, 1994, 1995). Because this study has only searched for periplasmic Cpx-inducible proteins, inner-membrane Dsb proteins (DsbB and DsbD) would not have been identified by this approach. Accordingly, an analysis of membrane proteins in Cpx-activated strains could be informative in identifying the remaining members of the Cpx regulon.

**Three classes of αE and Cpx regulatory targets**

Figure 7 shows a list of Cpx-regulated and αE-regulated loci. There are now three types of genes that are controlled by either the Cpx or αE regulons: (1) those that are controlled solely by Cpx [dsbA], (2) those that are controlled solely by αE (tpoH\textsubscript{pr}, tpe\textsubscript{rp}, fkpA), and (3) those that are jointly controlled by both Cpx and αE [degP] (Lipinska et al. 1988; Erickson and Gross 1989; Danese et al. 1995; Raina et al. 1995).

The simplest explanation for the interactions depicted in Figure 7 is that all interactions are direct. For example, CpxR would bind upstream of its regulatory targets (degP, orfA-dsbA) and stimulate transcription from the promoters of these genes. Pogliano et al. (this issue) have demonstrated footprinting of CpxR at the degP and dsbA loci, lending support to this model.

In sum, the results presented in this paper and in previous studies indicate that the Cpx two-component signal transduction system and the heat shock-inducible α-factor αE both control the synthesis of a periplasmic protease (DegP) and periplasmic enzymes that are capable of performing protein-folding functions (Lipinska et al. 1988; Erickson and Gross 1989; Danese et al. 1995; Raina et al. 1995). Given these two classes of proteins, it seems likely that the primary functions of the Cpx and αE systems are to monitor and mediate protein-folding and protein-turnover functions within the extracytoplasmic compartments of *E. coli*. We would not be surprised if many of the remaining unidentified members of the Cpx and αE regulons fall into these two classes of proteins.

**Materials and methods**

**Media**

Media were prepared as described (Silhavy et al. 1984). Liquid cultures were grown in Luria broth. The final concentration of ampicillin used in the growth media was 50 μg/ml. Standard microbiological techniques were used for strain construction and bacterial growth (Silhavy et al. 1984).

**Strains and phage**

All strains are derivatives of MC4100 (Silhavy et al. 1984). The genotypes of all strains used in this study are given in the legends to Figures 1, 2, 3, 5, and 6 in the text. The cpxA24 mutation was moved by P1 transduction, as described previously (Danese et al. 1995).

The ara74::cam mutation (gift of Leslie Pratt, Harvard Medical School, Boston, MA) confers upon MC4100 an arabinose-resistant and arabinose-minus phenotype [MC4100 is normally sensitive to growth in the presence of arabinose]. The ara74::cam mutation was introduced into strains when the arabinose-dependent induction of NlpE synthesis was required (Figs. 1b, 5c, and 6). The ara74::cam mutation was moved with a linked Tn10 insertion (zab::Tn10), and the resulting transductants were scored for the ability to grow in the presence of arabinose.

The zab::Tn10, cpxA::cam, cpxR::spc, degP::Tn10, and surA::kan (kind gift of Sara Lazar and Roberto Kolter) mutations were moved by P1 transduction, selecting for resistance to the appropriate antibiotic ([Tn10] tetracycline; [cam] chloramphenicol; [spc] spectinomycin; [kan] kanamycin). The degP::Tn10 mutation was assayed further for the conferment of temperature-sensitive growth at 42°C.
ARS88 has been described [Simons et al. 1987]. Lysogenization of ARS88(fkpA-lacZ) and ARS88(orfA-dsbA-lacZ) was performed as described by Simons et al. [1987]. All ARS88 operon fusions were shown to be located in single copy at the katt locus by P1 transduction.

**Plasmid construction**

All plasmids used in this study confer ampicillin resistance.

pND12, which overproduces $\sigma^F$, was constructed as follows: $rpoE$ was amplified from the chromosome of MC4100 by PCR using the RpoE3 primer (5'-CTATCCACGGTGTCAGACATCATCAT-3') and RpoE5 (5'-CCATGACAAAACAAAAACGGGATCCGGTACCGAAC-3') primers. SalI and BamHI restriction sites were incorporated into the RpoE3 and RpoE5 primers, respectively, to facilitate subcloning. The amplified DNA was then subcloned into the BamHI and SalI sites of pBR322, creating pND12. pND12 places $rpoE$ under its own transcriptional control. The sequence of the pND12 insert was confirmed by dideoxynucleotide sequencing.

pJE100 overproduces the outer-membrane protein OmpX [Mecsas et al. 1993]. The parent vector for pJE100 is pBR322. pND18 expresses the nlpE locus under the control of the arabinose-inducible pBAD promoter [Danese et al. 1995]. pBAD18, the parent vector for pND18, has been described [Guzman et al. 1995].

**Construction of ARS88(fkpA-lacZ)**

The FkpA5 (5'-CTTCAATGGTGTTTCTGCCAAG-3') and Fkpalac3 (5'-CTTTAAGGTCGTTGACCATCTCG-3') primers were used to amplify from the chromosome of MC4100, EcoRI and BamHI restriction sites were incorporated into the FkpA5 and Fkpalac3 primers, respectively, to facilitate subcloning. The amplified DNA was subcloned into the EcoRI and BamHI sites of pRS415, generating pND28. This amplified DNA includes nucleotides from position -286 with respect to the FkpA translational start site to position +55 with respect to this same site. The nucleotide sequence of the FkpA insert of pND28 was confirmed by dideoxynucleotide sequencing. The fkpA-lacZ fusion of pND28 was then recombined onto phage ARS88, and recombinants were used to lysogenize MC4100 as described [Simons et al. 1987].

**Construction of ARS88(orfA-dsbA-lacZ)**

The DsbA5 (5'-CGTCGCTACAGTCTTACCGATATCGC-3') and DsbA3 (5'-CAAGGGAAGCTTATGTTGATGCAAG-3') primers were used to amplify from the promoter region of orfA (the gene immediately upstream of dsbA) through to the beginning of the dsbA coding sequence. EcoRI restriction sites were incorporated into the DsbA5 and DsbA3 primers to facilitate subcloning. This DNA was amplified from the chromosome of MC4100 and subcloned into the EcoRI site of pRS415, generating pND31. The proper orientation of the insert was confirmed by restriction analysis. pND31 drives lacZ transcription from both promoters that are known to initiate transcription of dsbA, orfA, and pdsbA [Belin and Boquet 1994]. The amplified DNA contains nucleotides from position -422 with respect to the translational start site of orfA to position +86 with respect to the start site of translation of dsbA. The orfA-dsbA fusion of pND31 was then recombined onto phage ARS88, and recombinants were used to lysogenize MC4100 as described [Simons et al. 1987].

**$\sigma^F$ and Cpx control synthesis of protein-folding enzymes**

**$\beta$-Galactosidase assays**

Cells were grown overnight in Luria broth. Cells were then subcultured [1:40] into 2 ml of the same media and grown to mid-log phase. $\beta$-Galactosidase activities were determined using a microtiter plate assay [Slautch and Silhavy 1991]. $\beta$-Galactosidase activities are expressed as $U/[A_{600}] \times 10^3$, where U = mule of product formed per minute. Assays were performed on a minimum of four independent cultures of each strain, and the results were averaged to obtain the indicated activities. Error bars indicate the S.D. The absence of error bars indicates that the S.D. fell below the resolution limit of the graphing program.

**Preparation of periplasmic protein extracts**

All procedures were performed on ice, and all solutions were chilled on ice.

Periplasmic protein extracts were prepared as follows: Strains were grown in the appropriate media until they reached an OD$_{600}$ of ~1.0. One milliliter of each culture was harvested and resuspended in 250 $\mu$l of 0.2 M Tris-HCl (pH 8.0). Two hundred and fifty microliters of 0.2 M Tris-HCl, 1 M sucrose (pH 8.0) was then added to each suspension, and 2.5 $\mu$l of 0.1 M EDTA was added subsequently along with 7.5 $\mu$l of lysozyme (4 mg/ml). Then, 500 $\mu$l of distilled water was added, and the cell suspension was incubated on ice for 2 min. Twenty microliters of 1 M MgCl$_2$ was added to the cell suspension, and each mixture was incubated for 30 additional min on ice. After this incubation, each suspension was harvested at 14,000 rpm in a microcentrifuge to pellet spheroplasts. The remaining supernatant contained the bulk of periplasmic proteins. The periplasmic proteins were precipitated from these supernatants with trichloroacetic acid (TCA). The TCA-precipitated samples were resuspended in a milliliter volume equal to the initial OD$_{600}$ value. Twenty-microliter samples of these periplasmic protein extracts were resolved by polyacrylamide gel electrophoresis and subsequently stained with Coomassie brilliant blue [Sambrook et al. 1989].

**Amino acid sequence analysis of FkpA and DsbA**

Periplasmic protein extracts depicted in Figure 1 were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane. The membrane was stained with Coomassie brilliant blue, and the bands marked with arrowheads in Figure 1 were excised. The identity of the first 11 residues from each band was determined by Edman Degradation by the Princeton University Synthesis/Sequencing Facility.

**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 342-nucleotide-long fragment, spanning from position 883 to position 1175 of the published sxyD-FkpA sequence [Horne and Young 1995], was used to create a radioactive probe for S1 experiments depicted in Figure 3. This probe was used to determine the transcriptional start sites used to express the fkpA locus. A 425-nucleotide-long fragment, spanning from position 931 to position 1356 of the published orfA-dsbA sequence [Belin and Boquet 1994], was used to create a radioactive probe for S1 experiments depicted in Figure 6. This probe was used to quantify the amount of transcription from the orfA promoter described by Belin and Boquet [1994].
A 270-nucleotide-long fragment spanning the pspc promoter was used to create a radioactive probe for S1 experiments. The pspc transcript serves as an internal loading control for quantifying changes in the amount of transcription from the fkpA and porA promoters.

Each probe was phosphorylated with either $[\gamma-32P]ATP$ or $[\gamma-33P]ATP$ in the forward reaction as described (Sambrook et al. 1989). Sixty micromgrams of total RNA was used in each S1 assay, and the assays were performed as described in Sambrook et al. (1989). The DNA sequence of $\text{fkpA}$ and orfA-$\text{dsbA}$ was determined as described previously (Russo et al. 1993). The $\text{fkpA}$ and orfA-$\text{dsbA}$ sequencing reactions and S1 nuclease samples were resolved on 6% polyacrylamide sequencing gels and analyzed using the Phosphorlmager ImageQuant (Molecular Dynamics) analysis program.

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