The activity of σ^E, an *Escherichia coli* heat-inducible σ-factor, is modulated by expression of outer membrane proteins

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σ^E and σ^{32} are two heat- and ethanol-inducible σ-factors in *Escherichia coli*. The σ^{32} regulon is also induced by unfolded and misfolded proteins in the cytoplasm, and the function of many of the proteins in the σ^{32} regulon is to bind to cytoplasmic proteins and assist them in folding or unfolding. To further understand the function of the σ^E regulon, we searched for mutants that affected σ^E activity. Our results indicate that a signal generated by expression of outer membrane proteins modulates σ^E activity. Specifically, σ^E activity is induced by increased expression of OMPs and is reduced by decreased expression of OMPs. In addition, mutations that cause misfolded OMPs induce σ^E activity. This signal is generated after the fate of OMPs and periplasmic proteins diverge in the secretory pathway and is not the result of an accumulation of OMP precursors in the cytoplasm. Our results indicate that this effect of OMPs is specific to the σ^E regulon, because none of the above mutations affect σ^{32} activity. We propose that the σ^E regulon is involved in processes that occur in extracytoplasmic compartments and that these two heat-inducible regulons may have distinct but complementary roles of monitoring the state of proteins in the cytoplasm (σ^{32}) and outer membrane (σ^E).

[*Key Words*: σ-Factors; protein export; outer membrane proteins; heat shock; σ^E]

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In bacterial cells the σ-subunit directs RNA polymerase to initiate transcription at promoter sites on the DNA (Burgess et al. 1969). The primary σ-factor in the cell is responsible for transcription of most genes during exponential growth. In addition, alternative σ-factors direct transcription of sets of genes whose products are needed for specific functions, such as sporulation, nitrogen fixation, or flagella synthesis (Gross et al. 1992). Alternative σ-factors are often activated by changes in environmental or cellular conditions that generate morphological and/or molecular cues, signaling the need for the gene products in the regulon under control of a particular σ-factor. Elucidation of these signal-transduction pathways provides insights about global control of gene activity in prokaryotic cells.

The activity of two *Escherichia coli* alternative σ-factors, σ^{32} and σ^E (σ^{24}), increases after temperature upshift or exposure to ethanol (Grossman et al. 1984; Erickson et al. 1987; Straus et al. 1987; Erickson and Gross 1989; Wang and Kaguni 1989). RNA polymerase (E) containing σ^{32} ($E\sigma^{32}$) transcribes the heat shock genes with products that consist primarily of chaperones and proteases. These heat shock proteins function to process partially folded proteins in the cytoplasm and target them for secretion, proteolysis, or refolding (Straus et al. 1988; Hoffmann et al. 1992; Martin et al. 1992; Wild et al. 1992a); to aid in assembly and disassembly of complex protein structures (Alfano and McMacken 1989; Dodson et al. 1989; Zylicz et al. 1989); and to protect the cell from severe stresses (i.e., 10% ethanol and 50°C) (Neidhardt and VanBogelen 1987). Interestingly, under conditions of severe stress, only $E\sigma^E$ transcribes *rpoH* (Erickson et al. 1987), the gene encoding σ^{32}. Because σ^{32} is unstable (Straus et al. 1987), continued transcription of *rpoH* by $E\sigma^E$ is probably essential for cell survival under these conditions. Thus, σ^E was initially thought to be a supplementary heat shock σ-factor that functioned to maintain high concentrations of σ^{32} during severe conditions.

The only other *E. coli* gene known to be transcribed by $E\sigma^E$ is *degP* (*htrA*) (Erickson and Gross 1989), which encodes a periplasmic endopeptidase essential at temperatures above 42°C (Lipinska et al. 1988, 1989, 1990; Strauch and Beckwith 1988; Strauch et al. 1989). Although the physiological substrates of DegP have not been identified, it can degrade colicin A (Cavard et al. 1989). Because $E\sigma^E$ is the only form of RNA polymerase that transcribes *degP*, the requirement for DegP at 42°C is circumstantial evidence that σ^E plays an essential role in protecting cells from stress caused by high tempera-

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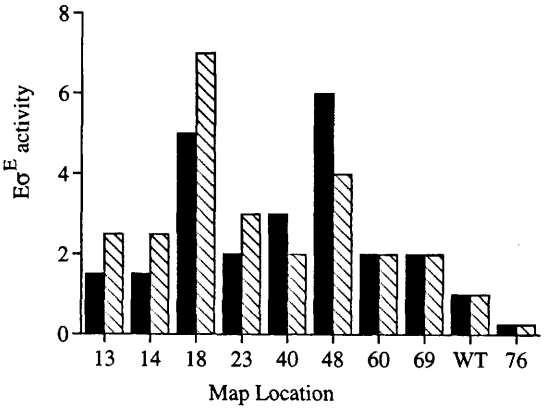

Figure 1. Changes in $E\sigma^E$ activity at the *rpoHP3* and *degP* promoters resulting from pISE plasmids or mini-Mu insertion (*dse*). $E\sigma^E$ activity at *rpoHP3* was assayed by monitoring β-galactosidase activity from a single copy Φλ[*rpoHP3–lacZ*] fusion (solid bars). $E\sigma^E$ activity at the *degP* promoter was assayed by S1 mapping of RNA (hatched bars). Assays were done in the CAG16052 strain background for the pISE strains (map positions 13, 14, 18, 23, 40, 48, 60, and 69) and in MC1061Φλ[*rpoHP3–lacZ*] for the *dse* allele (map position 76). The wild-type values were obtained from CAG16052 + pEG5005 for the pISE strains and MC1061Φλ-[*rpoHP3–lacZ*] for the *dse* strain. All values were normalized to wild type and are averages of measurements made at least twice.

tures. However, the periplasmic location of DegP suggests that the $E\sigma^E$ regulon may protect extracytoplasmic compartments from stress.

The experiments in this paper focus on identifying the signals that modulate $E\sigma^E$ activity. We find that the activity of $E\sigma^E$ increases when outer membrane proteins (OMPs) are overproduced and decreases when they are underproduced. Additional experiments suggest that the signal generated by OMPs is likely to originate in the periplasm or at the outer membrane (OM). This signal, unlike heat or ethanol, does not affect $E\sigma^{32}$ activity. We suggest that σ^E responds to signals in the extracytoplasmic compartments of the bacterial cell.

Results

Identification of positive regulators of $E\sigma^E$ activity

To elucidate the role of σ^E in *E. coli* cell physiology, we used two genetic strategies to identify potential positive regulators of $E\sigma^E$ activity. The first strategy was predicated on the rationale that increasing the copy number of genes encoding positive regulators of $E\sigma^E$ will increase $E\sigma^E$ activity. The second strategy was based on the rationale that loss-of-function mutations in positive regulators will decrease $E\sigma^E$ activity. To monitor $E\sigma^E$ activity, gene fusions containing only 37 bases of the *rpoHP3* promoter were used to express various reporter genes. Thus, changes in transcription from these fusions should result solely from changes in $E\sigma^E$ activity at the promoter.

To identify genes that increased $E\sigma^E$ activity when present in multicopy, a random plasmid library of *E. coli* DNA (Groisman and Casadaban 1986) was introduced into a reporter strain in which expression of chloramphenicol acetyltransferase is driven from *rpoHP3*. Cells growing on higher concentrations of chloramphenicol than the parental strain could contain plasmids that increased sigma E activity (pISE). The inserts on those plasmids were mapped to nine regions of the *E. coli* genome (Table 1) by hybridization to the Kohara phage gene bank (Kohara et al. 1987). Because most of the DNA regions were isolated only once, more genes may exist that increase $E\sigma^E$ activity when cloned on a multicopy plasmid. The increase in $E\sigma^E$ activity for one representative of each region was verified and quantified by examining expression from two different $E\sigma^E$ promoters. Plasmids caused a 1.5- to 7-fold increase in $E\sigma^E$ activity, and, in general, the increase in expression at *rpoHP3* and *degP* were similar (Fig. 1).

To identify genes that decreased $E\sigma^E$ activity when inactivated, transposon mutagenesis with mini-Mu (Castilho et al. 1984) was performed in a reporter strain in which expression of *galK* is driven from *rpoHP3*. Cells with normal expression of $E\sigma^E$ are red on MacConkey–galactose plates, whereas those with decreased $E\sigma^E$ activity should be white. In addition, cells with decreased $E\sigma^E$ activity should be temperature sensitive as a result of decreased transcription of *degP*, which is required for growth at high temperature. This screen identified one locus, *dse* (decreased sigma E), mapping to 76 min by P1 transduction and to Kohara phage 619–621 by hybridization (Table 1). The *dse* insertion caused a fourfold decrease in expression at both the *rpoHP3* and *degP* promoters (Fig. 1).

Table 1. *Mapping of loci altering $E\sigma^E$ activity*

Locus[a]	Number of isolates	Kohara phage[b]	Known genes[c,d]
ise13	1	160–162	***ompT***, *envY*, *appY*
ise14[e]	1	168	*lipA*
ise18	12	205–206	***ompX***[f]
ise23	1	225–226	
ise40	1	335–336	*htpX*, *prc*
ise48	2	373	***ompC***
ise60	1	458–459	*argA*
ise69	1	518–519	*mtrA*, *deaD*, *pnp*, *yhb*
dse76	16	619–621	***ompR***, ***envZ***, *bioH*, *pckA*

[a]The number indicates the map position of the locus on *E. coli* chromosome; (*ise*) increased σ^E activity; (*dse*) decreased σ^E activity.
[b]Plasmids were mapped to Kohara phage (see Materials and methods)
[c]Genes mapping to these phage were identified using GeneScape (Bouffard et al. 1992).
[d]Genes in boldface type encode proteins that are located in the outer membrane, or positive regulators of OMPs.
[e]Cells containing pISE14 were mucoid.
[f]*ompX* is described in J. Mecsas, R. Welch, J.W. Erickson, and C.A. Gross (in prep).

Overproduction of OMPs increases Eσ^E activity

A number of loci were identified that increased Eσ^E activity when present in multicopy. We initially focused on characterizing the insert on plasmid pISE18 because this region of the genome was isolated independently 12 times and gave one of the largest (five- to sevenfold) increases in Eσ^E activity. DNA sequence analysis of the region responsible for this phenotype indicated that the gene causing the increase in Eσ^E activity encodes an OMP, OmpX (J. Mecsas, R. Welch, J.W. Erickson, and C.A. Gross, in prep.). Because several other pISEs contained inserts that mapped to regions known to encode OMPs (Table 1), it seemed possible that overproducing a variety of OMPs could induce Eσ^E activity. To test this, multicopy plasmids encoding OM porins OmpF or OmpC, the OM protease OmpT, or OmpX were introduced into cells containing Φλ[*rpoHP3–lacZ*]. Overproduction of OmpC, OmpF, or OmpX caused an increase in Eσ^E activity at both *rpoHP3* and the *degP* promoters (Fig. 2A,B). Furthermore, Figure 3 shows that these multicopy plasmids caused the expected increase of these proteins in the OM. Overproduction of OmpT, which was tested only at the *rpoHP3* promoter, also caused a small but reproducible increase in Eσ^E activity (Fig. 2A), whereas pBR322 had no effect on Eσ^E activity. This suggests that overexpression of a variety of OMPs induces Eσ^E activity and that pISE13 and pISE48 were identified because they encode OmpT and OmpC, respectively.

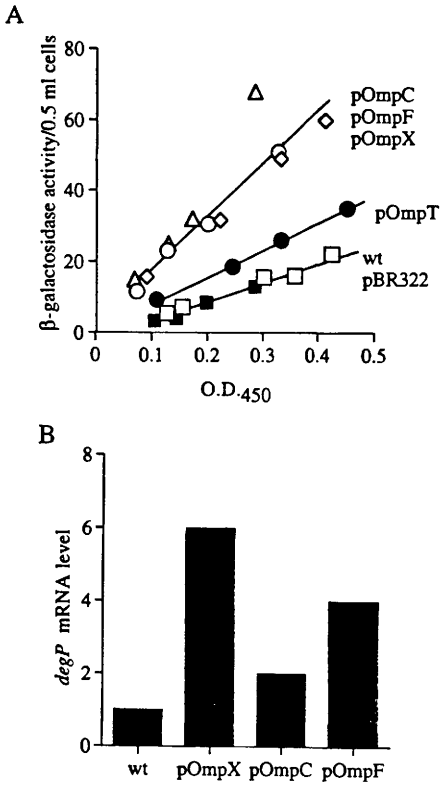

Figure 2. (*A*) Eσ^E activity in strains overexpressing OMPs from multicopy plasmids. β-Galactosidase activity in the parental strain (□) was compared with strains containing either pMY111 (OmpC, △), pMY222 (OmpF, ○), pJE100 (OmpX, ◇), pML21 (OmpT, ●), or pBR322 (■). The data shown are representative of at least three experiments. The increase in *rpoHP3* activity was 3- to 8-fold for strains overexpressing OmpC, OmpF, and OmpX and 1.4- to 2-fold for the strain overexpressing OmpT. The r^2 values (variance) for a linear fit of the points from each strain are all >0.975 for the data in this experiment. (*B*) *degP* mRNA levels in MC1061Φλ[*rpoHP3–lacZ*] and isogenic strains overexpressing OmpC, OmpF, or OmpX as assayed by S1 mapping. RNA was purified from cells grown at 30°C and harvested at an OD_{450} of 0.3–0.4. Values were normalized to wild type and are averages of two to five measurements.

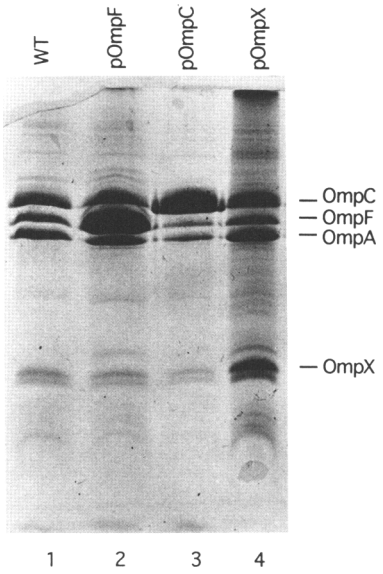

Figure 3. OMP profiles of MC1061Φλ[*rpoHP3–lacZ*] cells and isogenic strains containing multicopy plasmids expressing OMPs. Equivalent amounts of cells were loaded in each lane. (Lane *1*) Proteins from wild-type cells; (lane *2*) pOmpF (pMY222); (lane *3*) OmpC (pMY111); (lane *4*) OmpX (pJE100).

Underproduction of OMPs decreases Eσ^E activity

In the screen designed to generate loss-of-function mutations that caused a decrease in Eσ^E activity, one locus was identified 16 independent times (Table 1). To determine the gene inactivated by the mini-Mu transposon, one transposon with flanking sequences was cloned. Restriction mapping indicated that the insertion was located in *ompR*, a transcriptional activator of two major OM porins, OmpC and OmpF (Forst et al. 1988; Mizuno and Mizushima 1990). As expected, isolation of OMs from cells containing this insertion showed little OmpC and OmpF (data not shown). We considered two possible explanations for the decrease in Eσ^E activity of these mutants. Eσ^E activity could be responding to the decreased amount of OMPs present in the strain. Alternatively, Eσ^E activity could depend on a pathway that is dependent on OmpR transcriptional activity. These two possibilities can be distinguished by overexpressing OmpX in a Δ*ompR* strain. According to the first hypothesis, Eσ^E activity will be high, whereas in the second, it

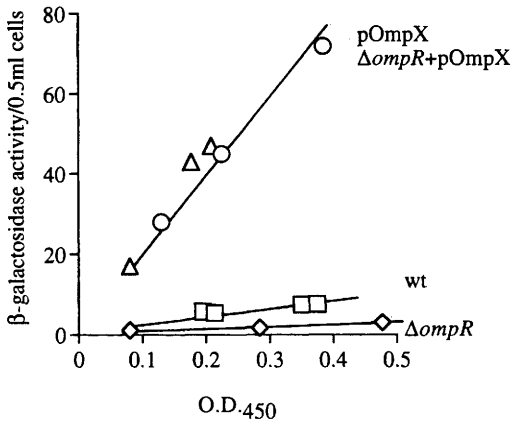

Figure 4. Overexpression of OmpX induces $E\sigma^E$ activity independent of *ompR*. β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) was compared with isogenic strains containing Δ*ompR* (◇), overproducing OmpX (△), or overproducing OmpX in a Δ*ompR* background (○). β-Galactosidase activity in Δ*ompR* strain was three- to five-fold lower than the wild-type strain, and activity in the Δ*ompR* + pJE100 strain was three- to six-fold higher than wild type in three experiments (r^2>0.96).

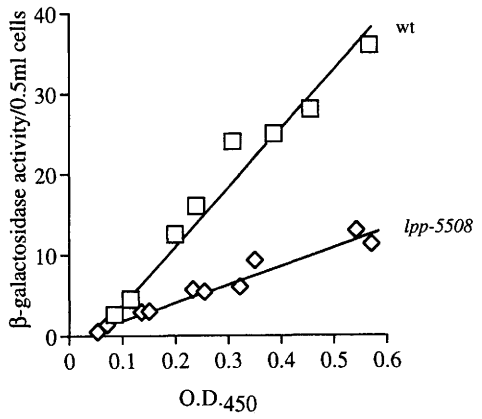

Figure 5. β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with an isogenic strain containing *lpp5508* (◇). The difference in β-galactosidase activity ranged from three- to fivefold in two experiments (r^2>0.95).

will remain low. As shown in Figure 4, $E\sigma^E$ activity is induced by overproduction of OmpX in a Δ*ompR* background. Thus, OmpR is not a transcriptional activator for $E\sigma^E$. Instead, $E\sigma^E$ activity appears to correlate with the amount of OMPs produced.

We asked whether decreasing the levels of other OMPs also affected $E\sigma^E$ activity. Lpp is the major OM lipoprotein in *E. coli* (Hirota et al. 1977). In an *lpp5508* background Lpp is undetectable, and the *E. coli* OM is less stable and more sensitive to low amounts of detergents and chelating reagents. $E\sigma^E$ activity was decreased in an *lpp5508* background (Fig. 5), supporting the idea that $E\sigma^E$ activity is reduced in cells with decreased OMP content.

Cellular localization of signal-inducing $E\sigma^E$ activity by overexpression of OMPs

To further understand how production of OMPs affects $E\sigma^E$ activity, the location of the signal generated by overproducing OMPs was determined. First, the location of the bulk of the overproduced OmpX was determined by fractionation of the cell into cytoplasmic, inner membrane, periplasm, and OM components. As expected, all detectable OmpX was in the OM (Fig. 6). This result, however, does not exclude the possibility that an undetected pool of OmpX was mislocalized or jammed in the secretion apparatus and, thus, caused the increase in $E\sigma^E$ activity. The following experiments were done to test these possibilities.

If OMP precursors in the cytoplasm generate a signal that induces $E\sigma^E$ activity, then cells that accumulate OMP precursors in the cytoplasm should have a high level of $E\sigma^E$ activity. In fact, the converse was observed. In a *secB* deletion strain, partially folded OMP precursors accumulate in the cytoplasm (Kumamoto 1991), but $E\sigma^E$ activity was reduced twofold (Fig. 7A). Thus, accumulation of OMP precursors in the cytoplasm does not induce $E\sigma^E$ activity.

Overproduction of OMPs could titrate components of the inner membrane secretion apparatus, thereby generating a signal to increase $E\sigma^E$ activity. Because periplasmic proteins and OMPs share some cytoplasmic and inner membrane components of the secretion apparatus, overproduction of periplasmic proteins should also titrate these components of secretion apparatus. $E\sigma^E$ activity, however, was unaffected when periplasmic proteins DegP or β-lactamase were overexpressed from high-copy-number plasmids (Fig. 7B). Western blot anal-

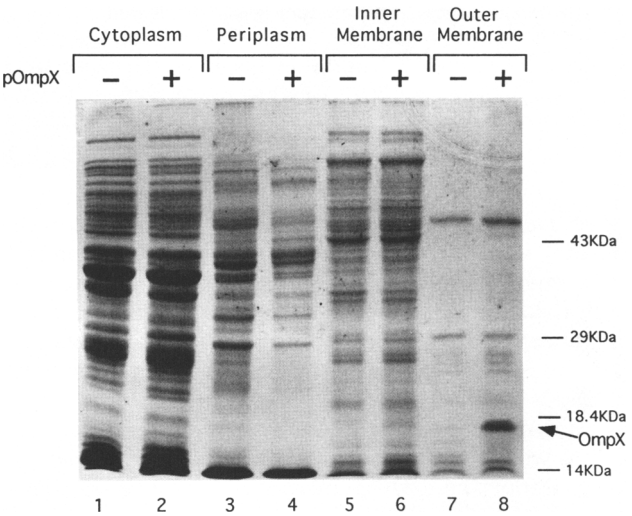

Figure 6. Protein profiles in different cell compartments in MC1061Φλ[*rpoHP3–lacZ*] wild-type cells and the otherwise isogenic strain overproducing OmpX (pJE100). Cells were fractionated as described in Materials and methods. Cytoplasmic (lanes *1,2*), periplasmic (lanes *3,4*), inner membrane (lanes *5,6*), and OMPs (lanes *7,8*), from wild type (lanes *1,3,5,7*) and cells containing pJE100 (lanes *2,4,6,8*) were electrophoresed on a SDS–polyacrylamide gel. The OmpC and OmpF proteins were not resolved because this gel did not contain urea.

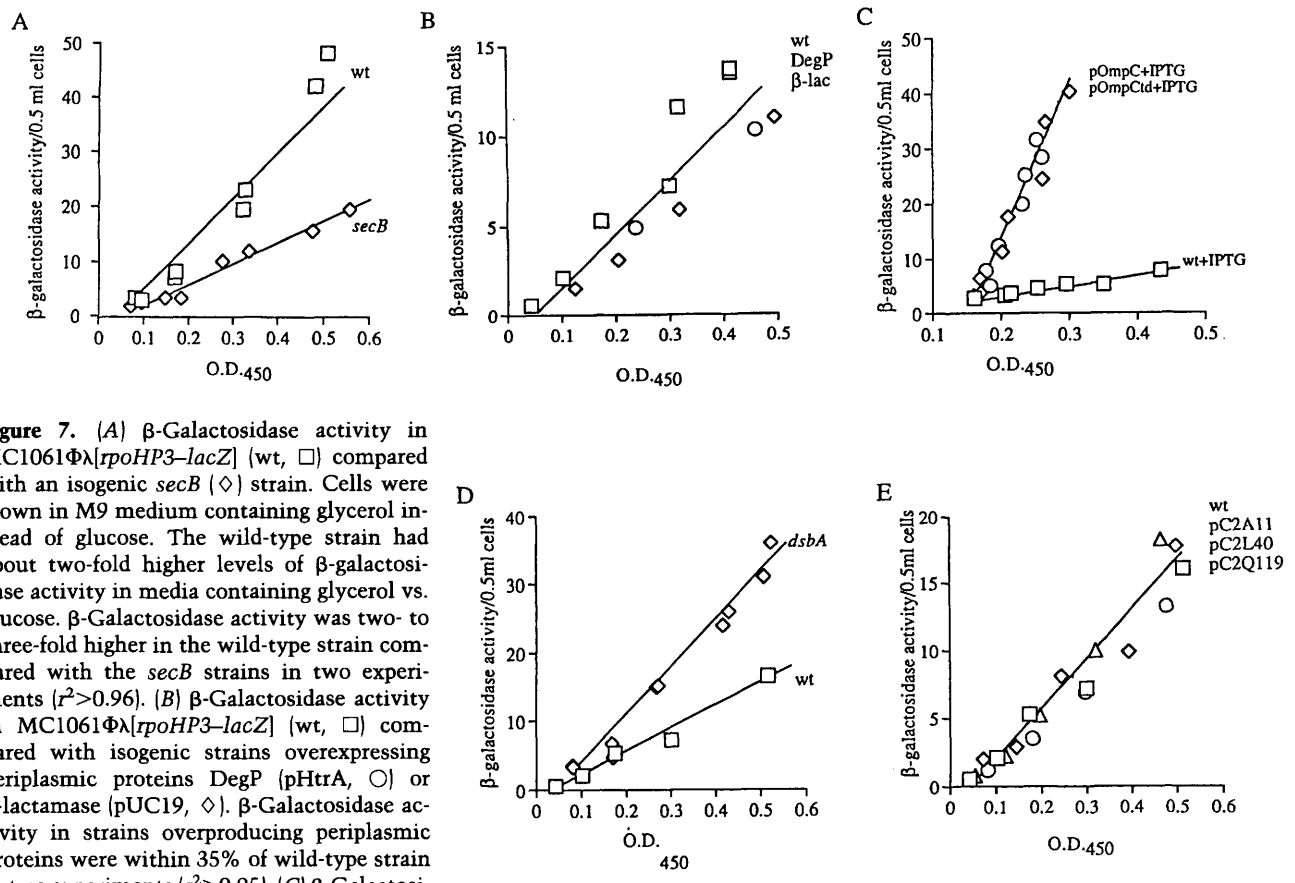

Figure 7. (*A*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with an isogenic *secB* (◇) strain. Cells were grown in M9 medium containing glycerol instead of glucose. The wild-type strain had about two-fold higher levels of β-galactosidase activity in media containing glycerol vs. glucose. β-Galactosidase activity was two- to three-fold higher in the wild-type strain compared with the *secB* strains in two experiments (r^2>0.96). (*B*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with isogenic strains overexpressing periplasmic proteins DegP (pHtrA, ○) or β-lactamase (pUC19, ◇). β-Galactosidase activity in strains overproducing periplasmic proteins were within 35% of wild-type strain in two experiments (r^2>0.95). (*C*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with isogenic strains overexpressing IPTG-inducible OmpC (pGMC1, ○) or OmpCtd (pKMCtd, △). Strains were grown to an OD_{450} of 0.15, and IPTG was added to a final concentration of 1 mM. After addition of IPTG, the rate of β-galactosidase synthesis in strains overexpressing OmpC and OmpCtd increased 10- to 15-fold compared with the wild-type strain in three experiments. Growth of the pOmpCtd overproducing strain stopped 35 min after IPTG addition, whereas growth of the OmpC overproducing strain slowed immediately after IPTG addition (r^2>0.91). (*D*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with an isogenic strain containing *dsbA* (◇). The *dsbA* strain had 1.5- to 2.5-fold higher β-galactosidase activity than the wild-type strain in three experiments (r^2>0.97). (*E*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with isogenic strains containing CycA–AP fusion proteins, pC2A11 (△), pC2L40 (○), and pC2Q119 (◇). β-Galactosidase activity in strains expressing CycA–AP fusion proteins were within 25% of the wild-type strain (r^2>0.93).

ysis of DegP confirmed that expression from a multicopy plasmid increased the level of both the precursor and processed forms of DegP (data not shown). In addition, overproduction of periplasmic proteins MalE and alkaline phosphatase had no effect on $E\sigma^E$ activity (data not shown). These experiments suggest that overproduction of OMPs does not generate a signal for $E\sigma^E$ activity by titrating components of the secretion apparatus that are shared with periplasmic proteins. Furthermore, $E\sigma^E$ activity is not induced by increases in periplasmic proteins.

After transport through the inner membrane, the secretion pathways of periplasmic and OMPs diverge. Therefore, overproduced periplasmic proteins may not be in the same environment as overproduced OMPs. To specifically ask whether $E\sigma^E$ activity is induced by OMPs that are mislocalized in the periplasm or jammed in the secretion apparatus, $E\sigma^E$ activity was examined in a strain containing an OmpC variant that lacks 2 amino acids in the mature portion of the protein, OmpCtd (Catron and Schnaitman 1987). This variant is secreted through the inner membrane but is poorly incorporated in the OM and inhibits incorporation of wild-type OmpC, OmpF, and OmpA into the OM. Induction of OmpCtd increases the activity of $E\sigma^E$ 10- to 15-fold (Fig. 7C). Thus, $E\sigma^E$ activity is induced under conditions that slow down the processing of OMPs after they have passed the inner membrane but before insertion in the OM.

Overproducing OMPs could cause them to aggregate or fold more slowly, resulting in accumulation of misfolded proteins. The *E. coli dsbA* gene product was shown recently to facilitate disulfide bond formation of proteins in both the OM (OmpA) and the periplasmic space (AP) (Bardwell et al. 1991). $E\sigma^E$ activity at the *rpoHP3* promoter in a Δ*dsbA* background was induced about twofold (Fig. 7D). A similar effect on a *degP–lacZ* fusion has been observed by P. Danese and T. Silhavy

(pers. comm.). This suggests that $E\sigma^E$ activity may be induced by misfolded OMPs or periplasmic proteins.

Finally, to test whether misfolded periplasmic proteins induce $E\sigma^E$ activity, $E\sigma^E$ activity was tested in strains expressing protein fusions between portions of *Rhodobacter sphaeroides* CycA, a periplasmic *c*-type cytochrome (not found in *E. coli*), and alkaline phosphatase. These protein fusions are exported to the *E. coli* periplasm, and the CycA portion is then rapidly degraded. This indicates that these hybrid proteins do not fold properly (Brandner et al. 1991). Expression of these misfolded periplasmic proteins did not alter $E\sigma^E$ activity (Fig. 7E).

Altering levels of the DegP protease has no effect on $E\sigma^E$ activity

Results with the Δ*dsbA* strain suggested that $E\sigma^E$ activity may be induced by misfolded OMPs. Although the substrates for the periplasmic DegP protease are unknown, these could include misfolded proteins or other molecules involved in signaling $E\sigma^E$ activity. Two experiments were designed to determine whether DegP levels, and presumably activity, influenced $E\sigma^E$ activity. A Δ*degP* strain should accumulate DegP substrates; however, $E\sigma^E$ activity was unaffected (Fig. 8A). This indicates that either accumulation of DegP substrates does not induce $E\sigma^E$ activity or that other uncharacterized periplasmic proteases compensate for the lack of DegP.

Overproduced OMPs that induce $E\sigma^E$ activity could also be potential substrates for DegP. Thus, an increase in DegP in an OmpC overproducing strain might decrease $E\sigma^E$ activity. However, $E\sigma^E$ activity remained high in a strain with both DegP and OmpC encoded by multicopy plasmids (Fig. 8B). Thus, the signal generated by overproducing OmpC is not directly affected by the level of DegP protease.

OMP levels do not affect expression from $E\sigma^{32}$ promoters

Environmental stresses known to induce $E\sigma^E$ activity (temperature upshift and exposure to ethanol) also induce $E\sigma^{32}$ activity. It was not clear, however, whether all conditions that increase $E\sigma^E$ activity also increase $E\sigma^{32}$ activity. To test this, $E\sigma^{32}$ activity was assayed in a strain containing the *rpoDpHS* promoter fused to *lacZ* on a prophage (Wild et al. 1992) under conditions that alter OMP levels. Neither the plasmids increasing OMP levels nor chromosomal mutations in *ompR* or *dsbA* had any effect on $E\sigma^{32}$ activity (Fig. 9A). As reported previously, a *secB* mutation induced $E\sigma^{32}$ activity fourfold (Wild et al. 1992a), presumably because a buildup of partially folded proteins in the cytoplasm activates the σ^{32} regulon (Fig. 9B). Thus, changes in the levels of OMPs affects only the $E\sigma^E$ regulon.

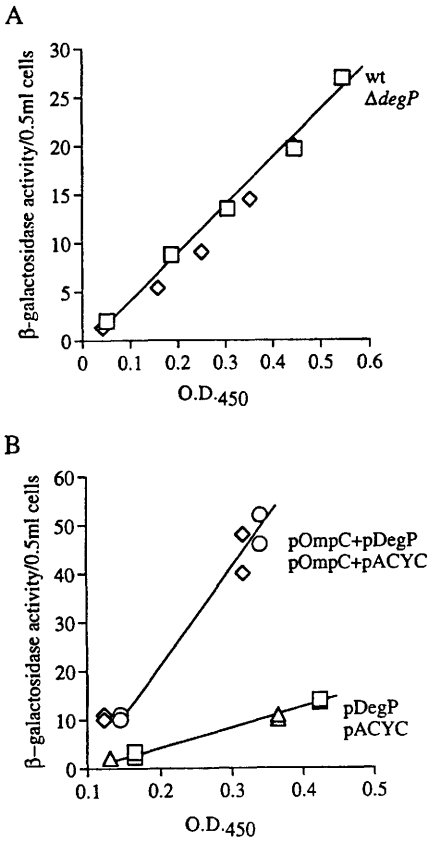

Figure 8. (*A*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] (wt, □) compared with an isogenic strain containing *degP* (◇). β-Galactosidase activity of these two strains was within 10% in three experiments (r^2>0.98). (*B*) β-Galactosidase activity in MC1061Φλ[*rpoHP3–lacZ*] isogenic strains overexpressing OmpC (pMY111, ◇), OmpC, and DegP (pMY111 and pKS17, ○) compared with cells overexpressing DegP (pSK17, △), or wild-type cells (pACYC184, □). β-Galactosidase activity between cells overproducing OmpC or OmpC and DegP was within 10%; these cells had four- to sixfold more β-galactosidase activity than cells overproducing DegP alone or wild-type cells (r^2>0.97).

Discussion

We describe the results of experiments designed to identify positive regulators of $E\sigma^E$ activity in *E. coli*. Initially, two genetic strategies were used to allow identification of a broad range of regulators because each approach has limitations. The screen for loss-of-function mutants fails to identify regulators that are essential, are not required for growth at high temperature, or have redundant functions. The multicopy selection fails to identify genes that cannot be cloned on multicopy vectors or gene products with activity that does not increase with increasing gene dosage. The initial results from these complementary approaches indicate that $E\sigma^E$ activity changes when the level of OMPs is altered. On the basis of these observations, we showed that increasing the amount of OmpX, OmpT, OmpF, or OmpC increases $E\sigma^E$ activity. Furthermore, mutations that increase the amount of misfolded OMPs or reduce the efficiency of OMP incorporation into the OM induce $E\sigma^E$ activity. Decreasing specific OMPs, Lpp or OmpF and OmpC, or decreasing

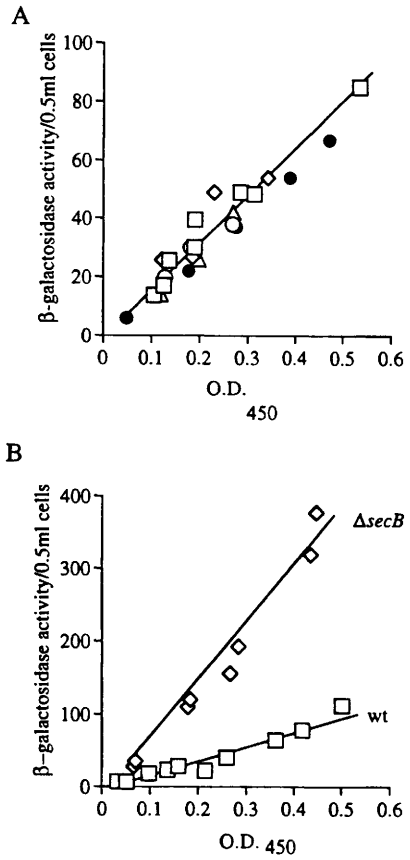

Figure 9. (*A*) β-Galactosidase activity in MC1061Φλ[*rpoDpHS–lacZ*] (wt, □) compared with isogenic strains overexpressing OmpC (pMY111, ◇) or OmpX(pJE100, △), or with insertions in *ompR* (○) or *dsbA* (●). Differences in β-galactosidase activity among these strains were within 20% (r^2>0.95). (*B*) β-Galactosidase activity in MC1061Φλ[*rpoDpHS–lacZ*] (wt, □) compared with an isogenic strain containing *secB* (◇). The *secB* strain had four-fold higher β-galactosidase activity than the wild-type strain in two experiments (r^2>0.94).

OMP levels indirectly by SecB limitation reduces $E\sigma^E$ activity. Thus, one signal that modulates $E\sigma^E$ activity is linked to production of OMPs.

Where in the cell do overproducing OMPs generate a signal?

Although all of the detectable overproduced wild-type OMPs are in the OM, the signal need not originate there. OMPs transverse several cellular compartments before insertion into the OM (for review, see Pugsley 1993). They are synthesized as precursor proteins containing an amino-terminal signal sequence, maintained in a translocation-competent form by interaction with cellular chaperones such as SecB, targeted to the membrane by interaction with SecA, transported through the cytoplasmic membrane by the SecY/E translocase, and cleaved by a signal peptidase as they exit the secretion machinery. These steps are common to both periplasmic proteins and OMPs. OMPs are then localized to the OM either through a periplasmic intermediate (Sen and Nikaido 1990) or by direct transfer to the OM at attachment sites (Bayer et al. 1982). This latter process is not well understood. The signal generated by gross changes in production of OMPs could be located in any of these compartments.

Our results suggest that the signal controlling $E\sigma^E$ activity is not generated during the early steps in the translocation of OMPs. A Δ*secB* strain accumulates precursors of periplasmic proteins and OMPs in the cytoplasm (Kumamoto 1991) but does not induce $E\sigma^E$ activity. This suggests that the signal generated by overproducing OMPs does not result from accumulation of cytoplasmic precursor proteins or from *secB* limitation. Overexpression of proteins normally located in the periplasm also does not induce $E\sigma^E$ activity. Because periplasmic proteins use the same translocase as OMPs (Pugsley 1993), it is unlikely that overproduced OMPs generate a signal by titrating the translocase. Finally, expression of foreign proteins localized to the periplasm or accumulation of DegP substrates does not alter $E\sigma^E$ activity. This suggests that a signal is not generated by aberrant periplasmic proteins. However, overexpressing OmpCtd (Catron and Schnaitman 1987), an aberrant OMP that is translocated but poorly incorporated into the OM, does induce $E\sigma^E$. On the basis of these observations, we propose that the signal for $E\sigma^E$ originates after the translocation pathways of OMP and periplasmic proteins diverge. The signal could be generated as the OMP exits the cytoplasmic membrane, in the periplasm, or in the OM. The extracellular location of the signal necessitates that a signal-transduction pathway exists to propagate the signal back through the inner membrane to influence the activity of $E\sigma^E$ in the cytoplasm.

What is the signal?

Although we have not quantitatively measured OMPs, it is known that the total amount of OMPs in the membrane can change by as much as 30% (Diedrich and Fralick 1982). Our data suggest that the total amount of OMPs produced directly correlates with $E\sigma^E$ activity. We consider two different ways in which varying the amount of OMP production could generate a signal that modulates $E\sigma^E$ activity. First, changing the amount of OMP production could change the composition of the OM. Alternatively, if we assume that a change in OMP levels reflects a change in their rate of synthesis, a change in the flow rate of OMPs through the system that targets OMPs to the OM could generate a signal.

In the first scenario, the composition of the OM could vary by changing either the absolute amount or the relative ratios of individual OMPs. Altering the absolute amount of OMPs could change the ratio of protein to either lipid or peptidoglycan and generate a signal, possibly by changing the structural integrity or fluidity of the cell envelope. However, to the extent that we have assayed this, $E\sigma^E$ is not sensing structural integrity of the cell envelope. Both the *lpp5508* mutation (Hirota et al. 1977) and overproduction of OmpX, OmpC, and OmpF

(data not shown) result in increased sensitivity to detergents and chelating reagents, yet these alterations have opposite effects on $E\sigma^E$ activity. Alternatively, the signal could be generated by a change in relative ratios of individual OMPs. Overproducing some OMPs leads to underrepresentation of other OMPs in the OM (Click et al. 1988). If one OMP negatively regulates $E\sigma^E$ activity, then overproducing other OMPs could decrease its level whereas underproducing other OMPs could increase its level. Although such a protein may exist, we were unable to identify one protein that varied consistently with $E\sigma^E$ activity (data not shown). For instance, OmpA increases in the Δ*ompR* strain and decreases in a Δ*secB* strain, yet both strains had decreased $E\sigma^E$ activity.

A second way of generating the signal modulating $E\sigma^E$ activity can be derived by considering the pathway that targets OMPs to the OM (Bayer et al. 1982; Sen and Nikaido 1990; Pugsley 1993). OMPs must be sorted from periplasmic proteins, transported to the OM, and folded properly. Although the specific components of this pathway are poorly understood, the pathway is likely to involve chaperones to maintain OMPs in appropriate protein conformations, molecules that target OMPs to the OM, and possibly molecules that insert OMPs in the membrane. A change in the flow rate of OMPs through this pathway could generate the signal modulating $E\sigma^E$ activity. For example, a chaperone involved in the transport of proteins to the OM could also interact with the signal-transduction pathway modulating $E\sigma^E$ activity. Overproduction of OMPs could titrate or alter the activity of this chaperone and signal for an increase in $E\sigma^E$ activity, whereas underproduction of OMPs would have the converse effect.

Why are both the $E\sigma^E$ and $E\sigma^{32}$ regulons induced by heat?

We believe that the answer to this question arises from a consideration of signals that uniquely induce each regulon. Conditions that increase the amounts of unfolded, partially folded, or misfolded proteins in the cytoplasm induce $E\sigma^{32}$ but not $E\sigma^E$. Conversely, conditions that increase OMPs in compartments outside the cytoplasm induce $E\sigma^E$ but not $E\sigma^{32}$. Two distinct regulons may exist to allow cells to respond to processes or signals that are unique to each compartment. Thus, just as the products of the $E\sigma^{32}$ regulon are involved in responding to environmental stimuli affecting the processing of cytoplasmic proteins, the products of the $E\sigma^E$ regulon could be involved in responding to environmental stimuli affecting the OM or the processing of OMPs. Although $E\sigma^E$ transcribes *rpoH*, transcription from the $E\sigma^E$ promoter accounts for <5% of total transcription of *rpoH* at 30°C (Erickson et al. 1987) Furthermore, $E\sigma^{32}$ activity is posttranscriptionally regulated (Straus et al. 1987, 1989). Thus, increases in $E\sigma^E$ activity do not necessarily increase $E\sigma^{32}$ activity at moderate temperatures.

In Gram-negative bacteria, the composition of the OM changes in response to many environmental conditions including temperature, osmolarity, dessication, starvation, and growth in other hosts. The products of the $E\sigma^E$ regulon could be involved in responding to environmental changes that affect processing of OMPs. In addition to the periplasmic location of DegP, circumstantial evidence exists for an extracytoplasmic role of the products of the $E\sigma^E$ regulons. $E\sigma^E$ recognizes promoters for two secreted hydrolytic enzymes (Erickson and Gross 1989), *dagA* (Buttner et al. 1988) and *phlA1* (Givskov et al. 1988) from *Streptomyces coelicolor* and *Serratia liquefaciens*, respectively. If σ^E homologs in *S. coelicolor* and *S. liquefaciens* are involved in transcription of these genes, the $E\sigma^E$ regulon may consist of extracytoplasmic proteins that are involved in altering the protein composition of the OM, periplasm, or extracellular space. The *degP* homolog in *Salmonella* (*htrA*) plays a role in virulence and appears to have an $E\sigma^E$ promoter (Johnson et al. 1991). This suggests the exciting possibility that $E\sigma^E$ activity may be induced during growth in and colonization of animals.

An appealing, but speculative, idea is that these two σ-factors may have complementary but distinct functions; both may be involved in protein processing—the $E\sigma^{32}$ regulon in the cytoplasm and the $E\sigma^E$ regulon in the extracytoplasmic compartments. It is clear that $E\sigma^{32}$ is induced by misfolded proteins in the cytoplasm; our data indicate that $E\sigma^E$ may be induced by misfolded OMPs in other compartments. We and others (P. Danese and T. Silhavy, pers. comm.) have shown that the Δ*dsbA* mutation, which creates misfolded or partially folded proteins in the periplasm and OM, induces $E\sigma^E$. A second $E\sigma^E$ inducer, OmpCtd, may cause accumulation of misfolded or improperly localized OMPs in an extracellular compartment. Finally, we have argued that overproducing OMPs may induce $E\sigma^E$ activity by saturating the pathway that inserts OMPs into the OM, thus increasing the pool of incompletely folded proteins in the extracytoplasmic compartments of the cell.

That two *E. coli* σ-factors may be induced by similar molecular cues in different cellular compartments is reminiscent of the situation in eukaryotic cells where unfolded or damaged proteins in different compartments or organelles induce chaperones by parallel but independent mechanisms. Unfolded proteins in the cytoplasm induce the heat shock response, possibly by titrating out Hsp70 (Craig and Gross 1991). Unfolded proteins in the endoplasmic reticulum induce a set of proteins residing in this compartment by interacting with BIP, an Hsp70 family member found exclusively in the endoplasmic reticulum (Kozutsumi et al. 1988; Mori et al. 1992). Future experiments addressing the exact nature of the signal that modulates $E\sigma^E$ activity and how the signal is transduced back to the cytoplasm should provide biologists with insights into how different cell compartments respond to similar environmental or physiological cues.

Materials and methods

Media and strains

All media were prepared as described (Miller 1972). Liquid cultures were grown in M9 glucose supplemented with vitamins

and all 20 common amino acids, except for experiments involving the *secB::kan* allele. In those experiments, both wild-type and *secB::kan* strains were grown in M9 glycerol supplemented with vitamins and all 20 common amino acids. Solid media used were MacConkey lactose, MacConkey galactose, LB, and M9 glycerol supplemented with amino acids and vitamins. Drug concentrations were 50 μg/ml of ampicillin, 30 μg/ml of kanamycin (Kan), 10 μg/ml of tetracycline (Tet), and 20–75 μg/ml of chloramphenicol (Cam).

CAG16028 Δ(*araCOIBA*, *leu*)*7696*, *araD139*, *galK*, *galU*, *hsdR*, Δ(*lac*)*X74*, *recA56*, *rpsL*, a derivative of MC1061 was used for making the RNA for the S1 assays. Most β-galactosidase assays were done in CAG16028 lysogenized with either the Φλ[*rpoHP3–lacZ*] resulting in CAG16037 or Φλ[*rpoDpHS–lacZ*] resulting in CAG16074 (Wild et al. 1992). CAG16038 was a derivative of C600 *galK* containing pJEK61 (Erickson and Gross 1989). CAG16040 was a derivative of C600 *galK* containing plasmid with PgalK driving *galK* expression. For identification and initial characterization of the pISE plasmids, M8820 MuCts (Groisman and Casadaban 1986) was lysogenized with λΦ[*rpoHP3–lacZ*] generating CAG16045 and transformed with P3CAT generating CAG16052 to select for increased $E\sigma^E$ activity. The *secB::kan* allele (Kumamoto and Beckwith 1985) was introduced into CAG16037 and CAG16074 using P1 transduction. To facilitate manipulation of the *lpp5508* allele (Hirota et al. 1977), a Tn*10*tet (*zdh-925*::Tn*10*) was linked to this mutation (Singer et al. 1989). The linked Tet^R was then transferred to CAG16037 or CAG16074 by P1 transduction. The presence of the *lpp5508* allele was determined by increased sensitivity to SDS and EDTA (Hirota et al. 1977). The *dsbA::kan* (Bardwell et al. 1991) and *degP:: kan* (Strauch and Beckwith 1988) alleles were transferred to CAG16037 and CAG16074 by P1 transduction.

Plasmids and phage

The following plasmids were used to express the proteins indicated in parentheses and have been described previously: pKS17 (DegP) (Strauch and Beckwith 1988), pMY111 (OmpC) (Mizuno et al. 1983), pMY222 (OmpF) (Ramakrishnan et al. 1985), pML21 (OmpT) (Grodberg et al. 1988), pC2A11, pC2L40, and pC2Q119 (CycA–AP fusions) (Brandner et al. 1991), pGMC1 (P_{tac}-OmpC) and pKMCtd (P_{tac}-OmpCtd) (Catron and Schnaitman 1987), and pHtrA (DegP) (Lipinska et al. 1988). To make pP3CAT, which was used in the multicopy selection, a 37-bp fragment of the P3 promoter of *rpoH* (Erickson and Gross 1989) was cloned into pKK232-8 (Pharmacia), resulting in pJM14. A 2.4-kb *Hgi*AI fragment of pJM14 was cloned into the 2.8-kb *Bsa*A1 fragment of pACYC184 resulting in pP3CAT. pJEK61 (Erickson and Gross 1989) was used in the screen for loss-of-function mutants. The pISE plasmids and the parental pEG5005 plasmids were generated as described (Groisman and Casadaban 1986). pJE100 is described (J. Mecsas, R. Welch, J. Erickson, and C.A. Gross, in prep.). The Φλ[*rpoHP3–lacZ*] was constructed by cloning the *SalI–Eco*RI fragment of pJEK61 into pRS415 and crossing the resulting plasmid with λRS45 as described (Simons et al. 1987). Φλ[*rpoDpHS–lacZ*] was described (Wild et al. 1992b).

Genetic selections

To select for genes that increase $E\sigma^E$ activity when overexpressed, a multicopy library was made using a mini-Mu system (Groisman and Casadaban 1986). CAG16052 was infected with a high-titer lysate containing this library, and appropriate dilutions were plated on LB plates supplemented with Kan and 20–75 μg/ml of Cam. Approximately 7000 Kan^R colonies were plated on a total of four plates at each Cam concentration. Unexpectedly, the presence of the pEG5005 vector sequences caused an increase in the resistance of CAG16052 to Cam; thus, the efficiency of plating was higher than expected. Cells that grew faster than the CAG16052 + pEG5005 strain were picked: Eighteen colonies were picked on 20 μg/ml; 14 colonies were picked on 40 μg/ml; and 8 colonies were picked on 50 μg/ml and 75 μg/ml. Colonies were purified twice; plasmid DNA was isolated and used to transform CAG16045 to show that the increase in $E\sigma^E$ activity was linked to the plasmid. Of the original 48 colonies picked, 22 resulted in an increase in $E\sigma^E$ activity at both the *rpoHP3* and *degP* promoters and were mapped to the Kohara phage.

To generate cells with insertions in positive regulators of genes for $E\sigma^E$ activity, mini-Mu transposon mutagenesis was performed as described (Castilho et al. 1984) using the *Mud*II1734 transposon. CAG16038 was infected with a mini-Mu lysate, plated on MacConkey plates containing galactose and Kan, and grown at either 20°C or 30°C. White Kan^R colonies were patched onto MacConkey–galactose plates and grown at 30°C and 44°C. Mutants that grew poorly at 44°C were taken from the 30°C plate, colony purified, and tested for Mu^S. To test that the insertion specifically reduced $E\sigma^E$ activity, P1 was grown on the white, temperature-sensitive, Mu^S cells and the lysate was used to transduce CAG16038 or CAG16040 to Kan^R. Only Kan^R insertions that conferred the $galK^-$ phenotype in CAG16038 (which carries *galK* under $E\sigma^E$ control) but not CAG16040 (which carries *galK* under $E\sigma^{70}$ control) were studied further. Approximately 22,500 Kan^R cells were screened (8000 at 20°C and 14,500 at 30°C); 3% of the Kan^R cells were white; 1% of the white cells were temperature sensitive; 50% of the temperature sensitive cells were Mu^S; 16 colonies had mini-Mu insertions that gave rise to white, Kan^R colonies when transduced into CAG16038 and red when transduced into CAG16040. The location of one insertion was mapped genetically using high frequency recombination F-factors (Hfrs) and linked Tn*10*tet (Singer et al. 1989). All of the other insertions were linked to the same Tn*10*. To identify the gene into which the mini-Mu inserted, chromosomal DNA from one insertion strain was isolated and digested with either *Hin*dIII or *Bam*HI. These enzymes do not cleave the kan^R gene allowing for cloning of adjacent chromosomal DNA. Digested DNA was ligated to pUC19 with either *Hin*dIII or *Bam*HI and transformed into DH5α. Plasmids from Kan^R colonies were analyzed with various restriction enzymes and compared with the restriction map of the *E. coli* chromosome between 74 and 76 min using the GeneScape program (Bouffard et al. 1992). The restriction pattern matched that of the *ompB* locus and showed that the *Mud*II1734 transposon had inserted 100 bp after the translational start site of the *ompR* gene.

Mapping pISE inserts and location of dse

A nylon membrane containing a subset of 466 phage from the collection of Kohara (Kohara et al. 1987) was obtained from Takara Biochemical. The plasmids were nick-translated, and hybridization, washing, and stripping of the membrane were done as recommended by Takara Biochemical.

β-Galactosidase assays

β-Galactosidase levels were measured as described (Miller 1972). Overnight cultures were diluted 1/1000 or to an OD_{450} of 0.004 in the appropriate medium and grown at 30°C. Cells (0.5 ml) were lysed with chloroform and one drop of 0.1% SDS. For line graphs, β-galactosidase activity was calculated by

$([A_{420(final)} - A_{420(initial)}]/(\text{min of reaction with ONPG}) \times 1000$ and plotted as a function of OD_{450} of the cells. Values in Figure 1 were divided by the A_{450} and normalized to the wild-type case. Although the level of β-galactosidase activity varied as much as threefold from day to day, all strains were affected equally; thus, the relative values remained constant. This unusually large variation could be the result of subtle changes in culture conditions that influence $E\sigma^E$ activity.

S1 mapping

pHtrA was used as the source of probe for the S1 mapping experiments as described (Lipinska et al. 1988). RNA harvesting (Barry et al. 1980) and S1 mapping (Maniatis et al. 1982) were performed as described. A constant amount of RNA was used in each assay. To control for losses during manipulations in the S1 assay, we used a second probe, pRL385, to a portion of the *rpoB* gene (Landick et al. 1990). Samples were electrophoresed on a 5% polyacrylamide–50% urea gel, and the gel was dried. To quantitate transcription from the *degP* promoter, radioactivity in the *degP* and *rpoB* transcripts was counted using an Ambis scanner. Changes in transcription from the *degP* promoter were calculated by [cpm $degP_{(mutant)}$/cpm $rpoB_{(mutant)}$]/[cpm $degP_{(wild\ type)}$/cpm $rpoB_{(wild\ type)}$].

Fractionation of cellular components

Protein composition of the inner membrane and OM was analyzed using their differential solubility in sarcosyl. Ten milliliters of cells growing in LB medium were harvested at an OD_{600} of 1. Cell pellets were resuspended in 500 μl of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA and transferred into an Eppendorf tube. Cell walls were digested with lysozyme (100 μg/ml) on ice for 10 min. $MgCl_2$ (10 mM) and DNase I (50 μg/ml) were added, and the spheroplasts were lysed by three freeze–thaw cycles. The lysate was centrifuged for 10 min at 15,000*g*. The supernatant (cytoplasmic and periplasmic fractions) was removed, and the pellet (crude membrane fraction) was washed with 500 μl of 20 mM $NaPO_4$ (pH 7.0). The inner membrane and OM were resolved by solubilizing specifically the inner membrane with 100 μl of 0.5% sarcosyl in 20 mM $NaPO_4$ for 30 min at room temperature. The insoluble OMs were pelleted by centrifugation for 10 min at 15,000*g*, washed with 100 μl of sarcosyl, centrifuged again, resuspended in SDS sample buffer, and boiled for 5 min. Proteins were analyzed on a 12% polyacrylamide–SDS gel. For Figure 3, the gel also contained 50% urea to resolve OmpC and OmpF. When periplasm and cytoplasm were separated, the cell wall was digested in 20% sucrose to prevent lysis of the spheroplasts (Brissette and Russel 1990).

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