EcfE, a new essential inner membrane protease: its role in the regulation of heat shock response in *Escherichia coli*

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We have identified a new protease in *Escherichia coli*, which is required for its viability under normal growth conditions. This protease is anchored in the inner membrane and the gene encoding it has been named *ecfE*, since it is transcribed by $E\sigma^E$ polymerase. Multicopy expression of the *ecfE* gene was found to turn down expression of both $E\sigma^E$ - and $E\sigma^{32}$ -transcribed promoters. Purified EcfE degrades both heat shock sigma factors RpoE and RpoH *in vitro*. EcfE has a zinc binding domain at the N-terminus, a PDZ-like domain in the middle and a highly conserved tripeptide, LDG, at the C-terminus. These features are characteristic of members of a new class of proteases whose activity occurs close to the inner membrane or within the inner membrane. Temperature-sensitive mutants of this gene were isolated mapping to the catalytic site and other domains that exhibited constitutively elevated levels of both heat shock regulons.

Keywords: EcfE/heat shock regulation/protease/RpoE/RpoH

Introduction

The heat shock response is a highly conserved phenomenon. In *Escherichia coli*, upon heat shock, synthesis of a number of proteins is induced rapidly. This response is under the control of two alternative heat shock sigma (σ) factors, RpoH and RpoE. RpoH controls the synthesis of >30 proteins, most of them involved in protein folding (DnaK/J, GroEL/S) and protein degradation, such as Lon and Clps proteins (for review, see Gross, 1996; Yura *et al.*, 2000).

The *rpoE* regulon is also induced by heat shock and controls the synthesis of >25 proteins (Dartigalongue *et al.*, 2001). Some of the products of RpoE-controlled genes are also involved in protein folding (*surA*, *fkpA*, *dsbC* and *skp*) and lipid biogenesis (*lpxD/A*, *rfaD* and others) (Dartigalongue *et al.*, 2001). It is now established that these two heat shock regulons seem to control and respond to protein misfolding in specific compartments. While *rpoH* responds to protein misfolding in the cytoplasm, *rpoE* responds to protein misfolding in the extracytoplasm (for review, see Missiakas and Raina, 1997; Raivio and Silhavy, 2000).

Both heat shock responses are tightly regulated at several levels. The heat shock response mediated by *rpoH* depends on the transient increase in the levels of RpoH. RpoH is normally very unstable with a half-life of <1 min (Tilly *et al.*, 1989; Morita *et al.*, 2000). Many factors contribute to this unusual instability. FtsH (HflB), an integral membrane protein, has been shown to be responsible for the proteolysis of RpoH (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995), based on the findings that the stability of RpoH is increased in *ftsH* mutants and that FtsH degrades RpoH *in vitro*. However, this FtsH-mediated degradation under *in vitro* conditions is extremely slow, and observed mostly for a 30-fold excess of protease over the substrate (Tomoyasu *et al.*, 1995). Further, it has been suggested that FtsH-mediated turnover required the C-terminus of RpoH (Blaszczak *et al.*, 1999). However, genetic experiments do not support a role for the C-terminus in its *in vivo* stability (Nagai *et al.*, 1994; Arsene *et al.*, 1999; Morita *et al.*, 1999).

Further genetic studies showed that *hslV/U* (*clpQ/Y*) gene products are also involved in modulation of the *rpoH*-controlled heat shock response (Missiakas *et al.*, 1996). HslV/ClpQ shares homology with β -type catalytic subunits of the 20S proteasome. It has been further shown that the HslV/U complex can degrade RpoH *in vitro* (Kanemori *et al.*, 1999). *In vivo*, HslV/U, ClpA/P and Lon participate in the turnover of RpoH (Kanemori *et al.*, 1997), but it is difficult to evaluate the relative contribution of these proteases (for review, see Yura *et al.*, 2000).

The availability of other alternative σ factors is also tightly controlled. For example, *rpoE* is controlled at the transcriptional level via two promoters. One of them is autoregulated (Raina *et al.*, 1995; Rouviere *et al.*, 1995). RpoE availability is also modulated at the post-translational level by interaction with a specific anti- σ factor, RseA. *rseA* is the second gene of the four-gene *rpoE rseA rseB rseC* operon (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). It has been reported that DegS controls the degradation of RseA, which could relieve the inhibitory effect of RseA on RpoE upon the induction of envelope stress (Ades *et al.*, 1999). However, under all growth conditions a stoichiometry of 2:5:1 for RpoE:RseA:RseB, respectively, has been observed (Collinet *et al.*, 2000). The mechanism responsible for this stoichiometry is unknown.

The present study addresses the role of additional gene(s) whose product(s) may be controlling this complex heat shock response. Here, we report identification of a gene designated *ecfE*, whose product modulates the levels of both RpoE and RpoH σ factors. *ecfE* was isolated because in multicopy it decreased the activity of both *rpoE*- and *rpoH*-transcribed promoters. We show that purified EcfE can degrade RpoE as well as RpoH *in vitro* in sub-stoichiometric amounts. We also show that *ecfE* is an essential gene. EcfE shares homology with the recently

identified protease S2P (Site-2 Protease), a membrane-anchored protease (Ye *et al.*, 2000). S2P protease cleaves the N-terminal domains of membrane-bound sterol regulatory element binding proteins (Brown *et al.*, 2000; Ye *et al.*, 2000). EcfE also shows some homology in the catalytic domain with *Bacillus subtilis* sporulation protein SpoIVFB. SpoIVFB has been shown to be necessary for the processing of σ factor σ^K (Rudner *et al.*, 1999). Sequence comparison with the available microbial genomes shows that EcfE homologues are present in all eubacteria except *Mycoplasma*.

Results

Identification of the *ecfE* gene

We have been interested in the σ^E regulon as well as regulation of *rpoE* activity and turnover of another heat shock σ factor RpoH. Here, we looked for multicopy clones in a p15A-based vector that reduced the activity of $E\sigma^E$ -regulated promoters. With this approach, we previously cloned the *rseA* gene, whose product negatively regulates RpoE activity, since it acts as an anti- σ factor specific to RpoE. To avoid recloning the *rseA* gene, we constructed a multicopy library using chromosomal DNA isolated from a strain carrying a null mutation in *rseA*. This library was used to isolate clones that turned off the $E\sigma^E$ -regulated promoters *htrA-lacZ*, *rpoEP2-lacZ* and *rpoHP3-lacZ*.

Since the heat shock response is elicited upon protein misfolding, we also tested whether these plasmids also affected the *rpoH* regulon. These plasmids were transformed into strains carrying $E\sigma^{32}$ -transcribed promoters, namely *groEL-lacZ*, *rpoDPhs-lacZ*, *ftsH-lacZ* and *lon::Mu53-lacZ*. Most of these plasmids were also found to similarly reduce the transcription of *rpoH* regulon. Analysis using different restriction enzymes identified 25 plasmids carrying identical DNA fragments. Three such plasmids were randomly chosen and sequenced. The sequence analysis of the three clones showed that they contained the *cdsA* gene (encoding CDP-diacylglycerol synthase) and a hypothetical ORF designated *yaeL*, which corresponds to the 4.3 min region of the chromosome.

We first tested whether the *cdsA* gene in multicopy was responsible for decreased $E\sigma^E$ or $E\sigma^{32}$ activity. We did not find any influence of clones that contained the *cdsA* gene alone on the multicopy plasmid (pSR3657) as compared with the parental plasmid (pSR3612) carrying the second ORF *yaeL*. We named this ORF *ecfE* after analysing its uncton and its transcriptional regulation. The *ecf* gene designation is based on its identification as part of the σ^E regulon (Dartigalongue *et al.*, 2001). Based on our transcriptional mapping of the *ecfE* promoter (Dartigalongue *et al.*, 2001) and previous work, it appears that *ecfE* is transcribed as a three-gene operon, in the order *uppS cdsA ecfE*.

Overall we observed that the basal level activity of RpoE-regulated promoters is reduced by a factor of 4- to 7-fold upon EcfE overexpression (Table I). *ecfE*-bearing plasmids reduced the activity of $E\sigma^{32}$ -transcribed promoters by a factor of 2- to 4-fold (Table I). The lesser effect was observed with the *groEL-lacZ* fusion-bearing strain. This promoter fusion also has a $E\sigma^{70}$ promoter, which masks the effect on the $E\sigma^{32}$ -dependent promoter.

Table I. The effect of *ecfE* overexpression on the heat shock response

Strains	Regulated by	Vector alone	p(<i>ecfE</i>)
<i>htrA-lacZ</i>	RpoE	100 $\pm$ 9	23 $\pm$ 5
<i>rpoHP3-lacZ</i>	RpoE	78 $\pm$ 7	19 $\pm$ 5
<i>rpoEP2-lacZ</i>	RpoE	350 $\pm$ 15	49 $\pm$ 6
<i>groEL-lacZ</i>	RpoH	720 $\pm$ 30	330 $\pm$ 17
<i>lon-lacZ</i>	RpoH	360 $\pm$ 21	53 $\pm$ 7
<i>rpoDPhs-lacZ</i>	RpoH	83 $\pm$ 7	21 $\pm$ 4
<i>ftsJ/H-lacZ</i>	RpoH	470 $\pm$ 37	140 $\pm$ 15
<i>htrP-lacZ</i>	RpoD	1510 $\pm$ 74	1570 $\pm$ 91
<i>htrL-lacZ</i>	RpoD	230 $\pm$ 19	225 $\pm$ 19
<i>dsp-lacZ</i>	RpoS	15 200 $\pm$ 520	16 000 $\pm$ 140
<i>pspA-lacZ</i>	RpoN	73 $\pm$ 7	69 $\pm$ 7

Isogenic cultures of bacteria carrying fusions to different RpoE-regulated promoters, RpoH-regulated promoters and those transcribed by other σ factors in the presence of a plasmid carrying *ecfE* were analysed for β -galactosidase activity (Miller units). Bacterial cultures were grown overnight in LB broth at 30°C, diluted 1:100 and allowed to reach an OD₅₉₅ of 0.2 for monitoring β -galactosidase activity. Samples were assayed in duplicate and the data presented here are the average of three independent experiments.

To confirm that the effect is specific to these heat shock regulons, we also tested four different promoters transcribed by other σ factors. We used *htrL-lacZ* and *htrP-lacZ* (RpoD transcribed), *pspA-lacZ* (RpoN transcribed) and *dps-lacZ* (RpoS transcribed). The data presented in Table I show no significant changes in the expression of these promoters upon *ecfE* overexpression. Among these fusions we deliberately used fusion to *pspA* and *dps* genes since it is known that *pspA* is also induced by a variety of stresses including heat shock but is not controlled by *rpoH*, and that *dps* is strongly induced in stationary phase under the control of *rpoS* (Altuvia *et al.*, 1997). RpoS, quite like RpoH, is an unstable protein and in this way we also rule out the possibility that the EcfE overexpression effect may be on labile proteins in general.

A minimal clone carrying the *ecf* gene under the arabinose-inducible promoter vector pBAD24 (pSR4173) was also tested. Introduction of this plasmid into strains carrying $E\sigma^E$ or $E\sigma^{32}$ promoters led to a decrease in the expression of the corresponding promoters depending on the amount of inducer used (Figure 1). Concentrations of arabinose >0.2% render *E.coli* very sick, and further such arabinose-dependent EcfE overproduction leads to complete growth arrest at temperatures >42°C.

Levels of RpoE and RpoH in vivo upon overexpression of *ecfE*

To verify that overexpression of *ecfE* was directly affecting the two heat shock regulons, we examined the levels of RpoE and RpoH in isogenic derivatives of strain LMG194 with pBAD24 vector either alone or carrying the *ecfE* gene. Expression of *ecfE* was turned on with the addition of inducer arabinose (0.01%) and after 2 h, cell extracts were analysed by western blotting using RpoE and RpoH antisera. As can be seen in Figure 2, the levels of RpoE and RpoH protein are reduced upon overexpression of *ecfE* while the amount of the control protein TrxA remains unchanged. A quantitative measurement shows an ~12-fold reduction in RpoE amount as compared with an ~6-fold reduction in the amount of RpoH.

Fig. 1. EcfE induction represses the heat shock response and suppresses the mucoidy of a *lon* mutant. Strains carrying promoter fusions under the control of RpoE (*htrA-lacZ*) or RpoH (*groEL-lacZ* and *lon::Mu53-lacZ*) were transformed with plasmid pSR4173 (pBAD24-*ecfE*⁺). Two independent transformants were streaked on LB plates containing X-gal to visualize reporter gene activity. *ecfE* expression was induced with 0.01% arabinose in the plate and is shown on the right side.

Fig. 2. Induction of *ecfE* leads to a reduction in the levels of RpoE and RpoH. Cultures of LMG194 strain carrying pBAD24 or pSR4173 (pBAD24-*ecfE*⁺) were grown in M9 medium with glucose at 30°C. At an OD₅₉₅ of 0.2, cultures were centrifuged, washed and resuspended in M9 medium containing 0.01% arabinose for ~2 h. Total cell extracts were analysed by SDS-PAGE, followed by western blotting with a mixture of anti-RpoH, anti-RpoE and anti-TrxA antibodies.

ecfE* is an essential gene under normal growth conditions in *E. coli

We recently reported that *ecfE* is an essential gene (Dartigalongue *et al.*, 2001). We constructed two null alleles by inserting an Ω Kan cassette at *Bsi*WI and *Sma*I sites. Such disrupted *ecfE* alleles were transferred to the gene replacement vector pKO3 (Link *et al.*, 1997). Recombination on to the chromosome occurred only in the presence of plasmid pSR4173 (pBAD-*ecfE*⁺) (Dartigalongue *et al.*, 2001). In order to establish that *ecfE* is essential, we constructed a merodiploid strain carrying *lecE*⁺ on the chromosome at the λ attachment site and then repeated the gene replacement experiment. Only in this situation could we resolve the *ecfE::\OmegaKan and obtained the null allele at the chromosomal location and the wild-type gene at *lecE*⁺. To rule out that essentiality was not due to any polar effect on the downstream gene, we inserted an Ω Kan cassette in the downstream ORF (*yaeT*, renamed *ecfK*; Dartigalongue *et al.*, 2001). We could easily transfer the null allele to the downstream gene using conventional genetic techniques. No obvious*

Fig. 3. Subcellular fractionation of EcfE. Strains carrying pSR4932 (pET22b-*ecfE*⁺) were grown at 37°C in M9 medium. Protein production was induced by the addition of 0.01 mM IPTG. After 30 min induction, cells were treated with rifampicin (200 μ g/ml) for another 30 min and then labelled with [³⁵S]methionine (50 μ Ci/ml) for 10 min. Cell extracts were submitted to fractionation as described in Materials and methods. Proteins were resolved by SDS-PAGE (12.5%) and visualized both by Coomassie Blue staining and by autoradiography of the dried gel (lanes 5–8). Lanes 1 and 5 represent total cell extract. Lanes 2 and 6 show the soluble fraction (soluble), and lanes 3 and 7, and 4 and 8 show inner membrane fraction (IM) and outer membrane fraction (OM), respectively. The molecular weight markers correspond to 110, 76, 49, 35.6, 29 and 19 kDa and were obtained from Bio-Rad.

phenotype was observed, hence the essentiality of the *ecfE* gene product has nothing to do with the downstream ORF *ecfK*.

Subcellular localization of EcfE

ecfE is predicted to encode a 450 amino acid polypeptide. The amino acid sequence examination of EcfE predicted it to be an inner membrane protein with five putative transmembrane protein helices spanning 5–24, 55–73, 99–123, 379–400 and 426–444 (predicted from the programs DAS and TMpred at EMBnet).

To confirm the inner membrane localization, we performed the following experiments. First, we induced the synthesis of *ecfE* by cloning *ecfE* under a tightly controlled T7 promoter expression system (pSR4932). The total cell extract was fractionated by sonication to separate soluble versus insoluble fractions (containing aggregates, inner and outer membrane). The inner membrane fraction was released by Sarkosyl treatment (Figure 3). As can be seen, most of the EcfE protein was solubilized by Sarkosyl (Figure 3, lanes 3 and 7). These results are further supported by the purification of EcfE from inner membrane fraction.

Mutational analysis of *ecfE*

Examination of the amino acid sequence of EcfE shows three interesting features. First, EcfE has a potential zinc binding 'H₂₂EFGH₂₆' motif. This motif is right at the end of the first transmembrane domain facing the cytoplasm. The second motif is a conserved PDZ domain spanning amino acid residues 208–280. PDZ domains are present in a variety of proteins with different functions (Pallen and Ponting, 1997; Bezprozvanny and Maximov, 2001). The third feature is characterized by the presence of a highly conserved C-terminal amino acid sequence N₃₉₄XXP XXXLDG₄₀₃ also found in the human S2P protease. H22, E23 and H26 of the zinc binding motif were changed to A using plasmid pSR4173 (pBAD-*ecfE*⁺) as a template. Based on sequence alignment of various PDZ domains, we chose to change by site-directed mutagenesis the G214, G239, D244 and R278 residues of EcfE to A. To test the

Fig. 4. Residues important for EcfE activity. Conserved residues in the predicted zinc binding domain, PDZ domain and undefined regions of EcfE were mutated using plasmid pSR4173 (pBAD24-*ecfE*⁺). Mutant plasmids were transformed in an appropriate background to examine their effect on the RpoE- and RpoH-dependent heat shock response (A). β -galactosidase activities were measured as explained for Table I. (B) Induction of heat shock response in *ecfE* chromosomal point mutants. All the point mutants were transferred to the chromosome in strains carrying either *htrA-lacZ* or *lon-lacZ* fusion and assayed for β -galactosidase activity.

role of conserved C-terminal amino acids we constructed the following changes: N394, P397, L401 and D402 to A and G403V. To check whether any of these residues play any critical role in the activity of EcfE, the following experiments were performed. First, we tested whether these plasmids encoding the respective *ecfE* mutants affected heat shock regulation. As shown in Figure 4A, none of the mutants could down-regulate either σ^E - or σ^{32} -transcribed promoters, unlike the wild-type *ecfE*, with the exception of G214 to A. Also overexpression of these mutant proteins did not result in toxicity or growth arrest.

Secondly, we tested whether they have a phenotype when transferred to the chromosome in single copy using the bacteriophage λ *red*-mediated recombination. The mutant alleles were transferred to the plasmid that carries the Ω Kan null inserted 700 nucleotides downstream of the *ecfE* gene stop codon. PCR-amplified DNA fragments carrying mutant alleles along with the Ω Kan were transformed into strain DY 392 (Yu *et al.*, 2000) and first selected for Kan resistance. The correct mutations were transduced into wild-type background carrying different $E\sigma^E$ - or $E\sigma^{32}$ -transcribed promoter fusions. We could transfer G214A to the chromosome without any obvious phenotype. However, other alleles behaved differently. DNA fragments carrying the respective changes H22, E23, G239, D244, N394, P397, L401 and D402 to A and G403V could be transferred to the chromosome but not the DNA fragment carrying R278A.

Fig. 5. Schematic presentation of various *ecfE* point mutants. All the point mutations generated either by site directed mutagenesis or by localized mutagenesis are shown. All the mutants are in a single copy on the chromosome. The thermosensitive phenotype is indicated as Ts and numbers in parenthesis indicate the number of times a particular mutant allele was isolated. The exact nucleotide change in each case is indicated.

Fig. 6. Growth curves of isogenic strains with *ecfE*⁺, *ecfE* H22A and *ecfE* conditional null alleles. Exponentially growing cultures at 30°C were diluted 1:100 into prewarmed LB medium and incubated at 30, 37 and 42°C. For *ecfE* conditional null, arabinose was depleted from the medium and replaced with 0.2% glucose. Bacterial growth was monitored by measuring the OD₅₉₅ after various time intervals.

Transfer of the R278A mutation to the chromosome was achieved only in a merodiploid strain. The rest of the mutants displayed a temperature-sensitive (Ts) phenotype and showed a constitutive, high basal level activity of the $E\sigma^E$ - or $E\sigma^{32}$ -transcribed promoter (Figures 4B and 5). The growth analysis of *ecfE* point mutant H22A was compared at 30, 37 and 42°C. The data presented in Figure 6 show clearly that the strain carrying the active site point mutant shows a decline in growth rate at both 37 and 42°C. This is more acute at 42°C as compared with the isogenic wild type and hence leads to lack of colony forming ability at high temperature. Also, the strain carrying the *ecfE* conditional null allele does not grow at any of these temperatures unless a wild-type copy is provided *in trans*. The data for the *ecfE* conditional are presented only at 30°C since they do not grow at any other temperature. These results argue that the putative zinc binding motif and NXXPXXXLDG motif are needed for EcfE activity in modulating heat shock response but are not essential for the viability of *E. coli* below 41°C.

Isolation of Ts mutant alleles in *ecfE*

In a complementary approach we further isolated Ts mutants of the *ecfE* gene on the chromosome. To achieve this, we used the *ecfK*::Kan null mutant strain (*ecfK* is

located downstream of *ecfE*) and isolated Ts mutants linked to this Kan marker that led to increased expression of the RpoH and RpoE regulons. This was achieved by hydroxylamine mutagenesis of P1 bacteriophage grown on *ecfK::Kan*. Such lysates were used in transduction of the Kan^R marker into strain using *rpoH*-regulated *rpoDPhs-lacZ* fusion. *rpoDPhs* is a relatively weak promoter and it was easier to score for Lac up mutants. We isolated 45 independent Ts mutants, which simultaneously show constitutively high β -galactosidase activity of *rpoDPhs-lacZ*. Only those mutants which were ~90% linked to Kan^R and the Ts phenotype were retained. Twenty-nine mutants were kept and checked for complementation by *ecfE* plasmid (pSR4173). Of them, 15 mutants were retained since they were fully complemented by the minimal *ecfE*-containing plasmid. All 15 mutants isolated were transduced into strains carrying *rpoE*-regulated promoters like *htrA-lacZ*, *rpoHP3-lacZ* or *rpoEP2-lacZ*. In all cases they showed a 5- to 7-fold induction of LacZ activity at 30°C (Figure 4B). We also transduced them into the strain carrying the *lon-lacZ* promoter fusion. Again an ~4-fold induction was observed (Figure 4B). The chromosomal DNA from these mutants was sequenced. Interestingly, some of the mutations were mapped to the catalytic HEXXH site and L and G residues of the NXXPXXLDG motif at the C-terminus (Figure 5). The Ts phenotype, heat shock expression (for some of the mutants) are shown in Figures 4B and 5. The results are summarized and the exact nucleotide changes depicted in Figure 5.

Purified EcfE degrades RpoH and RpoE

In multicopy, the *ecfE* gene down-regulates the two heat shock regulons, and point mutations in this gene have elevated heat shock levels. One explanation for this behaviour and consistent with the sequence prediction is that EcfE acts as a protease, degrading RpoE and RpoH. To test this, the EcfE-6His variant carrying a 6 $\times$ histidine tag fused to the C-terminus was purified. As mentioned earlier, overexpression of the *ecfE* gene is toxic, making it extremely difficult to purify the protein. EcfE-His₆ was recovered from the inner membrane fraction, by solubilizing using 0.3% Sarkosyl, dialysed in Tris-HCl 50 mM pH 7.5 containing 0.5% Triton X-100 and purified over Ni-NTA beads. The purified protein was pre-incubated in the presence of 100 μ M ZnCl₂ and assayed for its activity on purified RpoE, RseA and RpoH as substrates. One microgram of each protein substrate was added to 10, 20 and 30 ng of EcfE protein and incubated for 30 min at 25°C. The reaction mixture was analysed by loading samples on to a SDS-polyacrylamide gel followed by western blotting. RpoE, RpoH and RseA were visualized using specific antisera. As shown in Figure 7 there is a progressive decrease in the amount of RpoH or RpoE detected by western blot using specific antibodies (Figure 7A and B, respectively). The degradation of RpoE was somewhat more efficient than that of RpoH. Under similar conditions, we did not see any cleavage of the RseA anti- σ factor (data not shown). Thus, EcfE degrades the two σ factors RpoE and RpoH in sub-stoichiometric amounts. As a control we also purified the EcfE-H22A mutant protein (Figure 8). The mutant protein was inactive, further supporting the notion that the zinc binding motif is required for proteolytic activity

Fig. 7. Degradation of RpoE and RpoH using purified EcfE. One microgram of RpoE (A) or RpoH (B) was incubated with varying amounts of EcfE (0, 10, 20 or 30 ng) for 30 min at 25°C. The proteins were resolved by 12.5% SDS-PAGE, then transferred to a nitrocellulose membrane and analysed by western blotting with antibodies raised against RpoE (A) or RpoH (B). MW, molecular weight.

(Figure 8B and C). Purified RpoD and RpoS proteins were used as a control to rule out the possibility that EcfE degrades σ factors in a non-specific manner (Figure 8A and B). For *in vitro* proteolytic assay the best conditions for RpoH were found with incubation at 37°C when EcfE was supplemented with 0.5% Triton X-100 and 0.5% Tween-20.

The N-terminal domain of RpoH is susceptible to proteolysis

The exact region of RpoH contributing to its extreme susceptibility to proteolysis is not known. We used a newly developed *in vivo* assay for characterization of proteases and their substrates. This system is based on using an *E.coli* strain deficient in the *cya* gene [encoding adenylyl cyclase (AC)] and expression of the cognate gene from *Bordetella pertussis* to give rise to a Cya⁺ phenotype. If the two subdomains T25 and T18 of *Bordetella* AC are in separate molecules, the cells exhibit a Cya⁻ phenotype. The two subdomains are required for enzymic activity but can tolerate large in-frame polypeptide insertions without loss of enzymic activity. If a protein sequence inserted between the subdomains is cleaved by a protease, the cells will become Cya⁻. Two regions from RpoH (N-terminal residues M1-M156 and C-terminal residues T142-A284) were inserted between the subdomains of AC. Interestingly, fusion with the N-terminal fragment rendered the cells partly Cya⁻ indicating that they are likely to be impaired in AC activity. The β -galactosidase activity, which reflects the AC activity, declined 30-fold when an *ecfE*-encoding plasmid was provided *in trans* (Table II). Consistent with these results, when we tested the same

Fig. 8. Wild-type EcfE but not EcfE-H22A degrades RpoE and RpoH. The migration of EcfE, RpoD, RpoS, RpoH and RpoE is shown with arrows. (A) Lanes 1 and 2 show purified wild-type EcfE and EcfE-H22A proteins. Lane 3 shows purified RpoD. Lanes 4 and 5 show after 30 min incubation of RpoD with EcfE wild-type and EcfE-H22A protein at 25°C. (B) Lanes 3 and 5 show purified RpoH and RpoS, respectively. Lanes 2 and 4 show the protease assay with wild-type EcfE and RpoH or RpoS, respectively, after an incubation of 30 min at 37°C. Lane 1 shows results with incubation as above but with EcfE-H22A, using RpoH as the substrate. Lanes 2 and 4 show the protease assay with wild-type EcfE and RpoH and RpoS as the substrates. The asterisk on the left is for lane 2, which shows the accumulation of cleaved RpoH. (C) Lane 3 shows purified RpoE alone, lanes 2 and 3 show the protease assay with wild-type and EcfE-H22A proteins, respectively. Note the accumulation of degraded RpoE product near the bottom of the gel in lane 2 with wild-type EcfE, indicated by an asterisk. For (A) and (B), proteins were resolved by 12% SDS-PAGE and for (C) showing RpoE degradation, proteins were resolved by 15% SDS-PAGE. Proteins were visualized by Coomassie Blue staining.

N-terminal fusion in isogenic strains but carrying two mutant alleles, H22A and P209T, of *ecfE*, there was a marked stabilization of this fusion as reflected by the increased β -galactosidase activity and a *Cya*⁺ phenotype. In contrast, the fusion to the C-terminal domain was completely stable and cells remained *Cya*⁺ with the control plasmid. The introduction of the *ecfE* plasmid did not lead to a *Cya*⁻ phenotype and no reduction in β -galactosidase activity was observed (Table II). Similarly there was no effect on the *ecfE* point mutant

Table II. The N-terminal domain of RpoH is susceptible to EcfE-mediated proteolysis

Vector	β -galactosidase activity (Miller units)
pKAC	5500 $\pm$ 145
pKAC (N-terminal <i>rpoH</i>)	700 $\pm$ 50
pKAC (N-terminal <i>rpoH</i>) + p(<i>ecfE</i>)	25 $\pm$ 3
pKAC (N-terminal <i>rpoH</i>) + p(<i>ecfE</i> - P209T)	2500 $\pm$ 170
pKAC (N-terminal <i>rpoH</i>) + p(<i>ecfE</i> - H22A)	3700 $\pm$ 250
pKAC (C-terminal <i>rpoH</i>)	6500 $\pm$ 370
pKAC (C-terminal <i>rpoH</i>) + p(<i>ecfE</i>)	5900 $\pm$ 450
pKAC (C-terminal <i>rpoH</i>) + p(<i>ecfE</i> - P209T)	6700 $\pm$ 340
pKAC (C-terminal <i>rpoH</i>) + p(<i>ecfE</i> - H22A)	6100 $\pm$ 330

backgrounds. These results clearly argue that the N-terminal domain is the target for proteolysis *in vivo*.

Stabilization of RpoH and RpoE in *ecfE* mutants

It is known that RpoH is extremely unstable, with a half-life of ~30 s. Since our biochemical data show that EcfE can efficiently degrade RpoH *in vitro* at submolar concentrations, we examined the *in vivo* half-life of RpoH. As shown in Figure 9, the half-life of RpoH is increased in strains carrying mutant EcfE-P209T (SR4861) or EcfE-H22A (SR5070), from 45 s to ≥ 20 and 30 min, respectively (Figure 9A). The stabilization of RpoH might be even greater under non-permissive conditions.

We also determined the stability of RpoE in both wild type and the isogenic *ecfE* point mutants. RpoE is much more stable than RpoH. RpoE's half-life as measured by pulse-chase experiments show that in wild-type it is ~15 min. However, in the point mutants this half-life was increased to >120 min in the strain encoding EcfE-P209T and to >180 min in the strain encoding EcfE-H22A (Figure 9B). We did not detect any change in the half-life of the anti- σ factor RseA (data not shown) using the *ecfE* point mutants as compared with the isogenic wild-type.

Phenotypic characterization of EcfE point mutants

As mentioned above, we isolated *ecfE* point mutants on the basis of up-regulation of both heat shock regulons and temperature sensitivity. We examined these mutants for other phenotypes.

ecfE point mutants exhibited pleiotropic defects, including hypersensitivity to aminoglycoside antibiotics such as anikacin. *ecfE* point mutants did not grow at an anikacin concentration of >2 μ g/ml, compared with wild-type, which can grow in anikacin up to 10 μ g/ml. This probably reflects increased permeability of the outer membrane.

Effect of mutation in *ecfE* or depletion of EcfE protein on the overall protein profile

We performed several experiments to see the global effect on the protein profile. First, we performed two-dimensional SDS-PAGE analysis of isogenic strains MC4100 and SR4861 (*ecfE* point mutant). Culture were labelled with [³⁵S]methionine and analysed by two-dimensional SDS-PAGE. As can be seen (Figure 10) there is an increased accumulation of major heat shock proteins such

Fig. 9. Stability of RpoE and RpoH in strains carrying *ecfE* point mutants. Cultures were pulse labelled for 20 s (time 0) and chased with cold methionine. Samples were taken at different intervals and immunoprecipitated with anti-RpoH (A) and anti-RpoE (B) antibodies and analysed by SDS-PAGE. The amounts of RpoE and RpoH were quantified by densitometry using a PhosphorImager.

as DnaK and GroEL. However, we also see changes in the accumulation of many other proteins.

To further confirm these results, we carried out depletion experiments using the *ecfE* null allele on the chromosome and a wild-type gene present on a pBAD plasmid. Cells were depleted of EcfE by diluting out the arabinose contained in the growing medium. Samples were analysed by two-dimensional SDS-PAGE following various intervals of depletion. Protein spots were excised and analysed by tryptic digestion and MALDI-TOF analysis or by Edman degradation. The sequences of up-regulated as well as down-regulated proteins were compiled and analysed. The data presented in Table III show an increase in the accumulation of major outer membrane proteins (OMPs) upon depletion. Quantification of OMPs, which include OmpA, OmpF, OmpC and OmpX showed that they were up-regulated by at least 95%. In addition a protein involved in lipopolysaccharide (LPS) biogenesis, RfaD/HtrM, was also similarly increased in abundance upon the depletion of EcfE. The increased accumulation of RfaD/HtrM may be due to the induction of two promoters of the corresponding genes, which are under the control of $E\sigma^{32}$ or $E\sigma^E$ RNA polymerase (Raina and Georgopoulos, 1991; Dartigalongue *et al.*, 2001). We reported previously that the *rfaD* gene has a relatively strong *rpoE*-regulated promoter (Dartigalongue *et al.*, 2001). There was also increased accumulation of putative inner membrane proteins YfgM, YbgI, both of which, from the recent

Fig. 10. Global changes in protein profile in *ecfE* mutant. Isogenic wild-type and an *ecfE* mutant strain were grown at 30°C to OD₅₉₅ 0.3 in M9 medium supplemented with glucose. Cells were labelled with [³⁵S]methionine (50 µCi/ml) for 1 min. Cells were lysed and extracted with detergents (2% Nonidet P-40) and 8 M urea followed by several freeze-thawing cycles. The solubilized fraction was separated by charge electrophoresis using a mixture of ampholytes (1.6 and 0.4% in the pH ranges 5.0–7.0 and 3.5–10.0, respectively) in the first dimension and a 12.5% SDS-PAGE in the second dimension. Autoradiograms of the two-dimensional SDS-PAGE are shown: wild type (A) or *ecfE* P209T mutant (B). Circles in (A) identify proteins of lesser abundance in the *ecfE* mutant. Arrows in (B) identify proteins with increased abundance in the *ecfE* mutant. The letters K and EL identify DnaK and GroEL, respectively.

microbial genome sequence analysis, are highly conserved, although no function has been assigned to them. Another protein, which was prominently induced upon depletion was the periplasmic oligopeptide binding protein OppA. Some of the proteins were also down-regulated upon depletion, including periplasmic proteins such as cystine binding protein and D-ribose binding protein (see Table III for more details).

Induction of EcfE suppresses mucoidy of *Lon* mutants

It is known that mutations in the *lon* gene lead to a constitutive synthesis of colanic acid. This is due to stabilization of RcsA, a positive regulator for the transcription of *cps* genes, leading to overproduction of

Table III. Changes in protein profile upon depletion of EcfE

Protein	Protein level	Functions and localization
OmpA	up	major OMP
OmpC	up	major OMP
OmpF	up	major OMP
OmpX	up	major OMP
DnaK	up	protein folding, gene under σ^{32} control (cytoplasmic)
GroEL	up	protein folding, gene under σ^{32} control (cytoplasmic)
OppA	up	peptide binding protein (periplasmic)
HtrM/ RfaD	up	epimerase/LPS biogenesis (cytoplasmic)
ManC	up	colanic acid (cytoplasmic)
YfgM	up	highly conserved inner membrane protein
YbgI	up	highly conserved inner membrane protein
FecA	down	iron (III) dictrate transport (OMP)
FliY	down	cystine binding periplasmic protein (periplasmic)
GlyA	down	serine hydroxymethyl transferase—interconversion of serine and glycine— key enzyme in biosynthesis of lipids and purines (cytoplasmic)
PpcK	down	phosphoenol pyruvate carboxykinase (cytoplasmic)
RbsB	down	D-ribose binding periplasmic protein (periplasmic)
SyfA	down	phenylalanyl-tRNA synthetase α chain (cytoplasmic)
YeiP	down	belongs to elongation factor P family (cytoplasmic)

The proteins that are more abundant or decreased upon depletion of EcfE are indicated by 'up' and 'down', respectively. Only the major changes (induction or reduction by 40% or more) as observed on two-dimensional SDS-PAGE are reported.

colanic acid (Jubete *et al.*, 1996). During our analysis of EcfE overproduction effects on $E\sigma^{32}$ -driven heat shock promoters, the fusion used was *lon-lacZ*. This transcription fusion is inserted in the *lon* gene (*lon::Mu53-lacZ*) and gives a *Lon*[−] phenotype which includes a mucoid phenotype. However, when the *ecfE* gene is induced with arabinose, the strain becomes non-mucoid. We further analysed this phenotype by introducing a $\Delta lon::Tn10$ insertion in strain MC4100 carrying the *cpsB-lacZ* fusion. The data presented in Table IV show that overexpression of *ecfE* reduces *cps* expression in a *lon* mutant. As a control, we used another plasmid carrying *ecfE::ΩKan*, which is identical to pSR4173, or the *ecfE* plasmid derivative encoding EcfE-H22A. In both cases there was no reduction of *cps* gene expression as measured by *cps-lacZ* activity.

Discussion

EcfE is as a protease in *E. coli*

Degradation of RpoH. In this work, we have characterized a new essential protease in *E. coli*. The gene encoding this protease was obtained initially as a multicopy clone that reduced the expression of both $E\sigma^{32}$ and $E\sigma^E$ heat shock regulons. Interestingly, RpoH is a highly unstable protein and many proteases have been implicated in its turnover (Maurizi *et al.*, 1999; Morita *et al.*, 2000; Yura *et al.*, 2000). The most important role has been assigned to HflB (FtsH) protease based on *in vivo* and *in vitro* data (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995). In this study we show that RpoH is also partially stabilized *in vivo* in strains carrying mutations in the *ecfE* gene. In addition, we show that purified EcfE can catalytically degrade RpoH. Purified EcfE-H22A protein did not exhibit any proteolytic activity. Also this mutant elicited heat shock response *in vivo*. H22 belongs to the H₂₂EFGH₂₆ zinc binding motif, found in a variety of metalloproteases (Brown *et al.*, 2000). This motif is predicted to be at the end of the first potential

Table IV. Effect of EcfE overproduction on the transcriptional activity of the *cpsB* gene

Strains (MC4100 background)	β -galactosidase activity (Miller units at 30°C)
<i>cpsB-lacZ</i>	19 ± 2
<i>cpsB-lacZ</i> + pBAD24	21 ± 3
<i>lon::Tn10, cpsB-lacZ</i> + pBAD24	470 ± 23
<i>lon::Tn10, cpsB-lacZ</i> + pBAD24 (<i>ecfE</i> ⁺)	65 ± 6
<i>lon::Tn10, cpsB-lacZ</i> + pBAD24 (<i>ecfE::ΩKan</i>)	457 ± 29
<i>lon::Tn10, cpsB-lacZ</i> + pBAD24 (<i>ecfE</i> -H22A)	430 ± 33

transmembrane domain just near the cytoplasmic face. Earlier observations by Bukau *et al.* (1993) showed that a fraction of DnaK is always associated with the inner membrane. Since it is known that DnaK/DnaJ and RpoH interact physically (Gamer *et al.*, 1992; Liberek and Georgopoulos, 1993), it is likely that RpoH might be targeted to EcfE proteolysis via an RpoH-DnaK-DnaJ complex.

Degradation of RpoE. RpoE is the only σ factor other than the housekeeping σ factor RpoD that is essential for growth in *E. coli*. Interestingly, we have shown recently that *ecfE* itself is part of the σ^E regulon (Dartigalongue *et al.*, 2001). As pointed out, induction of *ecfE* transcription even under mild conditions leads to a reduction in RpoE activity as judged by the activity of reporter fusions *htrA-lacZ*, *rpoHP3-lacZ* and *rpoEP2-lacZ* as well as *in vivo* measurement of RpoE levels. These results cannot be an indirect effect on β -galactosidase degradation since the $E\sigma^{70}$ -transcribed promoters *htrL* and *htrP* were unaffected. In addition, RpoE half-life was increased by at least 8-fold in a strain encoding the EcfE P209 mutant and 12-fold in a strain encoding the EcfE-H22A mutant. Our *in vitro* studies confirmed that RpoE is a good substrate for EcfE.

How is RpoE targeted to membrane proteolysis by EcfE *in vivo*? RpoE interacts with a cognate anti- σ factor RseA

(De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). RseA is anchored to the inner membrane and a fraction of RpoE (36%) is always found to fractionate to the membrane in a RseA-dependent manner (Collinet *et al.*, 2000). Hence, RpoE could be targeted to proteolysis in complex with RseA.

Why is *EcfE* essential in *E.coli*?

Altering the amounts of functional EcfE overexpression drastically alters the heat shock response. Such changes are obviously going to be harmful to the cells. As pointed out, the three other σ factors seem to be unaffected as judged by transcriptional fusions to *rpoS*-, *rpoN*- and *rpoD*-regulated promoters. Further, no degradation of RpoD and RpoS was observed *in vitro* in the presence of purified EcfE. Misregulation of the heat shock response can in part explain why EcfE is essential. However, the effect on heat shock response may not be the sole reason for the essentiality of EcfE. Most of the OMPs are overproduced upon depletion of EcfE *in vivo*. Overproduction of OMPs is known to induce the *rpoE* regulon (Meccas *et al.*, 1993). We also see some LPS components to be affected like RfaD. We have already shown that essential genes like *lpxD/A fabZ* are partly subject to *rpoE* regulation. Hence in the absence of EcfE there may be an imbalance in the amounts of some components involved in LPS and lipid A biogenesis since not all of their cognate genes are under the control of the *rpoE* gene. Also a number of periplasmic proteins are either increased in their abundance or are decreased. This suggests an overall defect in balancing the amounts of exported proteins. Together they may all contribute towards the essentiality of the *ecfE* gene. It is also likely some cell wall/cell division apparatus may be affected. It may be relevant to point out that *ecfE* homologues are present in all sequenced bacterial genomes but not in *Mycoplasma*, which lacks a cell wall. Clearly more detailed analysis of cell wall biogenesis in the *ecfE* mutants is needed.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table V.

Media and chemicals

Luria-Bertani (LB) broth, M9 and M63 minimal media were prepared as described by Miller (1992). When necessary, the media were supplemented with ampicillin (Amp, 100 μ g/ml), tetracycline (Tet, 15 μ g/ml), kanamycin (Kan, 50 μ g/ml) or chloramphenicol (Cm, 20 μ g/ml). The indicator dye 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) was used at a final concentration of 40 μ g/ml in the agar medium. Different concentrations of arabinose were used depending upon experiment.

Genetic manipulation and cloning experiments to isolate the *ecfE* gene

First, a multicopy library was constructed using the p15A-based vector pOK12 (Vieira and Messing, 1991). Chromosomal DNA from strain SR3556 (*rseA*::Tn10Tet) was subjected to partial digestion with *Sau*3A. DNA fragments 2–5 kb in length were collected, purified and cloned into the *Bam*HI site of pOK12 vector. This was done to eliminate the possibility of recloning the *rseA* gene. This library was used to isolate clones that decreased *htrA-lacZ* expression, using the strain SR1458 (Raina *et al.*, 1995). The transformants were plated on X-gal-containing plates. White or pale blue colonies were retained and tested to verify that the promoter fusion *htrA-lacZ* was not lost. Plasmid DNA were transformed into strains carrying either *htrA-lacZ*, *rpoEP2-lacZ* or *rpoHP3-lacZ* fusions, to see if they bred true. About 70 such plasmids

were retained and used to transform two strains carrying σ^{70} -driven promoter fusions *htrP-lacZ* and *htrL-lacZ*, respectively, and three strains carrying different *rpoH*-regulated promoters, *groEL-lacZ*, *rpoDPhs-lacZ* and *lon::Mu53-lacZ*, respectively. These strains have been described previously (Raina *et al.*, 1995; Missiakas *et al.*, 1996). Thirty-seven plasmids that led to a strong repression of RpoE-regulated promoter fusions were also found to reduce the activity of all RpoH-regulated promoters tested. DNA restriction analysis of these 37 independent plasmids using various restriction enzymes showed that 25 of them contained a unique 2.3 kb *Sph*I–*Hinc*II DNA fragment. Three of these clones were used for hybridization to the ordered *E.coli* genomic library (Kohara *et al.* 1987) by random priming using [α - 32 P]dATP. The DNA isolated from these clones was sequenced and a Blast search revealed the presence of the promoter-less *cdsA* gene followed by *yaeL* and the beginning of a third ORF. The minimal 1493 bp *Kpn*I–*Acc*I DNA fragment carrying *yaeL/ecfE* exclusively was constructed. This clone, pSR3636, was found to reduce the transcriptional activity of both RpoH- and RpoE-regulated promoters. For controlled expression, the *ecfE* gene was subcloned in vector pBAD24 under the control of arabinose-inducible pSR4173. For protein purification *ecfE* was cloned using *Nde*I–*Xho*I restriction sites in pET-22b vector using primers 5'-CGGAAGGTCATATGCTGAGT-3' and 5'-CTAACTAAGTCGAGT-AACCGAGAG-3' (pSR4932).

The DDBJ/EMBL/GenBank accession number for the sequenced *ecfE* gene is AF407012.

Insertion of Ω Kan in the *ecfE* gene

The 2.3 kb *Sph*I–*Hinc*II DNA fragment carrying *ecfE* was subcloned in the Ts replicon vector pKO3 (Link *et al.*, 1997). This DNA fragment was made blunt by treatment with Klenow enzyme and inserted into pKO3 at *Sma*I site. The Ω Kan cassette was inserted into the unique *Bsi*WI and *Sma*I sites. The resulting clones were transformed into *recA*⁺ strain LMG194 and plated at 43°C for co-integrate formation. Gene replacement with the *ecfE*::Kan was obtained only when an extra copy of *ecfE* was provided *in trans*.

Mutagenesis

Site-directed mutagenesis was carried out using the Quik Change procedure as described by the manufacturer (Stratagene). Plasmid pSR4173 was used as a template with the appropriate oligonucleotides. Such *ecfE* alleles were either subcloned into pKO3 vector or transferred to a plasmid carrying a *kan* cassette downstream of the *ecfE* gene. Various mutant alleles were transferred to the chromosome in single copy, in the presence or absence of plasmid pSR4173 (pBAD-*ecfE*⁺). Some of the mutant alleles were transferred to the chromosome using a λ red-mediated system (Yu *et al.*, 2000).

Additional point mutants in the *ecfE* gene were obtained using the mutagen hydroxylamine

For this purpose, the gene immediately following *ecfE*, *yaeT/ecfK* (Dartigalongue *et al.*, 2001), was disrupted by inserting an Ω Kan cassette at *Nru*I site using conventional techniques. The null allele was recombined to the chromosome using the pKO3 vector as described earlier, and transduced into MC4100. Linked mutagenesis to the Kan^R marker was carried out by growing bacteriophage P1 on *ecfK*:: Ω Kan and mutagenizing it with hydroxylamine as described earlier (Raina *et al.*, 1995). This mutagenized lysate was transduced into strain SR3328 carrying *rpoDPhs-lacZ* and plated on agar containing X-gal (20 μ g/ml) and kanamycin. The *rpoDPhs-lacZ*-carrying strain appears pale blue on such plates, as *rpoDPhs* is a relatively weak promoter. Two hundred deep blue Kan^R transductants were retained. Of these, 45 were found to be >90% linked to the Kan^R marker as well as Ts above 41°C. To verify that such strains carried mutation in the *ecfE* gene, they were tested for complementation using the *ecfE*⁺-containing plasmid pSR4173. Of them, 15 were retained and the nature of the mutation was determined by directly sequencing the PCR-generated DNA using oligonucleotides covering the *ecfE* gene, using standard procedures.

Purification of EcfE

BL21 strain pLysE was used as the host to purify EcfE. EcfE with a C-terminal His₆ tag was expressed from vector pET-22b (pSR4932). This plasmid with EcfE appended to histidine tag did not interfere with EcfE activity, since it complemented *ecfE* point mutants just like the control plasmid (pSR4173). To induce the synthesis of the *ecfE* gene product, a 10 l culture of strain carrying pSR4932 was induced with 0.05 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at an OD at 595 nm (OD₅₉₅) of 0.2 for 2 h. Cultures were harvested spun at 6000 g, at 4°C. The pellet

Table V. Bacterial strains and plasmids

	Relevant characteristics	Reference or source
Strains		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) U169	our collection
BL21	<i>ompT lon</i> ⁻	our collection
BL21(pLysE)	<i>ompT lon</i> ⁻ Cm ^R	Invitrogen
DHT1	F <i>glnV44(AS) recA1 endA1 gyrA96 (Nalr) thi-1 hsdR17 spoT1 rfbD1 cya-854 ilv-691::Tn10</i>	Dautin <i>et al.</i> (2000)
GS043	MC4100 ϕ (<i>dps-lacZ</i>)	Altuvia <i>et al.</i> (1997)
PJD31	MC4100 ϕ (<i>pspA-lacZ</i>)	P.Model
SR933	MC4100 ϕ (<i>htrP-lacZ</i>)	Raina <i>et al.</i> (1991)
SR1208	MC4100 ϕ (<i>groEL-lacZ</i>)	Missiakas <i>et al.</i> (1993)
SR1421	MC4100 ϕ (<i>lon::Mu53-lacZ</i>)	Missiakas <i>et al.</i> (1996)
SR1458	MC4100 ϕ (<i>htrA-lacZ</i>)	Raina <i>et al.</i> (1995)
SR1588	MC4100 ϕ (<i>htrL-lacZ</i>)	Missiakas <i>et al.</i> (1993)
SR1710	MC4100 ϕ (<i>rpoHP3-lacZ</i>)	Raina <i>et al.</i> (1995)
SR2195	MC4100 ϕ (<i>rpoEP2-lacZ</i>)	Raina <i>et al.</i> (1995)
SR3328	MC4100 ϕ (<i>rpoDPhs-lacZ</i>)	this study
SR3556	MC4100 <i>rseA::Tn10</i>	Missiakas <i>et al.</i> (1997)
SR4496	MC4100 ϕ (<i>ftsJ/H-lacZ</i>)	this study
SR4861	MC4100 <i>ecfE</i> -P209T	this study
SR5070	MC4100 <i>ecfE</i> -H22A	this study
Plasmids		
pKO3	allelic replacement vector	Link <i>et al.</i> (1997)
pKAC	derivative of pSU40	Dautin <i>et al.</i> (2000)
pSR1628	pOK12 <i>rpoE</i> ⁺ (1.4 kb <i>Sau3A</i>)	Raina <i>et al.</i> (1995)
pSR3612	pOK12 (<i>ecfE</i> 2.9 kb <i>Sau3A</i> fragment)	this study
pSR3636	pOK12 (<i>ecfE</i> 1.5 kb <i>KpnI</i> - <i>AccI</i> fragment)	this study
pSR3640	pKO3 (<i>ecfE</i> with Ω Kan at <i>BsiWI</i>)	this study
pSR3641	pKO3 (<i>ecfE</i> with Ω Kan at <i>SmaI</i>)	this study
pSR3657	pOK12 (<i>cdsA</i> fragment)	this study
pSR4173	pBAD (<i>ecfE</i>)	this study
pSR4932	pET-22b (<i>ecfE</i> -His6)	this study
pSR 5096	pET-22b (<i>rpoS</i> -His6)	this study
pCD73	pBAD (<i>ecfE</i> -His6-myc)	this study
pCD951	pKAC (<i>rpoH</i> -N-terminal fragment M1-M156)	this study
pCD953	pKAC (<i>rpoH</i> -C-terminal fragment T142-A284)	this study

was resuspended in lysis buffer [50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole (buffer A) supplemented with lysozyme to a final concentration of 200 μ g/ml]. The mixture was incubated on ice for 20 min, sonicated and spun at 100 000 *g* for 30 min at 4°C. The insoluble pellet was resuspended in buffer A containing 0.3% Sarkosyl at 4°C for 45 min to solubilize the inner membrane proteins. Solubilized proteins were recovered by centrifugation at 100 000 *g* for 45 min at 4°C. The supernatant was dialysed against 50 mM Tris-HCl buffer pH 7.5 containing 0.5% Triton X-100 and 50 mM NaCl. Ten millilitres of this solution was applied to Ni-NTA beads (Qiagen), washed and eluted as recommended by the manufacturer. The mutant EcfE-H22A was purified in the same manner. RpoD-His₆ was expressed using plasmid pCL391 and purified as described by Sharp *et al.* (1999). RpoS-6His was expressed using plasmid pSR5096 in Rapid translation system RTS 500 *E.coli* HY kit as described in the Roche Molecular Biochemical manual. The translation product was applied to Ni-NTA beads and eluted with imidazole. Purifications of RpoH, RpoE and RseA have been described previously (Raina *et al.*, 1995; Collinet *et al.*, 2000).

Biochemical assays

The protease assay for EcfE protein was carried out at 25°C using increased concentration of this protease and various substrates at a concentration of 1 μ g each. The β -galactosidase assays were carried out as described by Miller (1992).

Stability of RpoE and RpoH

Bacterial cultures were grown in M9 medium supplemented with 0.2% glucose and labelled with 100 μ Ci/ml [³⁵S]methionine for 20 s and chased with 100 μ g/ml of cold methionine for various periods of time. Aliquots of cells were harvested at different intervals and immediately precipitated with 10% trichloroacetic acid. Protein samples were immunoprecipitated using either anti-RpoH or anti-RpoE antibodies. The complexes were concentrated by protein A-Sepharose beads (Pharmacia) and analysed by

12.5% SDS-PAGE. The incorporated radioactivity was quantified using a PhosphorImager (Molecular Dynamics).

Fractionation experiments

Cells were grown in LB broth at 37°C to an OD₅₉₅ of 0.5. Cells were resuspended in 100 mM Tris-HCl pH 7.5, 10 mM EDTA and incubated with 1 mg/ml of lysozyme for 20 min, followed by treatment with DNase I. Cells were lysed by multiple freeze-thaw. The lysate was centrifuged at 25 000*g* for 15 min. The supernatant containing all soluble proteins (periplasmic and cytoplasm) was removed. The pellet containing the membrane fractions and insoluble material was resuspended in 20 mM NaPO₄ (pH 7.0) containing 0.5% Sarkosyl for 30 min at 20°C and spun at 100 000 *g* at 4°C for 30 min. The supernatant containing the inner membrane fraction was removed. The pellet containing the OMPs was washed with the same buffer and resuspended in SDS-PAGE sample buffer. Fractions were analysed by loading on to SDS-polyacrylamide gels (12.5%).

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