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Phosphorylation-mediated regulation of heat shock response in *Escherichia coli*

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Summary

Escherichia coli **has two heat shock regulons under the transcriptional control of $E\sigma^{32}$ and $E\sigma^{E}$ RNA polymerases. These polymerases control the expression of genes, the products of which are needed for correct folding of proteins in the cytoplasm and the extracytoplasm respectively. In this study, we report that mutations in a tyrosine phosphatase-encoding gene led to decreased activity of these heat shock regulons. The activity of the tyrosine phosphatase is presumably co-ordinated with that of a cognate kinase. We show here that mutants deleted for the phosphatase-encoding gene accumulate phosphorylated RpoH. We find that RpoH is phosphorylated at amino acid position 260, which is located in the conserved region 4.2, and that this phosphorylation event attenuates RpoH activity as a sigma factor. The *rpoH* Tyr-260Ala mutation confers a temperature-sensitive phenotype that leads to an altered heat shock response. Additionally, we show that the antisigma factor RseA is phosphorylated at the N-terminally located Tyr-38 and that this phosphorylation presumably alters its binding affinity towards σ^{E}.**

Introduction

The heat shock response is universally conserved in evolution. It has evolved to detect and correct misfolding of polypeptides upon environmental changes, particularly those involving extreme temperatures. In *Escherichia coli*, heat shock response leads to the transcriptional activation of a subset of specific genes. The transcription of heat shock genes is controlled by two RNA polymerase species, $E\sigma^{32}$ and $E\sigma^{E}$. Misfolded polypeptides in the extracytoplasm of *E. coli* primarily induce the σ^{E} regulon. σ^{E} is transcribed by the *rpoE* gene. However, misfolding in the cytoplasm induces the principal heat shock sigma factor σ^{32} encoded by the *rpoH* gene. Heat shock proteins include chaperones, folding catalysts and proteases.

After a temperature upshift from 30°C to 42°C, the rate of heat shock protein synthesis increases immediately and reaches a maximum induction (10- to 15-fold above the level at 30°C) within 5 min (reviewed by Gross, 1996; Missiakas *et al*., 1996). This rapid induction is achieved by multiple mechanisms that co-ordinate the level of transcription and translation of the *rpoH* gene, and also control the stability and activity of σ^{32}. Temperature-controlled translation of *rpoH* mRNA is achieved by melting of an inhibitory secondary structure in this RNA, thereby selectively increasing the translation of σ^{32} (Morita *et al*., 1999). The activity of σ^{32} is controlled by DnaK/DnaJ chaperones. It has been proposed that a pool of free DnaK (Craig and Gross, 1991) and/or DnaJ (Bukau, 1993) acts as a 'cellular thermometer', which monitors the state of unfolded proteins and adjusts the amount of active σ^{32} and the expression of heat shock genes accordingly. Additionally, σ^{32} is turned over rapidly. σ^{32} is an extremely unstable protein *in vivo* with a half-life of 30–45 s (reviewed by Gross, 1996).

RpoE belongs to a family of highly conserved sigma factors designated as the extracytoplasmic function (ECF) group, as they respond to extracytoplasmic stimuli. During the last few years, it has become clear that many mechanisms of signal transduction have evolved to control the level and activity of different ECF sigma factors. For example, *rpoE* transcription is positively autoregulated through its P2 promoter, and is activated upon misfolding of OMPs and other envelope stresses (Raina *et al*., 1995; Rouvière *et al*., 1995). RpoE is also negatively regulated by RseA and RseB proteins, the cognate genes of which are co-transcribed with the *rpoE* gene (De Las Peñas *et al*., 1997; Missiakas *et al*., 1997).

RseA is an inner membrane protein with a single transmembrane domain spanning approximately the middle of a polypeptide. It is the N-terminal cytoplasmic domain of RseA that can bind σ^{E} and thereby sequester it from the RNA polymerase (Missiakas *et al*., 1997). RseA also car-

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ries a 10 kDa C-terminal domain located in the periplasm. This domain interacts with RseB, a periplasmic protein (Missiakas *et al*., 1997). RseB seems to act as a sensor and presumably, depending on the accumulation of misfolded peptides, RseB dissociates from RseA and thus mediates the release of σ^E, which is otherwise sequestered to the membrane (Missiakas *et al*., 1997; Collinet *et al*., 2000).

The chromosome of *E. coli* encodes many two-component signal transduction systems, all of which respond to specific signals. Among these, the CpxA CpxR two-component system, in conjunction with two serine/threonine phosphatases (PrpA and PrpB), has evolved in parallel to the σ^E stress regulon (Danese *et al*., 1995; Raina *et al*., 1995; Missiakas and Raina, 1997). CpxA CpxR regulates the transcription of many genes with products participating in the folding of envelope proteins (Dartigalongue *et al*., 2001). Interestingly, to a certain extent, the CpxA CpxR regulon overlaps that of the σ^E regulon because some genes carry promoters for both CpxR and the $E\sigma^E$ RNA polymerase (Danese *et al*., 1995; Dartigalongue *et al*., 2001).

In the present work, we isolated a number of temperature-sensitive (Ts) mutants exhibiting a reduced basal level activity at $E\sigma^E$-transcribed promoters that did not map to the *rpoE* gene. Surprisingly, a subset of these mutants also showed reduced levels of transcription at $E\sigma^{32}$-transcribed promoters. We found that these mutations map to a gene encoding for a tyrosine phosphatase. Genome analysis showed that this gene is co-transcribed with a gene encoding a tyrosine kinase. We found that the decreased transcription at $E\sigma^{32}$-dependent promoters, observed in the Ts mutants, could be explained by the accumulation of phosphorylated RpoH. We also identified a tyrosine residue in RpoH that is the target for this phosphorylation event. The decreased transcription observed at $E\sigma^E$-dependent promoters could not be explained by a similar mechanism, and no post-transcriptional modification of RpoE could be observed in the Ts mutants isolated. Instead, RseA was found to be phosphorylated. While this work was in progress, two groups independently characterized two clusters of genes that are located at approximately 46 min and 22 min on the *E. coli* chromosome. These two clusters of genes were investigated because of the obvious sequence homology to genes encoding phosphatases (*etp* and *wzb*) and kinases (*etk* and *wzc*) (Ilan *et al*., 1999; Vincent *et al*., 2000; Wugeditsch *et al*., 2001). The Etk kinase was found to be required for virulence and the production of exopolysaccharide in pathogenic *E. coli* (Ilan *et al*., 1999). The homologous kinase Wzc was found to be required for the assembly of the serotype K-30 group I capsule in *E. coli* O9a:K-30 (Wugeditsch *et al*., 2001). The *E. coli* K-12 counterpart Wzc was found to be connected with the production of colanic acid (Vincent *et al*., 2000). However, in no case examined so far have any direct protein substrate(s) been identified. In this work, we show that our mutations map to the tyrosine phosphatase-encoding gene *etp* located at region 22 min, which leads to the accumulation of RpoH in an inactive phosphorylated form.

Results

Isolation of point mutations with decreased transcription of heat shock genes

In this study, we isolated point mutations that render *E. coli* temperature sensitive (Ts) for growth at 43°C in strains carrying either *htrA–lacZ* or *rpoH*P3–*lacZ* transcriptional fusions. The *htrA* and *rpoH*P3 promoters are known to be transcribed by $E\sigma^E$ RNA polymerase. To rule out any indirect effect on overall protein synthesis, we transduced these mutations in strains carrying promoter fusions to housekeeping genes. A similar strategy was used previously to identify the *rpoE* gene (Raina *et al*., 1995). In the present case, we eliminated mutations mapping to the *rpoE* gene by checking for complementation with a pSC101-based plasmid carrying the wild-type *rpoE* gene (pSR2663). Fourteen out of 89 independently isolated mutants were retained because they met the above criteria and because they also showed 100% co-transduction between the Ts phenotype and low β-galactosidase activity of *rpoE*-transcribed promoters.

As both the heat shock regulons sense misfolded proteins, we tested whether such mutants might also alter the σ^{32}-dependent response. Interestingly, we found that five mutants out of 14 tested exhibited a reduced basal level of transcription at $E\sigma^{32}$-dependent promoters. RpoH and RpoE respond to misfolding of proteins in a compartment-specific manner (cytoplasm versus extracytoplasm); hence, these results were intriguing, and we decided to analyse them further. Quantitative analysis of these mutants showed that they had a similar reduction in the basal level activity of $E\sigma^E$- and $E\sigma^{32}$-transcribed promoters at about 40–50% of the wild type (Table 1). The reporter promoter fusions tested were P*groESL–lacZ* ($E\sigma^{32}$-transcribed) and *rpoD*Phs–*lacZ*, which carries both $E\sigma^{32}$- and $E\sigma^E$-transcribed promoters.

Mapping and characterization of point mutations

As the point mutations we isolated confer a Ts growth phenotype at 43°C, we looked for complementation, i.e. restoration of temperature resistance as well as full *lacZ* activity of reporter genes, with a cloned *E. coli* genomic library constructed in a p15A-based vector (Missiakas and Raina, 1997). Complementing clones for the Ts phenotype as well as for the restoration of *lacZ* activity were

Table 1. Loss of function mutation in the *etp* gene leads to basal level reduction in the transcriptional activity of the σ^E- and σ^{32}-dependent promoters at 30°C.

Relevant genotype	β-galactosidase activity (Miller units) at 30°C
SR1458 = *htrA–lacZ*	98 ± 11
SR1458 *etp* D54V	57 ± 7
SR1710 = *rpoH*P3–*lacZ*	73 ± 9
SR1710 *etp* D54V	40 ± 5
SR4499 = *groESL–lacZ*	710 ± 64
SR4499 *etp* D54V	390 ± 41
SR4653 = *rpoD*Phs–*lacZ*	103 ± 9
SR4653 *etp* D54V	50 ± 6

obtained. Based on the degree of suppression, we could divide the complementing clones into two groups. One group both conferred temperature resistance and also restored the transcriptional activity of the reporter transcriptional fusion. The second group gave a somewhat reduced complementation in terms of growth above 43.5°C. Restriction enzyme digestion patterns also revealed that these two groups of complementing clones had different genomic DNA fragments.

The DNA isolated from the complementing plasmids was subcloned into different cloning vectors to obtain the minimal size that restored the β-galactosidase activity to the wild-type level and that complemented the Ts phenotype. One representative plasmid from each of the groups was used further for mapping and DNA sequence analysis. Plasmid pSR3947, representing one group of clones that conferred full complementation, was found to carry DNA sequences from the 22 min region of the *E. coli* chromosome. The DNA from the plasmid representing the second group with a lower degree of complementation (pSR3952) was found to carry the DNA from the 46 min region of the *E. coli* chromosome. Examination of the sequence showed that plasmid pSR3947 contains two open reading frames (ORFs), designated *yccY* and *yccC*, and plasmid pSR3952 contains three ORFs designated *wza*, *wzb* and *wzc*. The published *E. coli* DNA sequence suggests that the *yccY* and *yccC* genes are co-transcribed. Further subcloning experiments showed that the *yccC* gene was sufficient to complement the Ts phenotype if provided with a heterologous promoter. These results further confirm that the *yccY* and *yccC* genes are co-transcribed. As the *yccC* and *yccY* genes have recently been cloned, we will hereafter refer to these genes as recently published: *etp* for *E. coli* tyrosine phosphatase and *etk* for *E. coli* tyrosine kinase are used instead of *yccC* and *yccY* respectively (Ilan *et al*., 1999; Vincent *et al*., 2000). The genomic organization of the *wza*, *wzb* and *wzc* gene cluster also shows that they are co-transcribed along with 16 other genes. The deduced amino acid sequence showed that both gene clusters at 22 min and 46 min regions encode proteins that are highly homologous to protein phosphatases (Etp and Wzb) and protein autokinases (Etk and Wzc).

To ascertain the exact gene(s) to which our mutations mapped, we performed two types of experiments. First, we constructed systematic disruptions of the *etp* and *etk* genes as well as the *wzb* and *wzc* genes on plasmids. We then transferred them onto the chromosome and analysed for different phenotypes. Consistent with the analysis of our point mutants, disruption of the *etp* gene conferred a Ts phenotype above 42°C. Only a modest Ts phenotype above 43.5°C was observed when the *etk* gene was disrupted. In both cases, a kanamycin cassette lacking transcriptional terminator sequences was used to avoid any polarity effect. Disruption of ORFs *wzb* and *wzc* conferred a Ts phenotype only at 43.5°C. We also transduced these null alleles to strains carrying single-copy promoter fusion to either *rpoE*-regulated promoters or *rpoH*-regulated promoters. Again, a null mutation in the *etp* gene showed a similar decline for σ^E- and σ^{32}-transcribed promoters (40–50%) (Fig. 1). Thus, the disruption of the *etp* gene leads to a very similar phenotype to that noticed for the point mutants. Surprisingly, the disruption of the *wzb* gene showed only a weak reduction of up to 20–30% for σ^E-transcribed genes and an even lesser effect on the activity for σ^{32}-transcribed promoters. No significant reduction in the σ^{32}- or σ^E-transcribed promoters was noticed when a non-polar null allele of the *wzc* gene was introduced in strains carrying the appropriate *lacZ* reporter fusion.

In the next step, we directly sequenced the chromosomal DNA, which was polymerase chain reaction (PCR) amplified using primers specific to the *etp*, *etk*, *wzb* and *wzc* genes. Interestingly, all the mutations mapped to the *etp* gene, which encodes a putative phosphatase. A non-polar deletion of the *wzb* gene conferred a weak phenotype when examined for σ^E or σ^{32} reporter gene expression. This explains why we did not obtain mutations in the *wzb* gene in our genetic screen. Four out of the five mutants had a single site change [Cys-22 TGC to TGG (Trp), Asp-54 GAT to GTT (Val), His-71 CAT to GAT (Asp), Asp-121 GAT to GTT (Val)], whereas one mutant had two point mutations and was not used further. These phosphatases belong to the family of protein tyrosine phosphatases for which the three-dimensional structures have been determined (Su *et al*., 1994; Zhang *et al*., 1997). Three out of the five mutations map to the homologous region of these phosphatases and are presumably part of the catalytic site.

Overlap in the function of etp *and* wzb *genes*

As described above, *etp* and *wzb* genes encode putative phosphatases and are similarly co-transcribed with genes encoding for protein autokinases. In addition, the deduced amino acid sequence of their corresponding genes showed

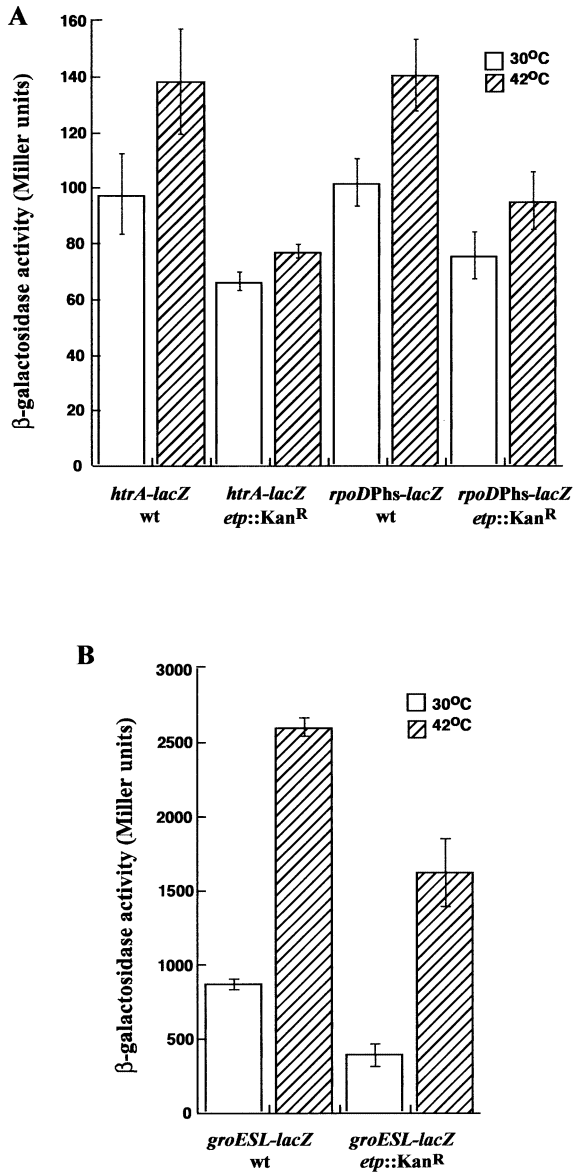

Fig. 1. Decreased activity of heat shock regulons in *etp* mutant bacteria.
A. A null mutation in the *etp* gene leads to a basal level reduction in the transcription of *htrA* (σ^E-regulated) and *rpoD* (with σ^E and σ^{32} promoters) genes.
B. The level of σ^{32}-dependent *groESL* promoter. Isogenic bacterial cultures were grown overnight at 30°C, diluted 1:100 and grown to an OD_{595} of ≈0.2. Half the culture was shifted to 42°C in prewarmed tubes and incubated for 20 min. Each sample was assayed thrice, and data presented are an average of three independent experiments. The standard deviation is shown as error bars. Values obtained at 30°C are represented by the grey bars and those obtained at 42°C by the hatched bars.

a very high degree of sequence conservation. Based on these observations, we examined whether a null mutation in the *etp* gene could be complemented by overexpressing the *wzb* gene and vice versa. A multicopy plasmid carrying the wild-type *etp* gene under the p*tac* promoter was able to restore both the Ts phenotype and the transcriptional defect observed with the *wzb* null mutant. However, overexpressing *wzb*$^+$ in a similar genetic set-up did not fully restore the defects observed with the *etp* null mutant. For example, no growth was observed above 43.5°C. These functional overlaps in cross-complementation can be explained by the very high degree of sequence homology between Etp and Wzb proteins. Nonetheless, these results also suggest that each phosphatase possesses a subset of specific substrates. Consistent with these results, the *wzb* gene was cloned as a multicopy suppressor of *etp* mutants in our initial search for complementation. Further, a double null combination of the *etp* and *wzb* genes has an additive effect in reducing expression of reporter Eσ^E- and Eσ^{32}-transcribed promoters. However, the Ts phenotype of the *etp wzb* double null mutant was more or less like that of the *etp* null mutant bacteria in that they were unable to form colonies above 42°C.

Heat shock response in etp *mutants*

As the *etp* mutants have decreased activity of σ^{32}- and σ^E-dependent promoters, we wondered whether they exhibit an altered heat shock response. Heat shock induction was compared between the isogenic wild-type and *etp* mutant strains carrying promoter fusions to either σ^{32}- or σ^E-dependent promoters. Two σ^{32}-dependent promoters, P*groESL* and *rpoD*Phs, were tested. A reduction of around 40–50% was observed at 30°C (Fig. 1). However, after heat shock, no difference in induction was observed between the wild type and the mutants. In the case of P*groESL*, a shift to 42°C led to a three- to fourfold induction in both cases. Thus, we conclude that heat shock induction is not altered, but that the basal level of transcription by σ^{32} is reduced in the *etp* mutant. Similar results were obtained when the *htrA–lacZ* reporter strain was used at 30°C. Lack of the *etp* gene led to a reduction in basal transcription (≈ 40%) at 30°C (Fig. 1A). However, upon temperature shift to 42°C, only a small increase in transcription was observed in the *etp* mutant compared with the isogenic wild type, which showed a 50% induction (Fig. 1A). It may be pertinent to point out that *rpoD*Phs has dual control of both σ^{32} and σ^E factors. Also, the σ^{32}-dependent promoter is relatively weak and, hence, the induction at 42°C is lower than that observed with the P*groESL* reporter (compare Fig. 1A and B). Thus, the σ^{32}- and σ^E-dependent heat shock responses seem to be differentially affected in the *etp* mutants, in terms of activation of transcription, after a shift from 30°C to 42°C.

Purified Etp and Etk display phosphatase and autokinase activity respectively

As the *etp* and *etk* genes are co-transcribed and encode

a predicted phosphatase and kinase, we tested the activity of their respective gene products. For this purpose, the *etp* gene was cloned in the pET-22b vector with a six-histidine tag (His_6-tag) appended at the C-terminus (pSR6013). Etp was purified using standard Ni-NTA affinity chromatography. The purified protein was assayed using *para*-nitrophenol phosphate (*p*NPP) as a substrate. As can be seen in Fig. 2A, Etp showed concentration-dependent *p*NPP phosphatase activity. The *etk* gene was cloned in the pBAD/His-C vector with a His_6-tag at the C-terminus. After induction with arabinose, the Etk protein was purified from the inner membrane fraction by Ni-NTA affinity chromatography (see *Supplementary material*, Fig. S1). Purified Etk (Fig. 2B) was incubated with [γ-^{32}P]-ATP, and this reaction mixture was loaded on SDS-PAGE, and the separated products were visualized by autoradiography. The results presented in Fig. 2B clearly demonstrated that Etk autophosphorylates quite efficiently. Autophosphorylated Etk was further incubated with purified Etp. This incubation resulted in the loss of ^{32}P-labelled Etk, suggesting that Etp was able to dephosphorylate Etk *in vitro* (Fig. 2C).

To establish whether phosphorylation and dephosphorylation were specific to a tyrosine residue, γ-^{32}P-ATP-labelled Etk was treated with either sodium hydroxide (NaOH) or tricholoroacetic acid (TCA) after incubation with the radioactive probe. It is known that phospho-Ser and phospho-Thr, but not phospho-Tyr, are sensitive to acid treatment, whereas phospho-Tyr is sensitive to alkaline treatment. Our results show that phosphorylation of Etk is only sensitive to the alkaline treatment (data not shown). Using phospho-Tyr monoclonal antibodies (P-Tyr-100; New England BioLabs), one can clearly see that phosphorylated Etk cross-reacts (Fig. 4B).

Etk phosphorylates σ^{32}

As we isolated mutations in the *etp* gene that led to a decrease in the activity of *rpoH*-regulated promoters, we suspected that, in the absence of Etp, Etk may be hyper-

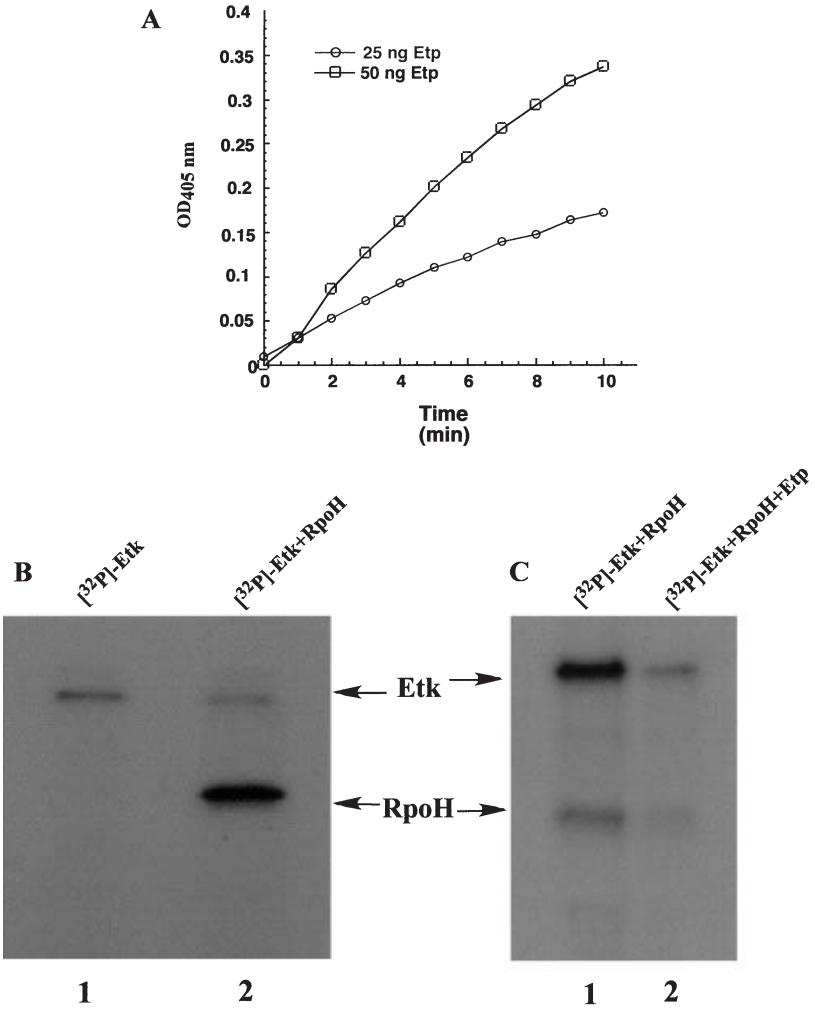

Fig. 2. Purified Etp possesses phosphatase activity, and RpoH is phosphorylated by Etk and dephosphorylated by Etp *in vitro*.
A. 25 ng (open circles) and 50 ng (open diamonds) of Etp was assayed in a 1 ml reaction volume for each assay. The assay buffer contains 50 mM Tris-HCl, pH 7.8, 20 mM *p*NPP. The increase in *p*-nitrophenol was monitored at 405 nm.
B and C. 0.2 μg of Etk was phosphorylated with [γ-^{32}P]-ATP (B, lane 1) and incubated further with RpoH at 25°C for 30 min (B, lane 2; C, lane 1). The same amount of protein as used for lane 1 (C) was incubated with 0.1 μg of Etp (C, lane 2).

phosphorylated *in vivo*, and it may in turn phosphorylate RpoH. To test this assumption, we prepared phosphorylated Etk *in vitro* and incubated it with purified RpoH at 25°C for 30 min in a 20 µl reaction. Half the reaction mixture was further incubated with purified Etp for another 30 min at 25°C. Both the samples were then separated by SDS-PAGE and subsequently subjected to autoradiography. Figure 2C shows one such example of phosphorylation and dephosphorylation. As can be seen, RpoH is a good substrate for phosphorylation by the Etk kinase, and this process is reversed when Etp is added to the reaction mixture (Fig. 2C, lane 2).

In order to address how much RpoH gets phosphorylated, we performed two-dimensional gel electrophoresis of non-phosphorylated and phosphorylated RpoH with or without incubation with ^{32}P-labelled Etk. The data, presented in Fig. 3A, show that approximately half the RpoH was phosphorylated, as seen by a shift in the isoelectric point to a single additional phosphorylated form. These results were confirmed further by autoradiography of the same two-dimensional PAGE (Fig. 3B). The more acidic spot is the only one that is radioactive. These experiments clearly demonstrate that Etk can phosphorylate RpoH *in vitro*. The appearance of a single radiolabelled spot suggested that phosphorylation occurred either at a single residue or uniformly at several sites. Furthermore, this phosphorylation is reversible, and dephosphorylation can be mediated by Etp (Fig. 2C).

Next, we examined the phosphorylated status of RpoH *in vivo*. Wild-type BL21(DE3) and a BL21(DE3) *etp*::Kan^R variant were transformed with a plasmid carrying the *rpoH* gene under an IPTG-inducible promoter (pSR6027). Both strains were induced for the expression of the *rpoH* gene. As the cloned *rpoH* gene carried a six-histidine tag, it was purified from total cell extracts using Ni-NTA affinity chromatography. Proteins were eluted with imidazole and examined by two-dimensional PAGE. The gels were silver stained, and the intensity of the spots was quantified by densitometry. As can be seen, the RpoH protein purified from the wild-type strain is present mostly in the non-phosphorylated form (Fig. 3C). RpoH extracted from *etp* mutant bacteria exhibited two isoelectric forms (Fig. 3D). The more acidic form (phosphorylated) constitutes >30% of the total RpoH protein. It may be mentioned here that

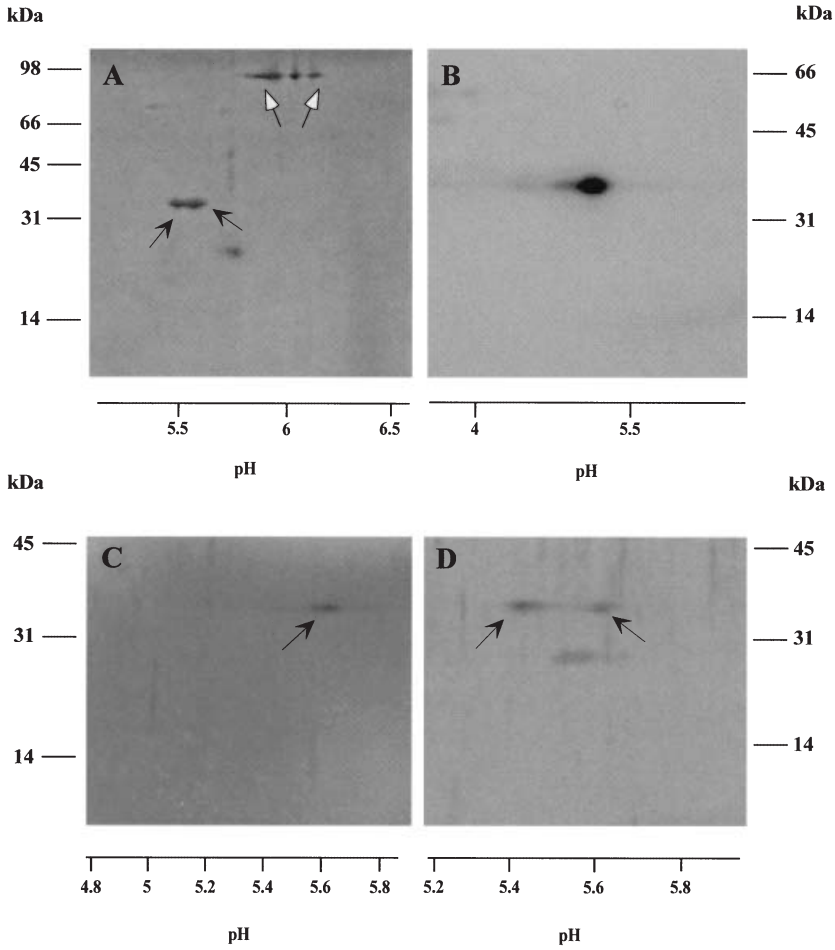

Fig. 3. Phosphorylation of RpoH. Two-dimensional equilibrium gel electrophoresis of RpoH with or without incubation with ^{32}P-labelled Etk. After 30 min incubation of RpoH (0.1 µg) with 0.1 µg of [^{32}P]-Etk at 25°C, samples were analysed by two-dimensional PAGE. These proteins were resolved in the first dimension on 1.6% (pH 5.0–7.0) and 0.4% (pH 3.4–10.0) ampholytes (Pharmacia) and by 12.5% SDS-PAGE in the second dimension.
A. Picture of Coomassie brilliant blue-stained dried gel with two spots of RpoH as shown by closed arrows. Isoforms of Etk are indicated by the open arrows.
B. Autoradiogram of the dried gel from (A).
C. Purified RpoH from the wild type without incubation with Etk and resolved on two-dimensional PAGE, followed by silver staining.
D. The relevant portion of two-dimensional PAGE of RpoH purified from an *etp* null strain. The picture of the silver-stained dried gel is shown. The estimated pH is shown below and the molecular weight standards used at the side.

the cellular concentration of RpoH is very low under steady-state conditions estimated at 10–30 copies per cell at 30°C (Craig and Gross, 1991), which necessitated the use of an overexpressing system for the phosphorylation studies.

Mapping of the phosphorylation site in RpoH

Examination of the RpoH amino acid sequence shows that it contains seven Tyr residues located at positions 19, 43, 68, 71, 116, 200 and 260. We performed a systematic site-directed mutagenesis of these residues and generated seven variants of RpoH individually lacking one Tyr. The amino acid substitutions made are described in Table 3. All the *rpoH* mutations introduced were verified by DNA sequencing. Each RpoH variant was purified, incubated with the phosphorylated Etk and analysed as described previously. Only the Tyr-260Ala variant was found to have lost the ability to become phosphorylated *in vitro* (Fig. 4A). Hence, we conclude that Tyr-260 is the site of phosphorylation in RpoH. This residue is located in the highly conserved region 4.2 of RpoH. As the extent of phosphorylation, as judged by the incorporation of radioactivity, was found to be the same as in the wild type versus the other Tyr variants (except Tyr-260Ala), it argues again that only one Tyr residue is the site of phosphorylation. These results were confirmed further by performing immunoblot experiments with different variants of purified RpoH proteins after incubation with phosphorylated Etk using phosphotyrosine-specific antibodies. As can be seen, only the RpoH Tyr-260Ala mutant protein does not cross-react with phosphotyrosine-specific antibodies (Fig. 4B) and hence is specific to this Tyr residue only.

RpoH is phosphorylated in vivo

As both kinase and phosphatase are present in the cell and because RpoH is present in very low amounts, it might be difficult to isolate the phosphorylated form of RpoH from whole-cell extracts. To maximize the chances of isolating such species, we labelled the isogenic strains BL21(DE3), carrying either *rpoH*$^+$ or *rpoH* Tyr-260Ala on the plasmid, using [^{32}P]-orthophosphate. The synthesis of RpoH was induced by the addition of 1 mM IPTG for 30 min, followed by incubation for an additional 30 min with 100 μCi ml^{-1} [^{32}P]-orthophosphate. Proteins were TCA precipitated and analysed by 12.5% SDS-PAGE.

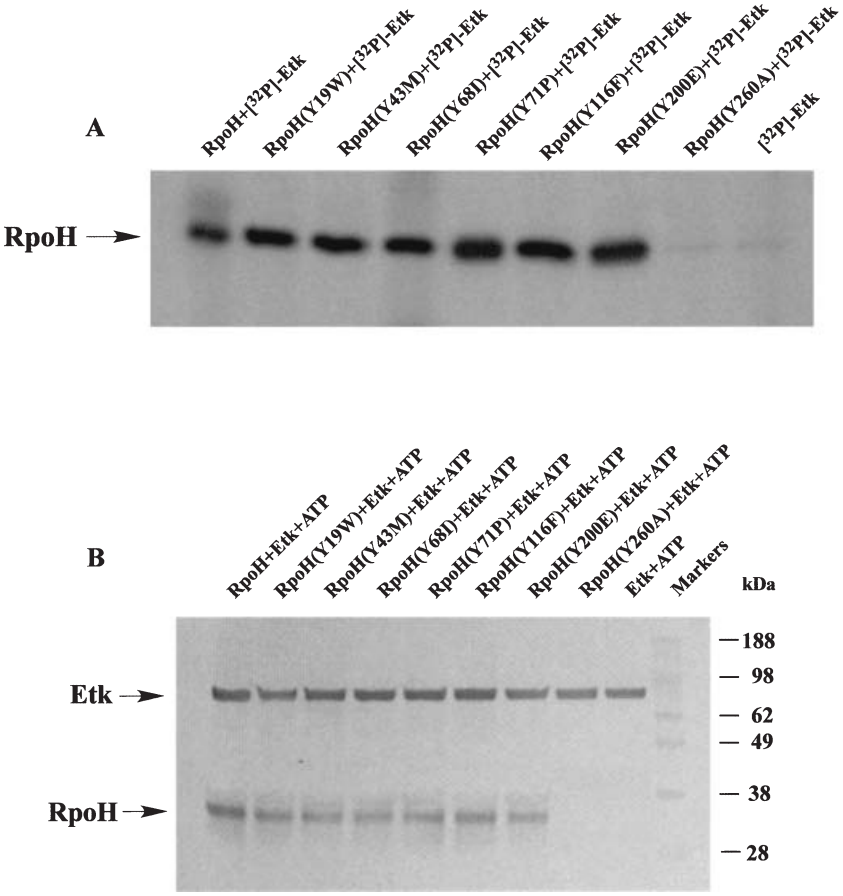

Fig. 4. RpoH is phosphorylated at Tyr-260 in the region 4.2.
A. All seven Tyr residues were mutated. The variant proteins were purified, and 0.1 μg of each protein per reaction was incubated with γ-^{32}P-labelled Etk as described in the legend to Fig. 2B. Proteins were separated by 12.5% SDS-PAGE, and radioactive species were revealed by autoradiography. The mutation introduced in each case is indicated.
B. The purified wild type and the seven variants of RpoH (0.1 μg in each case) were incubated with 5 mM ATP and an equivalent molar amount of purified Etk at 25°C for 30 min. Samples were resolved on a 12% Bis-Tris NuPAGE gel (Invitrogen), and a Western blot transfer was performed according to the manufacturer's instructions. The Western blot was treated with monoclonal antibodies against phosphotyrosine (P-tyr-100) and then detected by anti-mouse IgG, AP conjugate. Note that EtK and all the RpoH variants except for Tyr-260A show cross-reaction.

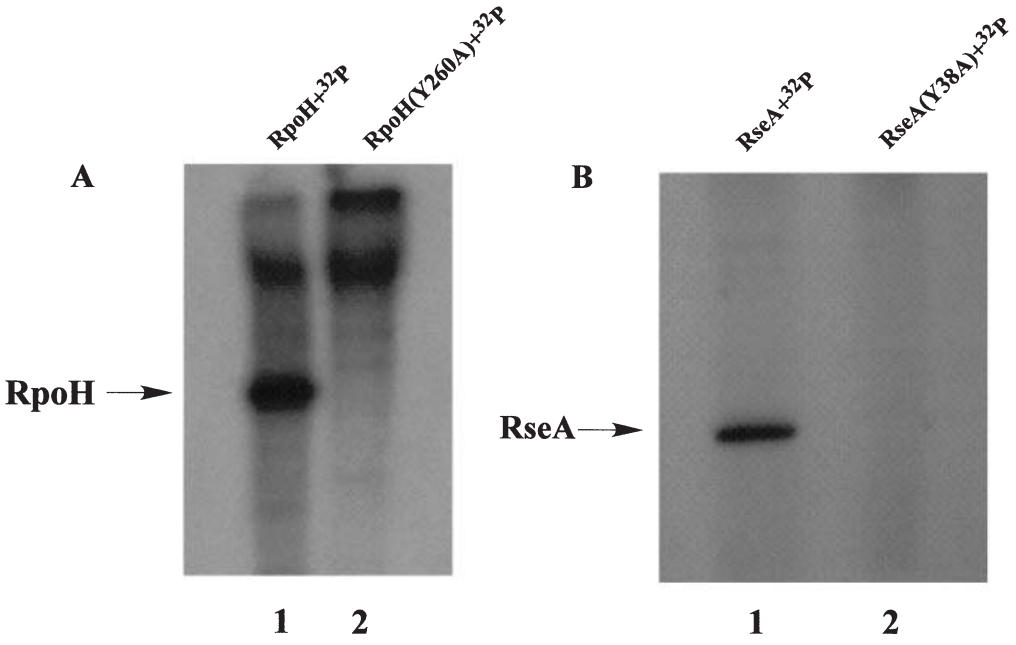

Fig. 5. *In vivo* phosphorylation of RpoH and RseA.
A. *In vivo* labelling of RpoH with [^{32}P]-orthophosphate. *E. coli* strain BL21 carrying pSR6027 (wild-type *rpoH*; lane 1) and pGK181 (*rpoH* Tyr-260Ala; lane 2) were induced with 1 mM IPTG for 30 min at 30°C, followed by labelling with 100 μCi ml^{-1} [^{32}P]-orthophosphate for 30 min at 30°C. Proteins from cell extracts were resolved on 12.5% SDS-PAGE, and an autoradiogram of the dried gel is shown.
B. *In vivo* labelling of RseA with [^{32}P]-orthophosphate. *E. coli* strain LMG194 carrying either wild-type *rseA* (lane 1) or *rseA* Tyr-38Ala (lane 2) on the pBAD/His C vector were used for the assay. Synthesis of wild-type and mutant RseA was induced by the addition of 0.1% arabinose for 1 h, followed by the addition 100 μCi ml^{-1} [^{32}P]-orthophosphate for 30 min at 30°C and analysed as in (A).

Figure 5A (lane 1) shows the presence of phosphorylated RpoH *in vivo*. No phosphorylated RpoH was detected in the extract obtained from the RpoH Tyr-260Ala-expressing strain (Fig. 5A, lane 2). These results argue that RpoH can be phosphorylated *in vivo* at Tyr-260.

Characterization of rpoH *Tyr-260Ala mutant*

As Tyr-260 can be phosphorylated *in vivo*, the question is what is the effect of phosphorylation on RpoH activity? Tyr-260 is located in the conserved region 4.2, which is needed for recognition of the –35 region of promoters. Tyr-260 is also located in the predicted helix–turn–helix motif needed for DNA binding. We asked whether a low-copy plasmid carrying the *rpoH* Tyr-260Ala variant could complement the *rpoH* null mutation. A null mutation in the *rpoH* gene leads to a Ts phenotype above 20°C and resistance to bacteriophage lambda (Kusukawa and Yura, 1988). *rpoH* Tyr-260Ala restored growth of the *rpoH* null mutation up to 37°C. However, no bacterial growth was observed at 42°C. *rpoH* Tyr-260Ala also supported bacteriophage lambda growth up to 37°C (Table 2). These

Table 2. *In vivo* characterization of RpoH Tyr-260Ala mutant.

	Growth at different temperatures			λ plating			β-Galactosidase activity (Miller units)
	30°C	37°C	42°C	30°C	37°C	42°C	30°C
Wild type ϕ (*groESL–lacZ*)	+	+	+	+	+	+	670 ± 72
*rpoH*ΔKan ϕ (*groESL–lacZ*)	±	–	–	–	–	–	120 ± 14
*rpoH*ΔKan ϕ (*groESL–lacZ*) pSR6027(*rpoH*$^+$)	+	+	+	+	+	+	730 ± 65
*rpoH*ΔKan ϕ (*groESL–lacZ*) pGK181(*rpoH* Tyr-260Ala)	+	±	–	+	+	–	325 ± 36

The symbol (–) indicates no growth or non-plating of bacteriophage λ, (±) poor growth, and (+) indicates growth or plating like the wild type.

results suggest that the RpoH Tyr-260Ala mutant protein is active *in vivo* up to 37°C.

Next, we looked at the heat shock response by pulse labelling the bacteria with [^{35}S]-methionine at 30°C and then shifting to 42°C for 5 min. The data presented in Fig. 6A show that induction of heat shock proteins at 42°C is significantly decreased in bacteria carrying the RpoH Tyr-260Ala mutant compared with wild type. It is possible that the interaction of RpoH Tyr-260Ala with core RNA polymerase is sensitive to high temperature or, alternatively, that the mutant protein is unstable at high temperature. We tested the latter possibility by Western blot analysis. Cell extracts prepared from the wild-type versus *rpoH* Tyr-260Ala mutant bacteria were probed with anti-RpoH antibodies. As can be seen in Fig. 6B, both strains accumulate the same amount of RpoH. Most importantly, both the wild-type RpoH and the mutant RpoH proteins accumulate to the same extent at 42°C (Fig. 6B).

Phosphorylated RpoH as well as RpoH Tyr-260Ala have reduced transcriptional activity

As phosphorylation does not affect the stability of RpoH, we tested whether it would alter the transcriptional activity of the protein. In this case, phosphorylation may alter the ability of RpoH to interact with core RNA polymerase or to recognize promoters. We performed *in vitro* run-off assays using the promoter region of the *lon* gene as a template. As can be seen in Fig. 7A, a significant decline was observed in the amount of transcript produced with the holoenzyme reconstituted with phosphorylated σ^{32} versus non-phosphorylated σ^{32}. Quantitative analysis of this experiment shows a reduction by about seven- to 10-fold when using phosphorylated RpoH. The reduction in the amount of transcript production was reversed when Etp was added to the phosphorylation mixture along with the core RNA polymerase.

We also performed *in vitro* run-off assays with the holoenzyme reconstituted with the wild-type phosphorylated σ^{32} versus non-phosphorylated σ^{32} and compared with the holoenzyme reconstituted with the σ^{32} Tyr-260Ala mutant protein. The results presented in Fig. 7B show that the σ^{32} Tyr-260Ala mutant protein is severely impaired in the initiation of transcription from the Eσ^{32}-transcribed promoter like that of the *lon* gene. Thus, modification of σ^{32} by either phosphorylation at Tyr-260 or alteration of Tyr-

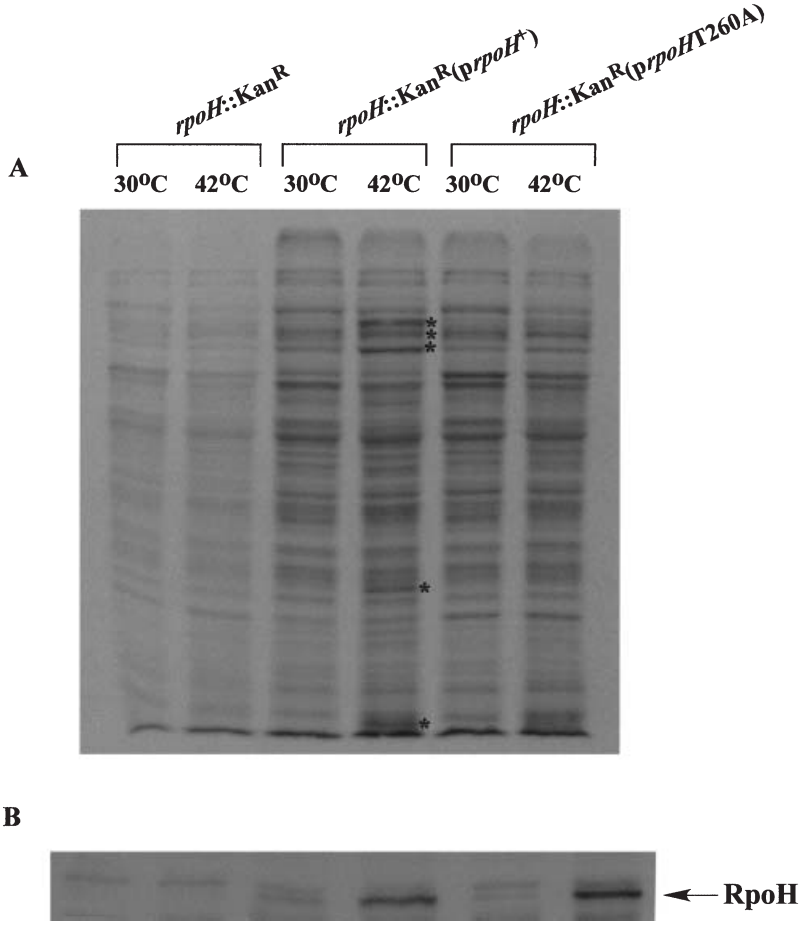

Fig. 6. The *rpoH* Tyr-260Ala mutant exhibits an altered heat shock protein profile at 42°C, which is a phenotype that is not reflective of its stability. Isogenic cultures of different strains with the relevant genotype shown were grown at 30°C in M9 minimal medium. Equivalent amounts of exponentially growing cultures were labelled for 5 min with [^{35}S]-methionine (50 μCi ml^{-1}) at 30°C or at 42°C. Bacterial cultures labelled at 42°C were preincubated for 5 min at 42°C before the addition of the radiolabelled methionine. Extracts were resolved by 12.5% SDS-PAGE.
A. Autoradiogram of the dried gel. The position of some of the major heat shock proteins that accumulated at 42°C is indicated by the symbol (*).
B. The total amounts of RpoH, as revealed by Western blot analysis, using RpoH-specific antibodies. The samples were loaded in the same order as in (A).

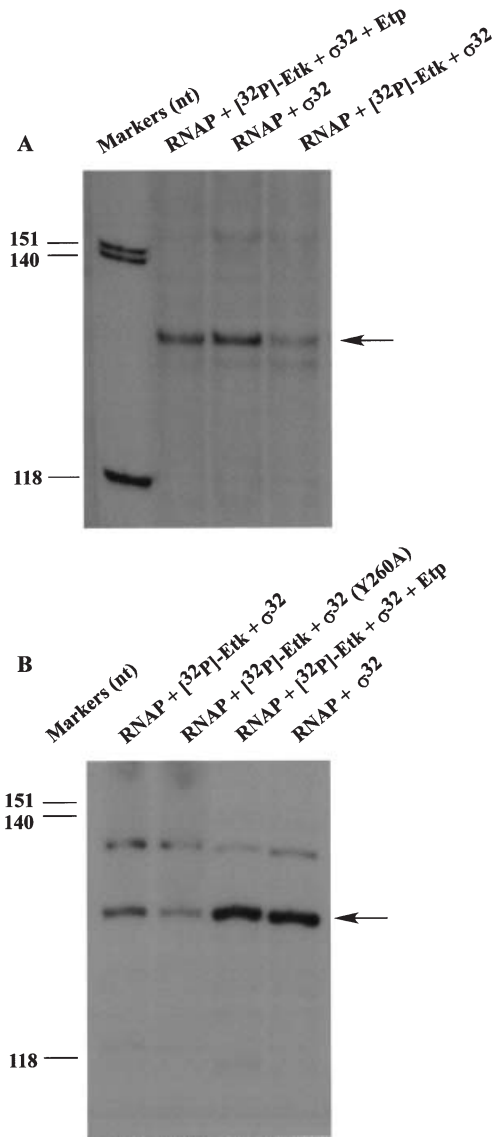

Fig. 7. Inhibition of the transcriptional activity of σ^{32} upon phosphorylation.
A. A 550 bp DNA fragment generated by PCR containing the promoter region of the *lon* gene was used as a template for *in vitro* run-off assay. For each reaction, 2 μg of the core RNAP was incubated with 1 μg of purified σ^{32}, which was either phosphorylated or non-phosphorylated as indicated. In a third reaction, the complex of RNAP + phosphorylated σ^{32} was incubated with 0.2 μg of Etp for an additional 30 min at 25°C before the start of RNA synthesis. In each case, the reaction was carried out at 37°C for 30 min in a 20-μl reaction. RNA samples (5 μl) labelled with [^{35}S]-UTP were loaded on a urea–acrylamide gel. As a control for the size of the product synthesized, radiolabelled DNA was prepared from the fragments created by the restriction endonuclease digestion of ϕX174 DNA with *Hinf*I. Autoradiogram of a dried gel is shown. The arrow shows the position of the run-off transcript.
B. The *in vitro* run-off assay was carried out as described above (A). In addition, under identical conditions, one more reaction was carried out using 2 μg of the core RNAP that was incubated with 1 μg of purified σ^{32} Y260A using the same template as in (A). The arrow shows the position of the run-off transcript. Note that the band migrating above the real transcript is a non-specific product from the end-to-end transcript.

260Ala causes a severe inhibition in its ability to initiate transcription as a sigma factor.

Is RpoE a substrate of Etk as well?

As *etp* mutants show a decreased activity of the σ^E regulon, it seemed reasonable to assume that RpoE may be phosphorylated as well. Purified RpoE protein was incubated *in vitro* with γ-^{32}P-labelled Etk. No phosphorylation of RpoE was observed. Increasing amounts of Etk and varying the ratios of Etk to RpoE yielded the same negative result. Hence, we conclude that RpoE is not a substrate of Etk *in vitro*.

Is RseA phosphorylated?

As RpoE and RseA interact *in vivo* as well as *in vitro*, we wondered whether RseA might be phosphorylated. Purified RseA was incubated with γ-^{32}P-labelled Etk. The reaction samples were analysed by 12.5% SDS-PAGE, and the products were revealed by autoradiography. As shown in Fig. 8A, RseA indeed appears to be phosphorylated.

Which domain of RseA is phosphorylated?

The antisigma activity of RseA is carried on the N-terminal domain of the protein. This domain is located in the cytoplasm. The RseA protein spans the inner membrane once, and the rest of the protein protrudes in the periplasm where it senses incoming signals. Overproduction of the RseA N-terminal domain, RseA^N, alone also inhibits RpoE activity (Missiakas *et al*., 1997). Because of its cytoplasmic location, we tested whether the N-terminal domain is phosphorylated. To avoid the toxicity caused by its overproduction, we constructed a plasmid containing the full-length *rpoE* gene along with the DNA sequence encoding RseA^N (1–96 amino acid residues). RseA^N co-purified with RpoE, and this complex was incubated with ^{32}P-labelled Etk kinase and separated on SDS-PAGE. As an internal control, full-length RseA was added to the phosphorylation reaction. Both RseA and RseA^N, but not RpoE, were radiolabelled, suggesting ^{32}P transfer from Etk to RseA (Fig. 8A). The phosphorylation reaction was also analysed by two-dimensional PAGE. As shown in Fig. 8B, the isoelectric point of the major spot corresponded to radioactive RseA.

Mapping the site of phosphorylation in RseA

Examination of RseA^N sequence shows the presence of a single Tyr residue at amino acid position 38. Tyr-38 was mutated to Ala, and the presence of the mutation was verified by DNA sequencing. This *rseA* Tyr-38Ala variant gene was subcloned in the expression vector pBAD/His

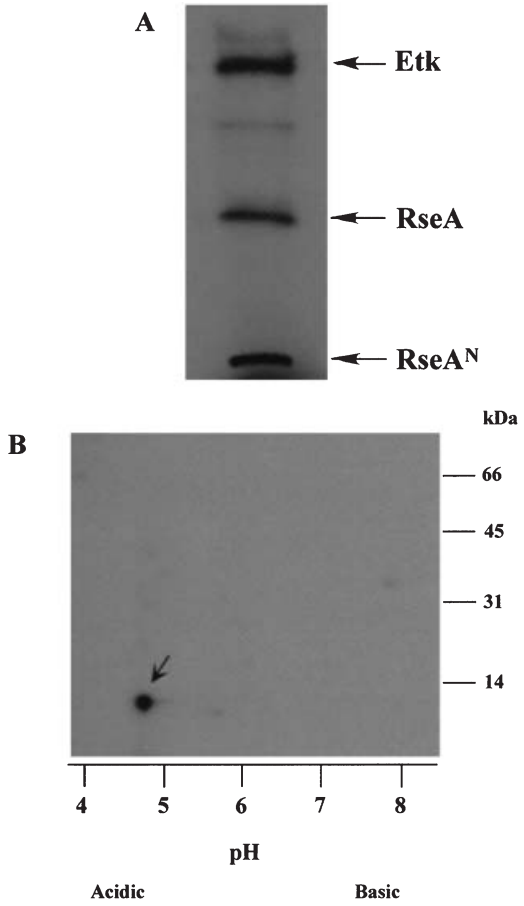

Fig. 8. Phosphorylation of RseA.
A. Etk can phosphorylate antisigma factor RseA at its N-terminus. Etk (0.2 μg) was phosphorylated with [γ-^{32}P]-ATP and incubated with 0.1 μg each of RseA and N-terminal domain (1–96 amino acid residues) for 30 min at 25°C. Proteins were separated by 15% SDS-PAGE, and radioactive species were revealed by autoradiography. The positions of Etk, full-length RseA and RseA^N are indicated on the right side of the autoradiogram.
B. Two-dimensional gel electrophoresis of phosphorylated RseA. RseA^N was phosphorylated as described above and analysed by two-dimensional PAGE as described in the legend to Fig. 4. Autoradiogram of dried gel is shown. The arrow points to the phosphorylated form of RseA^N, which migrates consistent with its predicted pI.

C for protein purification purposes. Bacterial cultures of strains carrying either *rseA* Tyr-38Ala or the wild-type *rseA* gene were labelled with 100 μCi ml^{-1} [^{32}P]-orthophosphate. The samples were analysed by 12.5% SDS-PAGE and revealed by autoradiography. Figure 5B shows that wild-type RseA is phosphorylated *in vivo* (lane 1), whereas RseA Tyr-38Ala is not (lane 2).

Global changes in the protein profiles upon induction of Etk tyrosine kinase or upon induction of Etp phosphatase

As the *etk* and *etp* genes are co-transcribed and possess antagonistic activities, it is likely that a particular stoichiometry must exist *in vivo* between phosphorylated and non-phosphorylated species of Etk. We reasoned that, in the absence of Etp, the phosphorylated form of Etk will accumulate *in vivo*. To verify this assumption, wild-type strain LMG194 and the isogenic *etp*::Kan^R variant carrying either pBAD/His C vector alone or pBAD/His C *etk*$^+$ were labelled using [^{32}P]-orthophosphate. Arabinose-induced Etk was clearly identified (Fig. 9, top, lane 2), and it appeared to be hyperphosphorylated in the *etp* mutant cell extracts (Fig. 9, top, lane 4). This is important as, in the *etp* mutant, the *etk* gene is expressed from its chromosomal copy. Additionally, several other phosphoproteins accumulated in the *etp* mutant (Fig. 9, top, compare lanes 1 and 2). We performed a similar experiment in which this time Etp was overproduced. Such overproduction clearly leads to a reduction in ^{32}P-labelled polypeptides, and no phosphorylated Etk is observed (Fig. 9, top, lane 3). We also analysed these samples on two-dimensional PAGE (Fig. 9, bottom). Again, the number of phosphoproteins accumulate when Etk is phosphorylated and present in excess (produced from the plasmid) (Fig. 9, bottom). Similarly, the extracts from *etp* mutant cells have increased abundance of phosphoproteins (Fig. 9, bottom, B, extracts from *wzb*; Fig. 9, bottom, C, extracts from *etp* mutant strains). These results suggest that Etk autophosphorylates *in vivo*, and Etp can dephosphorylate Etk.

Discussion

In this study, we have unravelled a new mechanism of heat shock regulation that involves phosphorylation-mediated inactivation of RpoH, the main heat shock sigma factor in *E. coli*. Mutants downregulated for Eσ^{32}- and Eσ^{E}-transcribed regulons were searched for and mapped to the 22 min region of the *E. coli* chromosome. These mutations were located in a gene encoding for a putative phosphoprotein phosphatase. Homology searches showed that this putative phosphatase belongs to the family of low-molecular-weight tyrosine phosphatases. During the progression of this work, two groups independently identified this gene, based on sequence homology, to this known family of tyrosine phosphatase (Vincent *et al*., 2000; Wugeditsch *et al*., 2001). In fact, *E. coli* has two such highly homologous phosphatases with a comparatively similar genetic organization. The second gene maps at 46 min and is designated *wzb* (Vincent *et al*., 2000). In both cases, the phosphatase-encoding gene is co-transcribed with a gene encoding for kinase *etk* (22 min) and *wzc* (46 min) respectively. Although the gene cluster mapping at 46 min is thought to be involved in colanic acid synthesis (Vincent *et al*., 2000; Wugeditsch *et al*., 2001), no physiological role has been assigned to the phosphatase mapping at 22 min. Despite a possible role in the

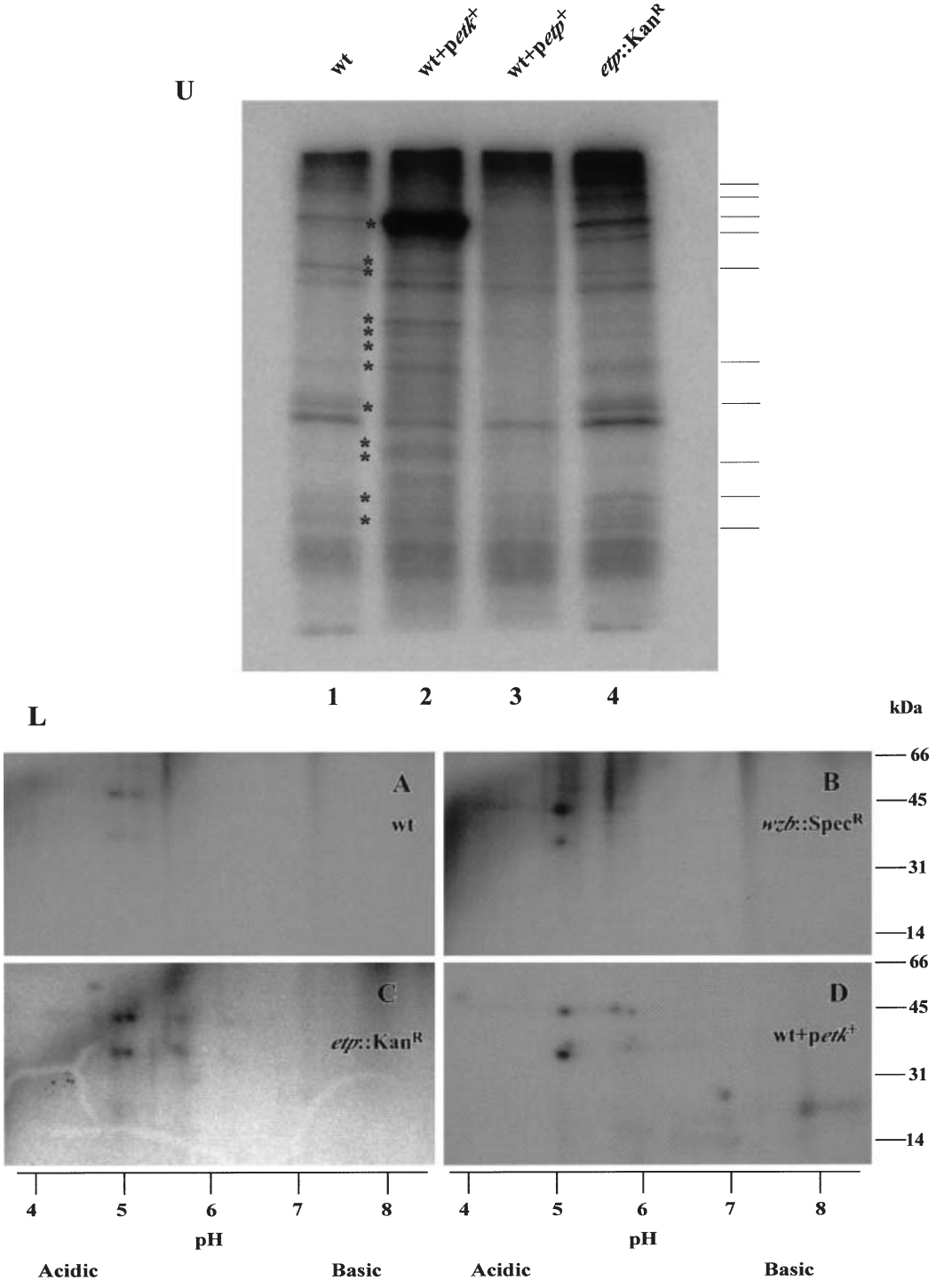

Fig. 9. Accumulation of phosphoproteins.
Top. Phosphoproteins of *E. coli* as visualized by autoradiography of SDS-PAGE. Cultures of isogenic bacterial strains, as indicated by the relevant genotype, were incubated for 30 min at 30°C with 200 μCi ml^{-1} [^{32}P]-orthophosphate. Samples were TCA precipitated, washed and separated on SDS-PAGE. The asterisks in between lanes 1 and 2 show the phosphoproteins accumulating upon the induction of the *etk* gene. The bars on the right side of lane 4 show the increased accumulation of Etk as well as the other phosphoproteins in bacteria strain LMG194 carrying the *etp*::Kan^R mutation.
Bottom. Phosphorylated proteins accumulate upon induction of Etk synthesis or lack of *wzb* and *etp* gene products. Proteins were separated on two-dimensional PAGE as described in the legend to Fig. 3. Autoradiograms of the dried gels are shown. Isogenic bacterial cultures were labelled with [^{32}P]-orthophosphate as described in the legend to Fig. 5. The estimated pH is shown below and the molecular weight standards used at the side.

production and export of capsule polysaccharide in pathogenic *E. coli* strains, no direct protein substrate has been shown for either Etk or Wzc.

As our mutations mapped to the *etp* gene (*yccY*), which is co-transcribed with the *etk* gene with the gene order *yccZ*, *etp* (*yccY*) and *etk* (*yccC*), we speculated that, in the absence of the functional *etp* gene, Etk might be hyperphosphorylated. This phosphorylated Etk could then potentially phosphorylate different substrates, which would subsequently either activate or inactivate them. As the mutants were isolated based on the reduced activity of σ^{32}- and σ^{E}-dependent regulons, we reasoned that these sigma factors might be phosphorylated and thereby would be less active. Up to now, very few proteins have been shown to be phosphorylated in prokaryotes at serine/threonine and tyrosine residues, although it is a very common post-translational modification involved in regulation in eukaryotes. For example, the activity of HSF1, the eukaryotic heat shock transcription factor, is regulated by phosphorylation (reviewed by Morimoto, 2002).

Here, we show that σ^{32} can be phosphorylated and dephosphorylated *in vivo* and *in vitro*. Furthermore, phosphorylated σ^{32} is found to have reduced activity *in vitro* as assayed in a run-off transcription experiment. It is worth mentioning that, when the gene encoding σ^{32} was initially cloned and expressed in minicells, two isoelectric forms were observed upon resolution by two-dimensional PAGE (Grossman *et al*., 1984). Consistent with the data reported here, these authors noticed that only the less acidic form that co-migrated with σ^{32} corresponded to the protein isolated from the active RNA polymerase complex (Grossman *et al*., 1984). These authors hypothesized that DnaK might be responsible for modifying σ^{32}. Phosphorylation of σ^{32} was reported in another study upon overexpression of bacteriophage T4 *mrh* gene (Mosig *et al*., 1998). As the deduced amino acid sequence of the *mrh* gene does not show any homology to any phosphatase or kinase, the effect observed might be indirect. The Mrh protein may modulate a putative phosphatase activity upon infection by T4 bacteriophage.

How does phosphorylation of σ^{32} modify its activity? Phosphorylation of σ^{32} occurs specifically at Tyr-260. Results of the *in vitro* transcription assay suggest that phosphorylation alters either core binding or promoter recognition. Phosphorylation did not appear to affect the stability of the protein at elevated temperature, as judged by Western blot analysis. During the purification of RpoH Tyr-260Ala, we noticed co-purification of both core RNA polymerase and the DnaK chaperone, suggesting that Tyr-260 does not play an important role in such interactions. More critical is the location of Tyr-260 in the conserved region 4.2. This region has been implicated in the recognition of the –35 region of the promoter element. Hence, phosphorylation may alter promoter recognition. Region 4.2 is the site of interaction with many regulatory proteins. In the case of RpoE, we have shown recently that region 4.2 is involved in its interaction with the antisigma factor RseA (Tam *et al*., 2002). Similarly, the antisigma factors AsiA and Rsd bind to region 4.2 of the σ^{70} subunit, thereby preventing the transcription of housekeeping genes (Jishage *et al*., 2001; Urbauer *et al*., 2001). However, none of these negative regulatory proteins is involved in any modification such as phosphorylation or dephosphorylation. Thus, the region 4.2, besides being needed for recognition of the –35 promoter element, seems to be the preferred target for regulatory proteins.

We have shown that *rpoH* Tyr-260Ala expressed on a plasmid complemented both bacteriophage λ growth and bacterial growth up to 37°C of an *rpoH* deleted strain. These results suggest that, at least up to 37°C, the transcriptional activity of RpoH Tyr-260Ala should be sufficient *in vivo*. However, *rpoH* Tyr-260Ala did not restore the lack of heat shock induction after a temperature shift from 30°C to 42°C in *rpoH* null mutant bacteria. It is quite likely that *rpoH* Tyr-260Ala does not recognize the –35 promoter element efficiently. This was further supported by our results showing highly impaired transcriptional activity from the *in vitro* run-off transcription experiment using holoenzyme reconstituted with RpoH Tyr-260Ala.

What is the physiological implication of σ^{32} phosphorylation *in vivo*? Our present model postulates that part of σ^{32} becomes phosphorylated under normal conditions and that this form is inactive. As phosphorylation is a reversible process, this would allow the cell to keep a pool of inactive σ^{32} molecules that can be readily dephosphorylated upon stress. This regulatory process may involve additional factors as well. It also implies that the amount or activity of phosphatase and kinase change with temperature or other stresses. Our model of the inactivation of RpoH activity by phosphorylation in the region 4.2 and yet a change in Tyr-260Ala leading to a Ts phenotype has an interesting parallel in the phenotype conferred by the mutation R178G (corresponding to region 4.2) in the *rpoE* gene. This R178G mutation totally abolishes the interaction of RpoE with the antisigma factor RseA. Also, quite like the Tyr-260Ala mutation in the *rpoH* gene, the *rpoE* R178G mutation also leads to reduced transcriptional ability of such RpoE protein to activate the transcription of $E\sigma^{E}$-driven promoters (Raina *et al*., 1995; Tam *et al*., 2002), although it does not involve any change in the post-translational modification such as phosphorylation as is the case with RpoH Tyr-260A.

Mutations in the *etp* gene also impaired the activity of σ^{E}. To our surprise, a different mechanism occurred in this particular instance, as no phosphorylation of σ^{E} could be observed. Instead, we found that RseA is the target for phosphorylation. Based on our *in vivo* and *in vitro* data,

we can show that RseA is phosphorylated by the Etk kinase. In addition, we demonstrate that the site of phosphorylation is Tyr-38 in the N-terminal domain, which carries the antisigma factor activity. Curiously, phosphorylation of RseA in the phosphatase mutant (*etp*) caused a reduced level of induction upon heat shock. These results are in contrast to those obtained for the $E\sigma^{32}$-mediated heat shock response in *etp* mutants. The heat shock response mediated by σ^{32} is unaltered, although the basal levels are reduced. Maybe some phosphoproteins, which accumulate in *etp* mutants, have an inhibitory effect on the RpoE activity.

At this point, we speculate that phosphorylation of RseA must increase its affinity for RpoE. This would prevent another possibility that phosphorylation alters the turnover of RseA. If the conformation of RseA is changed upon phosphorylation, it could limit its availability to proteases.

As a null mutation in the *etp* gene confers a Ts phenotype, we speculate that the Etk kinase/Etp phosphatase system has additional substrates and that their regulation by dephosphorylation is a limiting process for bacterial growth at high temperatures. The two-dimensional gel analyses already suggest that many proteins accumulate in the phosphorylated form when Etk is overproduced *in vivo* or in the absence of the *etp* gene product. Further studies at the genetic and biochemical levels are needed to answer these questions.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 3.

Oligonucleotides

See *Supplementary material*.

Media and chemicals

Luria–Bertani (LB) broth, M9 and phosphate-limiting minimal media were prepared as described previously (Missiakas and Raina, 1997). When necessary, the media were supplemented with ampicillin (Amp, 100 μg ml^{-1}), tetracycline (Tet, 15 μg ml^{-1}), kanamycin (Kan, 50 μg ml^{-1}) spectinomycin (Spec, 50 μg ml^{-1}) or chloramphenicol (Cm, 20 μg ml^{-1}). The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) was used at a final concentration of 40 μg ml^{-1} in the agar medium.

Isolation of trans*-acting mutations*

Trans-acting mutations, which reduce β-galactosidase activity from the *htrA–lacZ* and *rpoH*P3–*lacZ* fusions were isolated according to various genetic approaches, as described earlier (Raina *et al*., 1995). Briefly, P1 bacteriophage lysates of strain MC4100, carrying random mini-Tn*10*(Kan^R) transposons, were treated with either hydroxyamine or nitrosoguanidine. These lysates were used to transduce the Kan^R marker into strains SR1458 or SR1710 carrying single-copy promoter fusion *htrA–lacZ* and *rpoH*P3–*lacZ* respectively. These transductants were screened on Xgal-containing plates. Pale blue colonies, a phenotype reflecting reduced β-galactosidase activity, were retained and checked for Ts phenotype in the range 42–43°C. Only those transductants that showed 100% co-transduction between the Ts and reduced β-galactosidase activity phenotypes were retained. Using this screening, multiple mutations were isolated in the *rpoE* gene. To eliminate these candidates, the mutants were transformed with pSC101-based *rpoE*$^+$ plasmid pSR2663. Only those mutants that did not get complemented by plasmid carrying the *rpoE* gene were retained.

In the second round, P1 lysates were prepared on these mutants, and the Kan^R marker was transduced into strains carrying either P*groESL–lacZ* or *rpoD*Phs–*lacZ* single-copy promoter fusions on the chromosome. Five mutants were found to reduce the activity of these two σ^{32}-regulated fusions and were retained for further study.

Mapping and cloning strategies

We used a previously described p15A-based library to complement for Ts phenotype at 43°C and restore β-galactosidase activity. Four Ts mutant strains SR1594, SR1607, SR1851 and SR1854 were transformed with this library and plated directly at 43°C on plates containing Xgal. Plasmid DNA was extracted from 30 such clones and transformed back into the Ts mutants. Clones breeding true were retained. Fourteen such clones were subjected to analysis by restriction enzyme digestion. Surprisingly, two types of plasmids were identified. One group, consisting of nine plasmids, contained internal 2.7 kbp *Hin*dIII–*Pst*I fragments. The other complementing group, consisting of five plasmids, contained a unique 3.4 kbp *Bam*HI fragment. We analysed one plasmid from each group, pSR3947 and pSR3952, by DNA sequence analysis of these plasmids and compared them with the *E. coli* genome sequence. After determining the minimal clones by complementation analysis, we pursued our work with two ORFs from 22 min *yccY* (*etp*) and *yccC* (*etk*).

Overexpression of the *etk*, *etp* and *wzb* genes was achieved by amplifying their minimal coding regions by PCR using appropriate primers. This was followed by cloning into pBAD/His C expression vector (for the *etk* gene) and a T7 promoter-based expression vector pET-22b for the *etp* and *wzb* genes with an in frame His_6-tag at the C-terminus.

QuickChange mutagenesis (Stratagene) was used to substitute the seven Tyr codons in the *rpoH* gene, using appropriate oligonucleotides. The template used was plasmid carrying wild-type *rpoH* gene (pSR6027) with an N-terminal His_6-tag in the pIVEX 4.2 vector (Roche Diagnostics). The mutated *rpoH* gene was verified by sequence analysis. A similar strategy was used to generate the Tyr-38 to Ala change in RseA. Five original point mutants that exhibited a Ts phenotype and reduced basal levels of σ^E- and σ^{32}-transcribed promoters were also sequenced using the corresponding DNA. For this purpose, the corresponding

Table 3. Bacterial strains and plasmids.

Strains/plasmids	Relevant characteristics	Reference or source
MC4100	F⁻ *araD*139 Δ(*argF-lac*) U169	Our collection
LMG194	F⁻ Δ*lac*X74 *gal*E *thi rps*L Δ*pho*A Δ*ara*714 *leu*::Tn*10*	Invitrogen
BL21(DE3)	*ompT lon*	Invitrogen
BL21(pLysE)	*ompT lon* Cm^R	Invitrogen
SR1458	MC4100 ϕ (*htrA-lacZ*)	Raina *et al.* (1995)
SR1710	MC4100 ϕ (*rpoH*P3-*lacZ*)	Raina *et al.* (1995)
SR4499	MC4100 ϕ (*groESL-lacZ*)	This study
SR4653	MC4100 ϕ (*rpoD*Phs-*lacZ*)	Dartigalongue *et al.* (2001)
SR1594	SR1458 *etp*121 (D121V)	This study
SR1607	SR1458 *etp*71 (H71D)	This study
SR1851	SR1458 *etp*22 (C22W)	This study
SR1854	SR1458 *etp*54 (D54V)	This study
KY1621	*rpoH*::Kan^R	Kusukawa and Yura (1988)
SR4108	MC4100 *rpoH*::Kan^R ϕ (*groESL-lacZ*)	This study
SR4045	MC4100 *wzb*::Kan^R	This study
SR4500	SR4499 *wzb*::$Spec^R$	This study
SR4508	MC4100 *etp*::Kan^R	This study
SR4517	MC4100 *etp*::Kan^R *wzb*::$Spec^R$	This study
SR4757	LMG194 *etp*::Kan^R (pBAD/His C)	This study
SR4758	LMG194 *etp*::Kan^R (pSR4520 *etk*⁺)	This study
SR6268	SR4108 (pSR6027 *rpoH*⁺)	This study
SR6272	SR4108 (pGK181 *rpoH* Y260A)	This study
SR4756	BL21(DE3) *etp*::Kan^R	This study
GK183	SR4499 *etp*::Kan^R	This study
GK187	SR4653 *etp*::Kan^R	This study
GK189	SR1458 *etp*::Kan^R	This study
Plasmids		
pIVEX 4.2	T7 promoter based expression vector	Roche Diagnostics
pBAD/His C	Expression vector	Invitrogen
pTXB1	Intein tag fusion protein expression vector	New England BioLabs
pSR2663	pWSK30 *rpoE*⁺ *resA*⁺ (1697 bp *Ssp*I–*Msc*I)	Missiakas *et al.* (1997)
pSR3563	pAED-4 carrying the minimal *wzb*⁺ ORF (*Nde*I–*Eco*RI)	This study
pSR3947	pWSK30 *etk*⁺ *etp*⁺ (2.5 kbp *Sau*3AI)	This study
pSR3952	pWSK29 *wzb*⁺ (2.7 kbp *Bam*HI–*Pst*I)	This study
pSR3563	pAED-4 carrying the minimal *wzb*⁺ (*Nde*I–*Eco*RI)	This study
pSR4520	pBAD/His C carrying the minimal *etk*⁺ ORF (*Nde*I–*Xho*I)	This study
pSR6013	pET-22b carrying the minimal *etp*⁺ ORF (*Nde*I–*Xho*I)	This study
pSR3969	pSR3952 *wzb*::Kan^R	This study
pSR4590	pTXB1 (*rpoE*⁺ + *rseA*^{N})	This study
pSR6178	pIVEX 4.2b carrying the DNA sequence encoding for the last C-terminal 235 aa of Etk	This study
pSR4170	pSR3947 *etp*::Kan^R	This study
pSR3970	In frame deletion of *etk* in pSR3947	This study
pSR6027	pIVEX 4.2b carrying the minimal *rpoH*⁺ ORF (*Nde*I–*Sma*I)	This study
pGK106	pSR6027 *rpoH*19 (Y19W)	This study
pGK171	pSR6027 *rpoH*43 (Y43M)	This study
pGK173	pSR6027 *rpoH*68 (Y68I)	This study
pGK175	pSR6027 *rpoH*71 (Y71P)	This study
pSR6107	pSR6027 *rpoH*116 (Y116F)	This study
pGK179	pSR6027 *rpoH*200 (Y200E)	This study
pGK181	pSR6027 *rpoH*260 (Y260A)	This study
pGK177	pGK178 *rseA*38 (Y38A)	This study
pGK178	pBAD/His C carrying the minimal *rseA*⁺ ORF (*Nco*I–*Xho*I)	This study

chromosomal DNA was used as a template for PCR, using primers specific to the *etp* and *etk* genes. The PCR products were sequenced directly.

Disruption of etp, etk *and* wzb *genes*

To construct a null allele of the *etp* gene, a kanamycin cassette without terminators from the pUC4 plasmid (Pharmacia) was introduced at the unique *Pst*I site of the *etp* gene. Similarly, an in frame deletion was introduced in plasmid pSR3947 by removing a 550 bp fragment internal to the *etk* gene, resulting in plasmid pSR3970. These linear DNA fragments were introduced into the *recB recC sbcB* strain selecting for Kan^R. Colonies that were Kan^R but had lost the vector marker were retained. The correct location of the insert was verified by sequencing the PCR-amplified DNA, using appropriate oligonucleotide primers. Similarly, the disruption of the *wzb* gene was constructed by inserting a non-polar spectinomycin cassette at the unique *Nco*I site. The

null *wzb*::Spec^R allele on the chromosome was obtained as described above.

Purification of Etk, Etp and Wzb proteins

LMG194 carrying *etk*$^+$ cloned in the pBAD/His C vector with a C-terminal His_6-tag (pSR4520) was induced with 0.5% arabinose at an OD_{595} of ≈0.2 for 4 h. Cultures were spun at 6000 *g* at 4°C. The pellet was resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (buffer A)], supplemented with lysozyme to a final concentration of 200 μg ml^{-1}. The mixture was incubated on ice for 20 min, sonicated and spun at 45 000 *g* for 30 min at 4°C. The insoluble pellet was resuspended in buffer A containing 0.1% Triton X-100 at 4°C for 2 h to solubilize membrane proteins. Solubilized proteins were recovered by centrifugation at 100 000 *g* for 45 min at 4°C. This solution (20 ml) was applied over Ni-NTA beads (Qiagen), washed and eluted as recommended by the manufacturer. *etp* and *wzb* genes cloned into the T7 promoter expression vectors were transformed into the BL21(pLysE) strain. Strains (2 l cultures) carrying either pSR6013 (*etp*$^+$) plasmid or pSR3563 (*wzb*$^+$) plasmid were induced with 1 mM IPTG at an OD_{595} of ≈0.2 for 4 h. The harvested cultures were spun at 6000 *g* at 4°C and resuspended in buffer A supplemented with lysozyme, and the soluble fraction was collected after ultracentrifugation and applied over Ni-NTA beads as described above. Purified proteins were eluted with buffer A containing 200 mM imidazole, and the fractions were assayed directly for their phosphatase activity using *p*NPP as the substrate, as described for PrpA and PrpB (Missiakas and Raina, 1997). Purification of the wild-type RpoH and different variants with tyrosine mutation was performed as described previously (Raina *et al*., 1995). Purification of the wild-type RseA and RseA Tyr-38Ala was performed as described by Missiakas *et al*. (1997) and Collinet *et al*. (2000).

Labelling with [^{32}P]-orthophosphate and two-dimensional gel analysis

Bacterial cultures were grown in LB medium at 30°C up to an OD_{595} of ≈ 0.2 and, when required, supplemented with appropriate antibiotics. Cultures were spun and washed twice with minimal medium lacking phosphates. Cultures were diluted 1:10 and grown again up to an OD_{595} of ≈ 0.2 at 30°C. To induce the synthesis of Etk, 0.2% arabinose was added to this growth medium. Bacterial culture (5 ml) was labelled with 200 μCi of [^{32}P]-orthophosphate. Cultures were incubated further for 30 min. Two-dimensional gel electrophoresis was performed as described previously (Missiakas and Raina, 1997).

Biochemical assays

Phosphatase assays for Etp and Wzb were performed as described previously for PrpA and PrpB (Missiakas and Raina, 1997). Phosphorylation of Etk and other proteins was performed using 1.5 mM [γ-^{32}P]-ATP, 0.25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 25 mM $MnCl_2$. Etk (1 μg) was added to a 30 μl reaction and incubated at 25°C for 1 h. Phosphorylation assay with RpoH, RseA and RpoE was performed using 10 μl aliquots of ^{32}P-labelled Etk from the above reaction added to the substrates at 25°C and incubated for 30 min. The proteins were resolved by SDS-PAGE and revealed by autoradiography. Dephosphorylation of either [^{32}P]-Etk or other proteins was performed with the purified Etp as described previously (Missiakas and Raina, 1997). After the incubation of phosphorylated [^{32}P]-Etk and [^{32}P]-RpoH with Etp, samples were analysed by SDS-PAGE and revealed by autoradiography.

Purification of RNAP core and σ^{32} for run-off assays was as described previously (Raina *et al*., 1995). The template used was a PCR-amplified region containing the promoter region of the *lon* gene. The *in vitro*-synthesized products were separated on 3% acrylamide gel containing 8 M urea. The β-galactosidase assays were carried out as described previously (Missiakas and Raina, 1997).

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3449/mmi3449sm.htm

Fig. S1. Purification of Etk.

Table S1. Oligonucleotides used in this study.

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