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Signal transduction pathways in response to protein misfolding in the extracytoplasmic compartments of *E.coli*: role of two new phosphoprotein phosphatases PrpA and PrpB

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It is now well established that the σ^E regulon of *Escherichia coli* is induced by misfolding of proteins in the periplasm and the outer membrane. *htrA* belongs to this regulon and encodes a periplasmic protease involved in the degradation of misfolded proteins. *htrA* transcription is also under the positive control of a two component signal transduction system CpxR CpxA. Closer examination of the putative signal transduction pathway modulating *htrA* transcription has led us to the identification of two new genes. Biochemical and genetic evidence shows that these two genes encode two phosphoprotein phosphatases, designated PrpA and PrpB. These are the first examples of typical serine/threonine and tyrosine phosphatases described in *E.coli*. PrpA and PrpB are involved in signaling protein misfolding via the CpxR CpxA transducing system. In addition, both PrpA and PrpB modulate the phosphorylated status of some other phosphoproteins in *E.coli*. Finally, we show that PrpA is a heat shock protein.

Keywords: *cpxR cpxA* operon/*E.coli* extracytoplasm/phosphoprotein phosphatases/protein misfolding/*rpoE* regulon

Introduction

In *Escherichia coli*, the heat shock response is regulated overall by two alternative sigma factors, σ^{32} and σ^E/σ^{24}, encoded by the *rpoH* and *rpoE* genes, respectively. Transcription of these two regulons is induced by various stresses and by protein misfolding in general. The stimuli arising from misfolded proteins are even more dramatic at high temperatures since proteins are more prone to aggregation. It is therefore not surprising that most of the protein chaperones and proteases are encoded by heat shock regulated genes. It is quite interesting that each of the two heat shock regulons have evolved to respond to protein misfolding in a defined cell compartment. Transcription of the *rpoH* regulon is induced by protein misfolding in the cytoplasm whereas transcription of *rpoE*, that was first believed to be induced exclusively by misfolded outer membrane proteins (Mecsas *et al.*, 1993), appears to respond to the accumulation of any exported protein that is unstable or misfolded (Missiakas *et al.*, 1995, 1996b; Raina *et al.*, 1995). Many $E\sigma^{32}$-transcribed genes have been shown to encode repair functions such as chaperones and proteases (reviewed by Missiakas *et al.*, 1996a). The role of the σ^E regulon in preventing protein misfolding in the extracytoplasmic compartments is not yet fully deciphered. Interestingly, the elevated temperatures can induce both heat shock regulons. This is easily explained by the fact that the $E\sigma^E$ polymerase transcribes both the *rpoE* and the *rpoH* genes (Erickson and Gross, 1989; Wang and Kaguni, 1989; Raina *et al.*, 1995; Rouvière *et al.*, 1995). *htrA* (*degP*) is the third known σ^E-transcribed gene (Lipinska *et al.*, 1988; Erickson and Gross, 1989). This gene encodes a periplasmic protease which appears to degrade specifically unstable proteins. How is the need for HtrA in the periplasm signaled to the σ^E regulon? We have addressed this question genetically by constructing a transcriptional fusion between the *htrA* promoter and the *lacZ* gene and searching for *trans*-acting mutations affecting *lacZ* expression. Many mutations were found to map to the *rpoE* gene, but not all of them (Raina *et al.*, 1995). It appeared that transcription of *htrA* is also regulated by a second sensing system, the CpxR CpxA couple (Danese *et al.*, 1995; Raina *et al.*, 1995). CpxA encodes a protein with homology to the classical histidine kinases of the two component systems (Weber and Silverman, 1988) such as EnvZ or CheA (see review by Parkinson, 1993). These kinases are embedded in the inner membrane and upon activation by an 'outside' stimulus they undergo autophosphorylation to activate, in turn, a cognate DNA-binding regulatory protein by the transfer of the phosphate group. Based on sequence homologies with other regulatory proteins of two component systems, such as OmpR or CheA, it seems that CpxR (Dong *et al.*, 1993) could be activated through phosphorylation by CpxA. In this study, we demonstrate that, in fact, it is a network of phosphorylation and dephosphorylation processes which fine-tunes the transcriptional induction of *htrA*. This is quite similar to the signal transduction pathways observed in higher eukaryotes which occur through a cascade of events involving both kinases and phosphatases. Indeed, genetic analyses presented here reveal that *E.coli* encodes at least two type I phosphatase activities. In conjunction with the CpxA kinase, they modulate the transcription of some cellular components which may be important for protecting the cell upon accumulation of misfolded proteins in the periplasm. They do so by specifically signaling for induction of *htrA* transcription. PrpA phosphatase activity seems to be important for the general heat shock response of *E.coli* since overexpression of PrpA leads to the accumulation of the heat shock proteins.

Results

We have previously described two complementary approaches which led to the identification of the *rpoE*

gene (encoding σ^E) as well as other genes which could encode modulators of σ^E activity (Raina *et al.*, 1995). One of these approaches was based on isolating *trans*-acting mutations which down-regulate the expression of reporter–promoter fusions of *lacZ* to *rpoH*P3 and *htrA* promoters. Another approach was based on identifying those genes which, when present on multicopy plasmids, were able to positively affect the transcription of such promoter fusions. Both led to the identification of the *rpoE* gene itself (Raina *et al.*, 1995) as well as a few other additional genes. In this study, we specifically analyzed those loci which affect primarily *htrA* transcription in order to understand the different levels of sensing and signaling protein misfolding in the periplasm.

Isolation of mutations affecting htrA transcription

Among the original 64 Lac-down mutants isolated earlier (Raina *et al.*, 1995), at least three complementation groups were found to affect only the transcription of *htrA–lacZ* activity but not *rpoH*P3–*lacZ* (Figure 1A). Most of these mutants were simultaneously temperature sensitive for growth above 43°C. One group comprising six Lac-down mutants was complemented by cosmids which hybridized to λ Kohara phages 334, 335 and 336 corresponding to the 41 min region of the *E.coli* chromosome. Another group of seven mutants was found to be complemented by cosmids hybridizing to λ 449 and 450 (61.5 min). The last group of four mutants was complemented by cosmids hybridizing to λ 539, 540 and 541 (88.5 min). These map positions were further verified by bacteriophage P1-mediated transduction using known genetic markers. The location of mutations mapping at 41.5 min was confirmed by an observed 70% linkage with *eda*::Tn*10* (CAG18486, Singer *et al.*, 1989). Mapping of mutations located at 61.5 min was confirmed by linkage to *mutS*::Tn*10* (90% linkage) and to *rpoS*::Tn*10* (~75% linkage). The assignment of the third complementation group (88.5 min) was achieved using *clpQ*::ΩCm (DM1674) as the linked marker (Missiakas *et al.*, 1996c). This last group was complemented by clones carrying the two-gene operon *cpxR cpxA* and therefore corresponded to a group reported earlier (Raina *et al.*, 1995). The other two groups carried genes which were designated as *prpA* (41 min) and *prpB* (61.5 min) after further characterization which are described in the following sections.

Increased htrA transcription can be achieved by overexpression of the prp genes

In our previous studies (Missiakas *et al.*, 1993; Raina *et al.*, 1995), we had also observed that multicopy expression of genes not mapping to *rpoE* could induce transcription from the *htrA* gene and not *rpoH*P3 or *rpoE*P2 (the *rpoE* gene has two promoters, P2 is recognized by $E\sigma^E$; Raina *et al.*, 1995). These clones, selected from a genomic library constructed in a p15A-based vector (see Materials and methods), were analyzed more closely. It was found from their restriction pattern that 12 of them carried a common 2.8 kb *Sau*3A DNA fragment (pDM506, *prpA*$^+$). These 12 clones were mapped on the *E.coli* chromosome and were shown to hybridize to bacteriophage λ 336 (19H3) of the Kohara library (Kohara *et al.*, 1987). Interestingly, this corresponded to the 41 min region on the *E.coli* DNA chromosome to which one of the

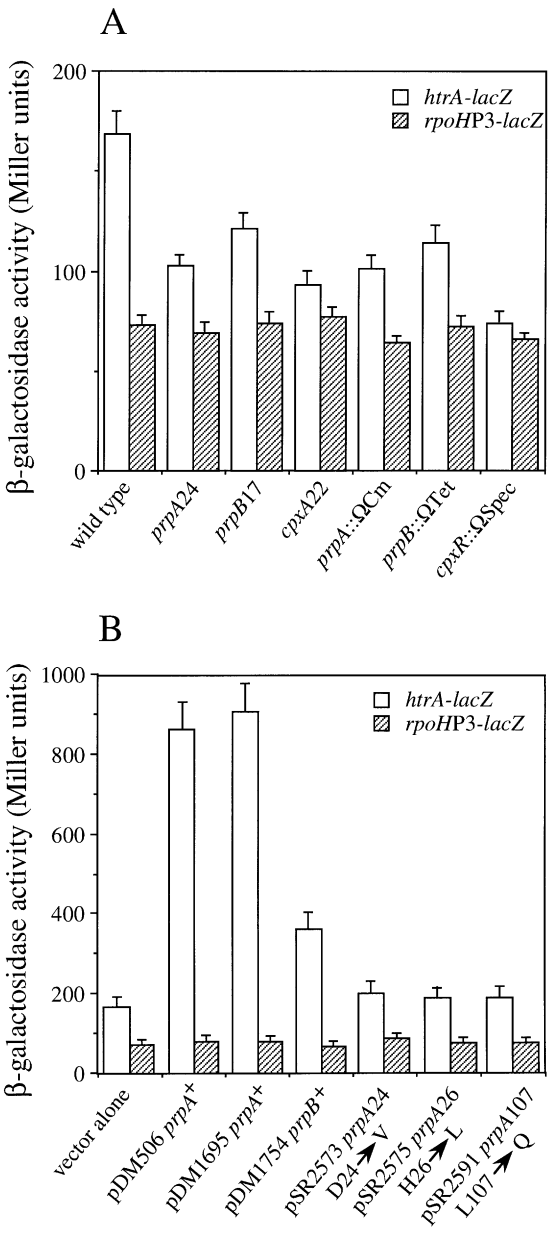

Fig. 1. (**A**) Mutations in *prpA*, *prpB* or *cpx* genes lead to a decrease in *htrA* transcription. (**B**) Overexpression of wild-type PrpA and PrpB lead to an increase in *htrA* transcription. β-galactosidase activities were determined as described by Miller (1992). Cells were grown overnight at 30°C, diluted 1:100 and grown to an OD of 0.3 at 595 nm. Each sample was assayed thrice and the data presented are an average of five independent experiments. The standard deviation is shown as error bars. Activities are depicted in Miller units.

complementation groups comprising six *trans*-acting mutations was also mapped. Further characterizations and subcloning experiments identified a 900 bp *Pvu*II–*Acc*I DNA fragment in pDM1695 (*prpA*$^+$), which was found to induce the *htrA–lacZ* expression to the same extent as the original construct pDM506 (Figure 1B).

This multicopy cloning approach did not lead to the re-isolation of the second locus mapping at 61.5 min, identified in the previous screening of *trans*-acting mutations. To verify whether an induction of *htrA* could be observed with *prpB*-carrying clones, we used the minimal DNA fragment subcloned from the cosmid com-

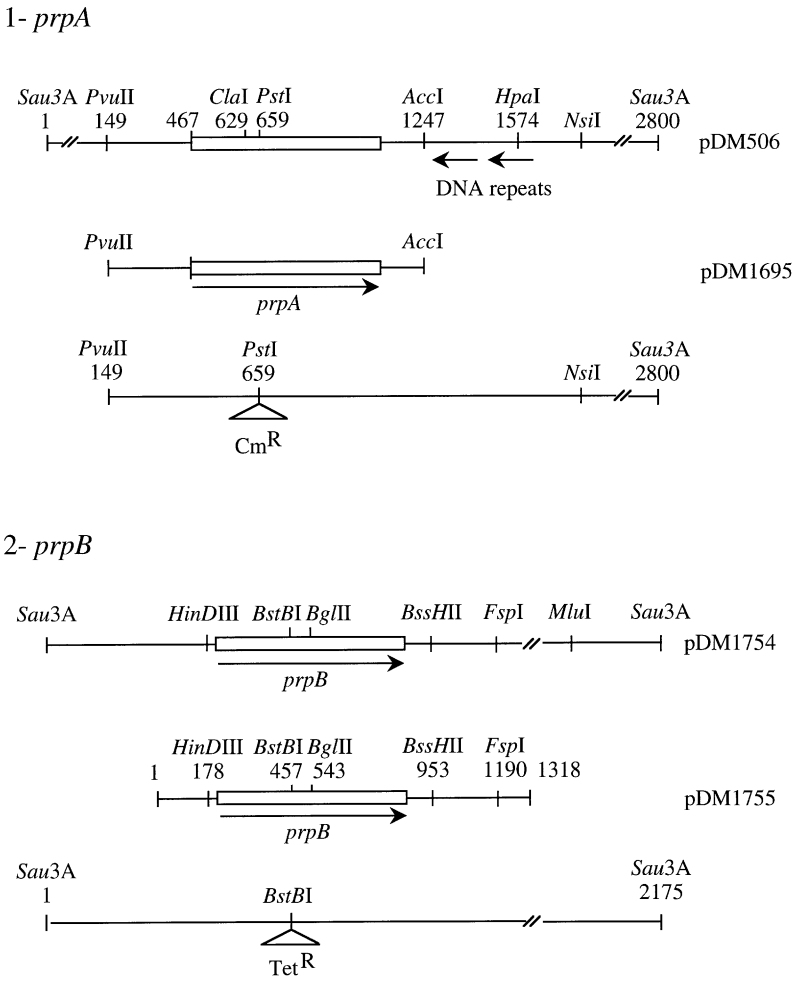

Fig. 2. Restriction maps of the *prpA* and *prpB* genes with surrounding DNA sequences. The arrows indicate the position of DNA repeats homologous to DNA stretches found at the end of the *dic*ABCD region.

plementing mutations at this locus and cloned it in the same vector used to construct the multicopy library. This new clone pDM1754 (*prpB*$^+$) was assayed for β-galactosidase activity of the *htrA–lacZ* fusion-carrying strain. As shown on Figure 1B, a comparatively smaller induction of *htrA* transcription was detected using pDM1754 (*prpB*$^+$). However, if cloned in a higher copy number vector (pBR322 based pSE420) pDM1757 *prpB*$^+$, an ~6-fold induction of *htrA* transcription was observed (Table II).

PrpA and PrpB proteins have typical serine/threonine phosphatase signatures

Sequence analyses of the two clones revealed an ORF of 654 nucleotides (nt) long for both *prpA* and *prpB* genes. In the case of *prpA* (pDM506), the ORF is predicted to start with an ATG at nt position 467 and terminating with TAA at nt position 1123, and could encode a 24 257 Da polypeptide with a predicted pI of ~7. These predictions are consistent with the observed pI and molecular weight as shown in Figures 6A and 11A (migration on 2D gel). Downstream of the *prpA* ORF are small stretches of DNA sequence which show significant homology to small stretches of DNA sequence found in the *dicABCF* region (Figure 2; Faubladier and Bouché, 1994). In addition, the *prpA* ORF is surrounded by large non-coding regions ~500 bp upstream and >1 kb at its 3′-end. This is quite unusual in the *E.coli* genome where ORFs are generally tightly packed. In the case of *prpB* (pDM1755), the ORF also starts with an ATG at nt position 202 and terminates with a TAG at nt position 856. It is located 108 nt downstream of the stop codon of the *mutS* gene and is transcribed in the same orientation. The *prpB* gene seems to have its own promoter since clones not carrying *mutS* can still complement a *prpB* mutant allele. *prpB* ORF is predicted to encode a 25 082 Da polypeptide. This is again consistent with the observed size of the protein (see later protein purification data, Figure 6A). Sequence comparison with GenBank release 91 indicated no similarity with any known *E.coli* proteins but a significant homology to the serine/threonine family of type I eukaryotic phosphatases. An even higher degree of homology was shared with the phosphatase from bacteriophage λ (referred to in this study as the λ-PP protein, λ*nin* ORF221; Cohen *et al.*, 1988) (Figure 3). Because of these observations, we designated the two genes as *prp* for **pr**otein **p**hosphatase.

What appeared particularly noteworthy was the presence of most of the conserved residues shown to be part of the active site. Among those are the residues involved in metal binding. From the known phosphatase sequences and 3D structure (Goldberg *et al.*, 1995), these residues

Fig. 3. Alignment of the amino acid sequences of full-length PrpA, PrpB and λ-PP (Plam). Domains of two eukaryotic protein phosphatases [RdgC Ser/Thr phosphatase from *Drosophila* (accession number P40421) and HPP1 *Homo sapiens* phosphoprotein phosphatase (Barker *et al.*, 1990)] exhibiting significant homology with prokaryotic phosphatases are also shown. Bold characters depict highly conserved residues.

would correspond to D24, H26, D53 and H80 for PrpA and D22, H24, D51 and H78 for PrpB (Figure 3). The only region with lower homology between PrpA, PrpB and λ-PP sequences consists of a small stretch of amino acids located between amino acids 106 and 122. This region in PrpA is predicted to contain a coiled-coil domain and might be important for interaction with the substrates. Consistent with its activity on *htrA* transcription as a phosphoprotein phosphatase, sequence analyses of four of the Lac-down mutants isolated in the *prpA* gene showed changes in the highly conserved residues. One of the mutations was D24 to V (GAT to GTT) and two others corresponded to H26 to N (CAC to AAC) and H26 to L (CAC to CTC). The fourth sequenced mutation corresponded to a change L107 to Q (CTG to CAG). L107 lies in the predicted coiled-coil domain of PrpA. Unlike wild-type *prpA*, neither of these cloned *prpA* mutant alleles was able to induce *htrA–lacZ* activity when provided on a same-copy-number plasmid (Figure 1B). These results further confirm that it is the phosphatase activity of PrpA which influences the transcription of *htrA*. We also sequenced one of the mutant variants of *prpB*, i.e. *prpB*17. This variant was found to carry the mutation H78 to N (CAC to AAC). This histidine residue is again conserved in all type I phosphoprotein phosphatases (Figure 3).

Phenotypic analyses of the prpA and prpB null mutants

The multicopy effect observed with the two genes mapped at 41 min (*prpA*) and 61.5 min (*prpB*) led us to presume that the corresponding *trans*-acting mutations isolated were the result of a loss of function of both PrpA and PrpB proteins. To confirm that this was indeed the case, the null alleles were constructed as described in Materials and methods and transduced into SR1458, the *htrA–lacZ*-carrying strain. Their effects on *htrA* transcription, as judged from the results presented in Figure 1A, were similar to those obtained with the point mutations. For both genes, *prpA* and *prpB*, all the alleles isolated as either null or point mutations conferred a slightly temperature-sensitive (Ts^-) growth phenotype above 43°C. However, such effects were not bactericidal. Only a mild synergistic effect was observed when mutations in *prpA* and *prpB* were combined. In addition, null mutations in *prpA* or *prpB* genes conferred a slow growth phenotype, even at permissive temperatures. For example, the doubling time of *prpA* null mutant bacteria is ~80 min at 30°C and that of *prpB* null mutant bacteria is ~65 min as compared with a 45 min doubling time for the isogenic wild-type bacteria at 30°C.

The prpA gene is heat shock regulated

A primer extension analysis was carried out with RNA isolated from wild-type bacteria under a range of growth temperatures. The results of this analysis showed that *prpA* mRNA has at least one defined 5′-end located 417 nt upstream of the putative ATG initiation codon (Figure 4A). The –10 (CACC) and –35 (GGCGAA) boxes of this start site present a good homology to the promoter consensus sequences of $E\sigma^{32}$-transcribed genes (Figure 4B) particularly in the –10 region. However, it may be noted that this promoter is quite weak compared with classical heat shock promoters. This may be due to a somewhat lower homology in the –35 region. In addition,

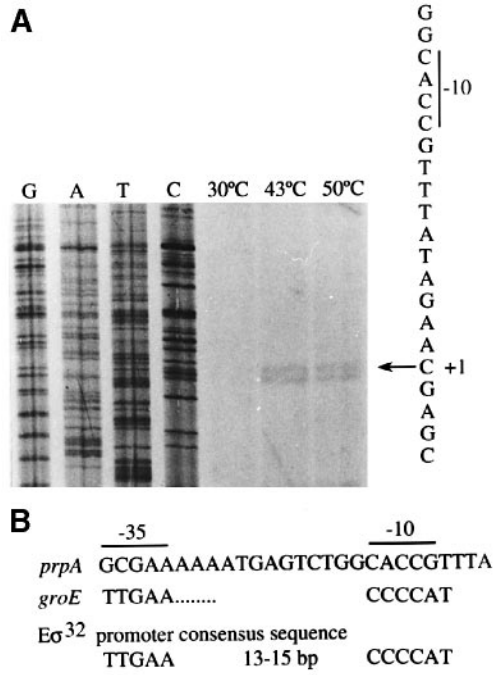

Fig. 4. (**A**) Mapping of the 5′ terminus of *prpA* transcripts. Primer extension reactions were performed using total cellular RNA and a ^{32}P-labeled oligonucleotide probe complementary to the *prpA* coding sequence. RNA was extracted from wild-type strain MC4100 at 30°C, or shifted to either 43 or 50°C for 10 min. Lanes labeled G, A, T and C correspond to the dideoxy sequencing reactions carried out with the same oligonucleotide as primer. (**B**) Promoter sequence alignment of heat shock genes.

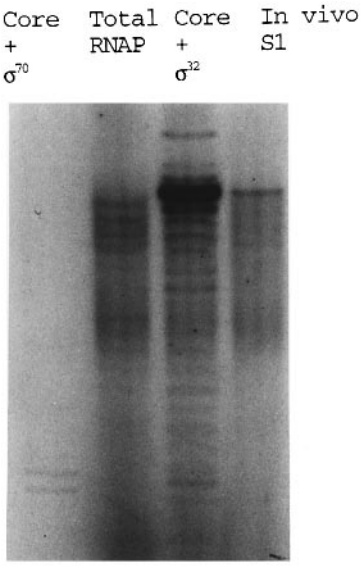

Fig. 5. *prpA* is transcribed by the Eσ^{32} polymerase. The *in vitro* run off transcripts were analyzed along with RNA made *in vivo* by S1 nuclease protection experiments. The S1 protected probe fragment co-migrates with the RNA obtained from *in vitro* experiments.

prpA-specific transcripts accumulated at 50°C (Figure 4A). It is known that, at such temperatures, heat shock genes or stress-inducible *psp* genes (Weiner *et al.*, 1991) are the only ones whose transcripts accumulate. To further substantiate that *prpA* is indeed transcribed by the Eσ^{32} holoenzyme, an *in vitro* run-off assay was carried out using a linear 659 bp *Sau*3A–*Pst*I DNA fragment as a template. As shown in Figure 5, purified Eσ^{32} holoenzyme initiates transcription at the same site as observed from *in vivo* RNA extracted from bacteria shifted to 50°C. Taken together, these results provide good evidence that *prpA* transcription is positively regulated by heat shock.

Purified PrpA and PrpB proteins exhibit phosphoprotein phosphatase activities in vitro

In order to compare the activities of the two putative *E.coli* phosphatases to some other known phosphatases, we decided to use the recently described bacteriophage λ phosphatase (λ-PP) as a control (Cohen and Cohen, 1989; Barik *et al.*, 1993; Zhuo *et al.*, 1993). A profile of the three purified proteins is shown in Figure 6A. PrpA, PrpB and λ-PP appeared to migrate to approximately the same distance as the molecular weight marker corresponding to 24–25 kDa. Surprisingly, λ-PP migrates on SDS–PAGE as a slightly higher molecular weight species than the PrpA and PrpB proteins despite its similar predicted mass. During all purification steps, the phosphatase activity was measured using the classical phosphatase substrate *p*-nitrophenol phosphate (pNPP) and was correlated with fractionation of a 25 kDa species (this activity is referred as pNPPase activity). All biochemical assays presented have been performed in triplicate using each protein separately. Among the three phosphatases, PrpA exhibited a rather good pNPPase activity at high temperatures, retaining ~90% of its activity at 65°C as compared with 20% for PrpB. This might be consistent with a role of the *prpA* gene product during the heat shock response and the finding that the gene is regulated by heat shock. The effect of various inhibitors was also tested on the pNPPase activity of the three enzymes (Table I). Despite their high sequence homologies, the pNPPase activities of the three enzymes are not inhibited to the same extent, depending on the salt or anion used. The pNPPase activity of PrpB was unaffected by most of the inhibitors except by zinc (Table I).

We also assayed the ability of the PrpA and PrpB phosphatases to dephosphorylate protein substrates at specific residues such as serine/threonine or tyrosine. For this purpose, two proteins, casein and myelin basic protein (MyBP), were phosphorylated using eukaryotic kinases which specifically phosphorylate at either serine/threonine or tyrosine. The assays were all performed under the same conditions of temperature, pH and phosphatase concentration (Figure 6B and C). In such conditions, it appeared that PrpA, like λ-PP, is able to dephosphorylate casein and MyBP at both serine/threonine and tyrosine residues (Figure 6B and C). The phosphoprotein phosphatase activity of PrpB was best observed with the phospho-MyBP substrates (Figure 6C). In this case again as with pNPPase activity, despite the high degree of homology between the three enzymes their catalytic properties appear to be slightly different. Hence like λ-PP, *E.coli* phosphatases PrpA and PrpB have dual specificities in that they are both serine/threonine and tyrosine phosphatases.

The PrpA- and PrpB-mediated increase in htrA transcription is dependent on the presence of functional CpxR and CpxA proteins

The genetic data presented here show that multiple unlinked mutations affecting *htrA* transcription could be

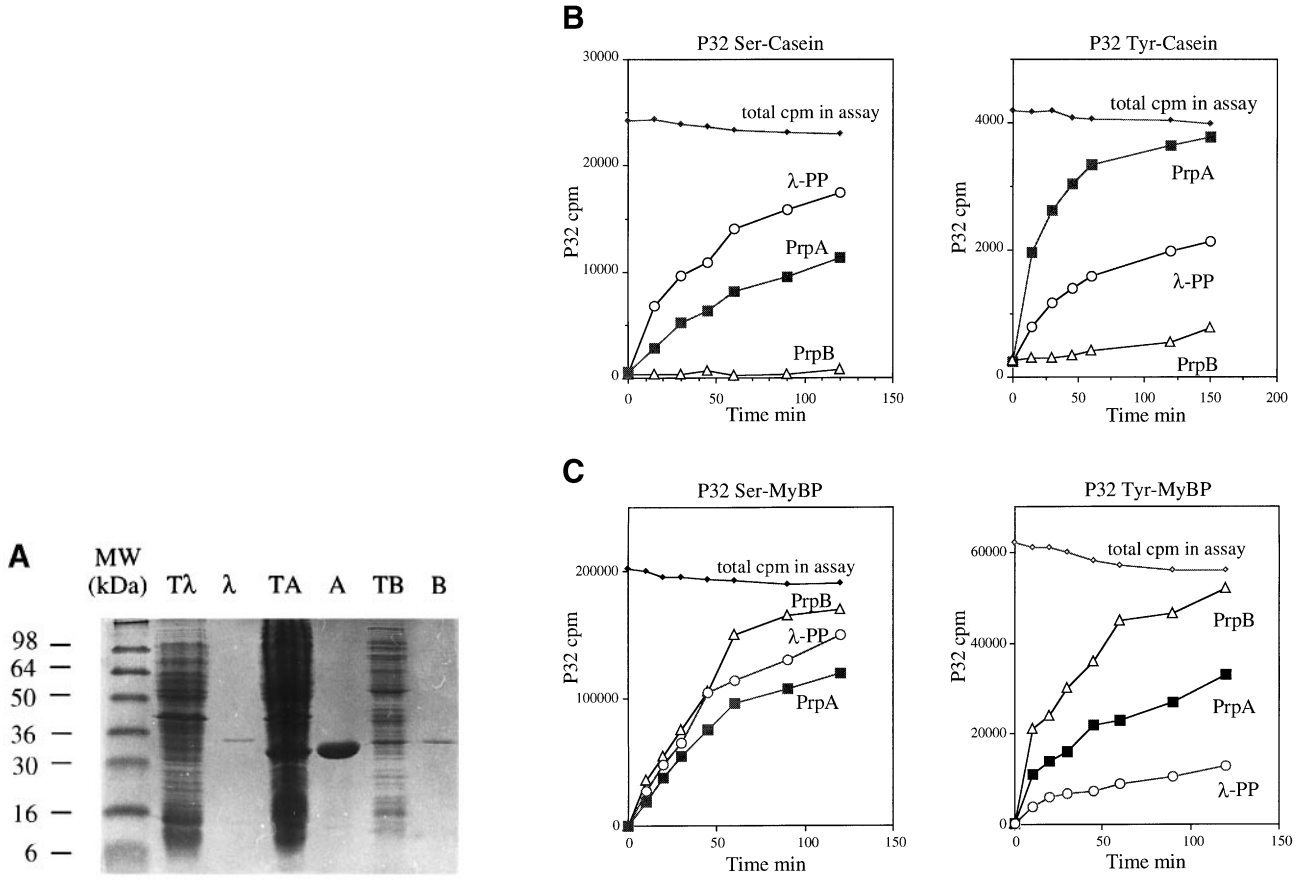

Fig. 6. (**A**) Purified λ-PP, PrpA and PrpB proteins. Proteins were purified as described in Materials and methods and electrophoresed on 12.5% SDS–PAGE. A gel stained with Coomassie Blue is shown. Lane M: pre-stained protein molecular weight standards (Bio-Rad). Lanes labeled as Tl, TA, TB: proteins from total cell extracts from strains carrying pSR3040 (λ*nin* ORF221), pDM1574 (*prpA*$^+$) or pDM1757 (*prpB*$^+$) respectively. Lanes labeled as λ, A, B: purified proteins, λ-PP, PrpA and PrpB respectively. (**B**) Dephosphorylation assays of [^{32}P]casein at 25°C. Left panel: [^{32}P]Ser/Thr-casein (0.1 mg/ml) was treated with 0.1 μg/ml of either PrpA or PrpB or λ-PP as indicated on the figure and described in Materials and methods. Right panel: [^{32}P]Tyr-casein (0.1 μg/ml) was treated with 0.2 μg/ml of either PrpA or PrpB or λ-PP. No autohydrolysis of [^{32}P]casein was observed during the course of the experiment. (**C**) Dephosphorylation assays of [^{32}P]MyBP at 30°C. Left panel: [^{32}P]Ser/Thr-MyBP (0.3 mg/ml) was treated with 0.1 μg/ml of either PrpA or PrpB or λ-PP. Right panel: [^{32}P]Tyr-casein (0.3 mg/ml) was treated with 0.2 μg/ml of either PrpA or PrpB or λ-PP. No autohydrolysis of [^{32}P]MyBP was observed during the course of the experiment.

isolated, including: *rpoE*, *cpxR*, *cpxA*, *prpA* and *prpB*. We tried to analyze the contribution of each locus to *htrA* transcription and whether the different mutations were epistatic, the aim being to understand the sequence of molecular events leading to *htrA* induction. To achieve this, we combined various mutations and examined their synergistic effect using the reporter LacZ activity from the *htrA* promoter. We first examined the combination of *rpoE* mutations with others. Double *rpoE cpxR* or *rpoE prpA* null mutations proved to affect *htrA* transcription most dramatically (Table II, reduction from 150 to ~14 Miller units). This was not the case with the double null mutation *prpA cpxR* or *prpB cpxR* (Table II, most of them displayed ~100 Miller units for their activity). Hence RpoE appears to act independently of PrpA as well as PrpB and CpxR/CpxA. This was further supported by the findings that the multicopy effect of *prpA* on the transcription of the *htrA* gene was still observed in an *rpoE* null background (induction from 38 to 94 Miller units). However, in strains lacking either the CpxR or CpxA protein no significant such induction was observed (Table II, 95 units in the *cpxR* mutant versus 109 for the isogenic *cpxR* mutant carrying the *prpA* gene onto the plasmid). Although the values for β-galactosidase activities scale up quite differently (Table II), clearly transcription of *htrA* which is severely reduced in the *rpoE* mutant background, can still be stimulated 2- to 3-fold upon overexpression of either *prpA* or *prpB*. A partial depend-

Table I. Biochemical properties of PrpA and PrpB phosphatases as compared with λ-PP

Compound	Concentration (mM)	Relative pNPPase activity (%)		
		λ-PP	PrpA	PrpB
$ZnCl_2$	10	14	56	35
$ZnCl_2$	20	7	43	23
Sodium molybdate	20	8	29	82
Vanadate	0.02	15	19	89
Fluoride	5	94	68	102
Tartaric acid	0.02	115	90	80
P-serine	5	90	108	97
Sodium sulfate	20	98	84	99

50 ng of each phosphatase was used to start each assay in a 1 ml reaction volume. Activities were assayed in 50 mM Tris–HCl pH 7.8, containing 20 mM pNPP and the various ions and compounds as indicated in the table, at 25°C.

Table II. PrpA and PrpB multicopy effect on *htrA* transcription requires the presence of a functional CpxR protein

Genetic backgrounds	β-galactosidase activity (Miller units) at 30°C
MC4100 *htrA–lacZ*	
wild type	147 ± 14
prpA::ΩCm	102 ± 11
prpB::ΩTet	114 ± 12
cpxR::ΩSpec	95 ± 11[a]
rpoE::ΩTet	38 ± 7[b]
prpA::ΩCm *rpoE*::ΩTet	15 ± 4
prpA::ΩCm *cpxR*::ΩSpec	92 ± 10
rpoE::ΩTet *cpxR*::ΩSpec	14 ± 5
prpB::ΩTet *cpxR*::ΩSpec	96 ± 12
MC4100 *htrA–lacZ*	
vector pOK12 alone (p15A-based)	152 ± 14
vector pSE420 alone (pBR322-based)	155 ± 15
pDM1695 *prpA*$^+$ (p15A-based)	911 ± 45
pDM1755 *prpB*$^+$ (p15A-based)	393 ± 27
pDM1757 *prpB*$^+$ (pBR322-based)	830 ± 33
cpxR::ΩSpec/pDM1695 *prpA*$^+$	109 ± 15[a]
cpxR::ΩSpec/pDM1757 *prpB*$^+$	119 ± 12[a]
rpoE::ΩTet/pDM1695 *prpA*$^+$	94 ± 7[b]
rpoE::ΩTet/pDM1757 *prpB*$^+$	83 ± 9[b]

[a]95 units for *cpxR* null is basal and hence no significant induction is observed upon overexpression of either PrpA or PrpB.
[b]Note the induction from 38 units in *rpoE* null to ~90 units upon overexpression of *prpA* or *prpB*.

ence on the presence of functional *rpoE* for the induction of *htrA* transcription by the *Cpx* pathway has also been observed by Danese *et al.* (1995). Similarly, the induction of *htrA* transcription by the overexpression of PrpB was also abolished in a *cpxR* null mutant background (Table II).

Protein misfolding in the extracytoplasm is sensed by a global Prp Cpx pathway

We then examined directly the involvement of a putative *cpx prp* pathway for sensing protein misfolding in the periplasm. Keeping in mind that *htrA* transcription *in vivo* is mainly induced by the accumulation of misfolded exported proteins, we took advantage of our knowledge of the Dsb proteins which are, so far, the best characterized folding catalysts. We have previously observed that mutations in the *dsb* genes, whose gene products catalyze the correct oxidation and folding of exported proteins (Missiakas *et al.*, 1995), led to a 2- to 3-fold induction of *htrA* transcription (Missiakas *et al.*, 1995; Raina *et al.*, 1995). Figure 7 compares the levels of inducibility of *htrA* transcription in a *dsbD* mutant background. Induction of *htrA* transcription is fully optimized only when functional CpxR/A and PrpA proteins are present (Figure 7). Clearly, part of the misfolding events is sensed by the whole σ^E regulon since transcription is induced from both *htrA* and *rpoH*P3 (Figure 7A). Interestingly, in an *rpoE* null mutant background, the accumulation of misfolded proteins associated with *dsbD* null mutation is still reflected by a minor induction of *htrA* transcription. No such increase is observed when using the *rpoH*P3–*lacZ* fusion in an *rpoE* null mutant background since *rpoH*P3 is exclusively transcribed by Eσ^E (Figure 7B). Also, introducing either *prpA* or *cpxR* null mutation does not affect transcription from the *rpoH*P3 promoter (Figure 7B). It

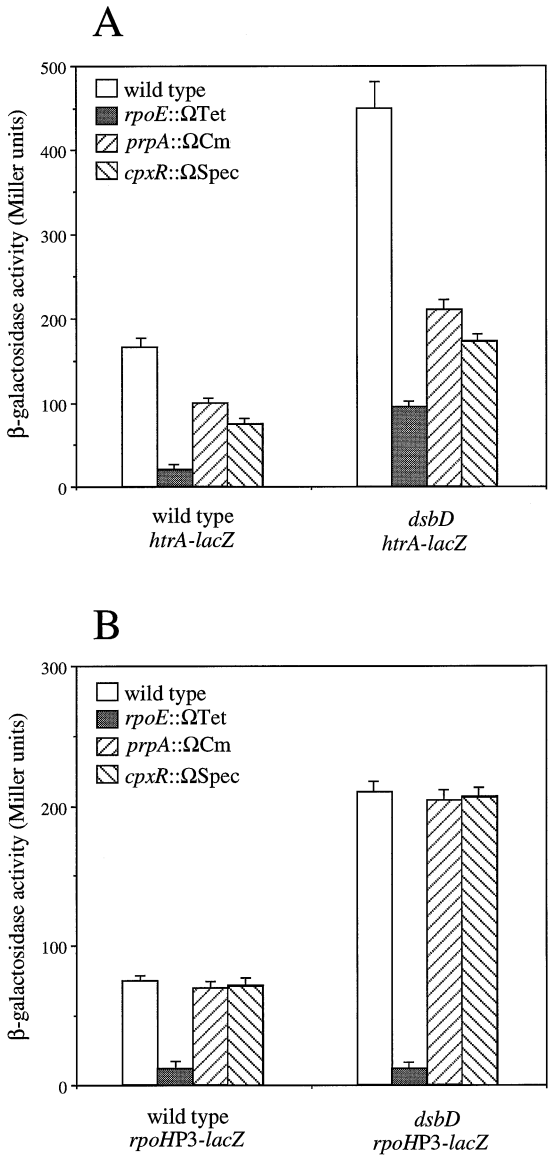

Fig. 7. Signaling for more *htrA* transcription in a strain deficient in DsbD periplasmic folding catalyst is dependent on the presence of both *prpA* and *cpxR* wild-type genes. Experimental conditions were the same as those described in the legend to Figure 1. Data regarding induction of *htrA–lacZ* fusion are shown (**A**). The *rpoH*P3–*lacZ* fusion was used in (**B**) and serves as a control since the *rpoH*P3 promoter is strictly transcribed by Eσ^E.

may be pointed out that, although the induction of *htrA* transcription is reduced from a factor of 3-fold in the wild type to 2-fold in *cpx* and *prp* mutants, it is reproducible and significant. It is particularly important since it is well established that a combination of *htrA* mutation is additive with any of the known mutations in *dsb* genes in terms of induction of the σ^E regulon (Missiakas *et al.*, 1995; Raina *et al.*, 1995).

P~(CpxA CpxR) is a target of PrpA phosphatase action in vivo

From the genetic evidence presented, it seemed likely that CpxA and/or CpxR could be the targets of the phosphatase activity of PrpA. Bacteria carrying the *cpxR cpxA* operon on a plasmid under the control of an inducible *ptac*

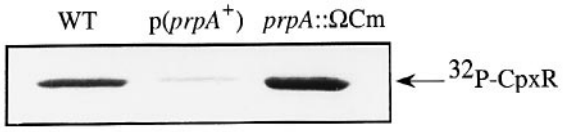

Fig. 8. Effect of PrpA activity on the ^{32}P~CpxR CpxA system. Cultures were grown in LB to OD 0.5 at 595 nm, washed and resuspended in low phosphate minimal medium containing all amino acids. Bacterial cultures were incubated for 2 h at 30°C with [^{32}P]orthophosphate (150 μCi/ml). The CpxR protein was immunoprecipitated from BL21 bacteria carrying the *cpxA* cpx*R* operon on the pOK12 plasmid. Lanes correspond to isogenic strains carrying either: (i) vector alone (WT); (ii) pDM1574 (*prpA*$^+$) or (ii) *prpA*::ΩCm.

promoter were incubated in the presence of radioactive inorganic phosphate ($^{32}P_i$). Incorporation of $^{32}P_i$ by the CpxR CpxA system was estimated by immunoprecipitating CpxR from total *E.coli* extracts. This phosphorylation status of the CpxR protein was used to monitor the phosphorelay activity between CpxA and CpxR. Incorporation of $^{32}P_i$ by the two component system was assayed in three isogenic backgrounds: wild-type bacteria, bacteria co-expressing the *prpA* gene on a plasmid with its own promoter, and bacteria lacking the functional PrpA protein (Figure 8). These results suggest that either the phosphate transfer between CpxA and CpxR or the stability of ^{32}P~CpxR are modulated by the presence of PrpA. Therefore, PrpA which has the signature of a typical type I phosphatase is able to dephosphorylate at serine and tyrosine residues but also at either histidine or aspartic acid residues. For some two component systems, an 'aspartyl-phosphatase' activity such as that of CheZ has been found to specifically dephosphorylate the P~CheY response regulator, providing a means to enhance the response time to signals issued by the chemotaxis transduction system (Parkinson, 1993). However, CheZ does not share any homology with typical phosphoprotein phosphatases. Hence we hereby provide the first evidence to date that type I phosphatases exist in *E.coli* and that they can modulate signal transduction pathways.

Overexpression of PrpA and PrpB relieves the envelope toxicity due to abnormal proteins

It has been previously reported that induction of the Cpx pathway can relieve the envelope toxicity otherwise observed in the presence of hybrid protein fusions like LamB–LacZ–PhoA (Danese *et al.*, 1995). This has been shown to be dependent on the induction of *htrA* transcription mediated via the Cpx pathway (Danese *et al.*, 1995). The expression of this hybrid fusion can be induced with maltose. Such an induction is toxic to the cell and makes bacteria sensitive to SDS (0.4%) unless HtrA is overproduced from a plasmid (Cosma *et al.*, 1995). When such a fusion-carrying strain was transformed with the plasmid pDM1695 containing the *prpA*$^+$ gene, the toxicity was relieved to the same extent as when *htrA* was provided on a plasmid with a similar copy number (Table III). Overproduction of PrpB also helped to relieve this toxicity but only when the *prpB* gene was present on a plasmid with a higher copy number as observed by the decrease in the zone of growth inhibition (Table III). These results correlate overall with the relative induction of *htrA* transcription by the *prpA*$^+$ or *prpB*$^+$ gene on multicopy plasmids.

Table III. Suppression of maltose and SDS sensitivities of *lamB–lacZ–phoA* strains upon overexpression of the *prpA, prpB* or *htrA* genes

Relevant genotype	Diameter of growth inhibition (mm)	
	Maltose (7%)	SDS (10%)
lamB–lacZ–phoA/		
pOK12 (p15A-based)	15.7 ± 2.1	12.7 ± 1.9
pDM1695 (*prpA*$^+$, p15A-based)	0	2.9 ± 0.4
pDM1755 (*prpB*$^+$, p15A-based)	4.3 ± 0.9	6.5 ± 0.7
pDM1757 (*prpB*$^+$, pBR322)	0	3.4 ± 0.6
pSR2828 (*htrA*$^+$, p15A-based)	0	3.1 ± 0.4

pOK12 is a p15A-based vector. Approximately 15 copies of this vector are present per cell (Vieira and Messing, 1993).

Table IV. Effect of PrpA overexpression on the transcriptional activity of the two component RcsB RcsC system

Genetic backgrounds	β-galactosidase activity (Miller units) at 30°C
MC4100 *cps–lacZ*	21 ± 3
MC4100 *cps–lacZ*/pOK12	30 ± 4
MC4100 *dsbA*::Tn*10 cps–lacZ*	96 ± 7
MC4100 *dsbB*::Tn*10 cps–lacZ*	103 ± 9
MC4100 *dsbA*::Tn*10 cps–lacZ*/pDM1695 (*prpA*$^+$)	52 ± 5
MC4100 *dsbB*::Tn*10 cps–lacZ*/pDM1695 (*prpA*$^+$)	47 ± 5

Influence of PrpA on other two component systems

The lack of two major periplasmic catalysts, DsbA and DsbB, leads to the induction of *htrA* transcription. In addition, *dsbA* and *dsbB* mutants are highly mucoid meaning that a third of the transcriptional machinery, the RcsB RcsC two component system, is also induced upon misfolding in the extracytoplasmic compartments. Mucoidy results from the production of colanic acid capsular polysaccharide. Capsule synthesis is promoted by proteins encoded by genes of the *cps* operon. Transcription of the *cps* genes is induced by two positive regulators, RcsA and phosphorylated RcsB. Synthesis of the capsule has been shown to be adaptive under certain external stresses such as desiccation, when strengthening of the cell envelope becomes an important mechanism of defense. Under normal conditions, capsular polysaccharide biogenesis is shut off since RcsA is rapidly degraded by the Lon protease. RscB, on the other hand, is part of the two component signaling system RcsB RcsC and is activated by phosphorylation, in a RcsC-dependent manner (Gottesman and Stout, 1991). Overexpression of *prpA* in *dsbA* or *dsbB* backgrounds greatly reduces the mucoidy of the mutant cells. Quantification of such an RscB-dependent transcriptional activity was also analyzed using *cps–lacZ*-carrying strains. As can be seen in Table IV, a 2- to 3-fold reduction of *cps–lacZ* activity was observed in *dsbA* or *dsbB* mutant strains carrying the multicopy *prpA* plasmid. It is likely that in this case, signaling for increased transcription of *cps* genes was inhibited by the phosphatase activity of

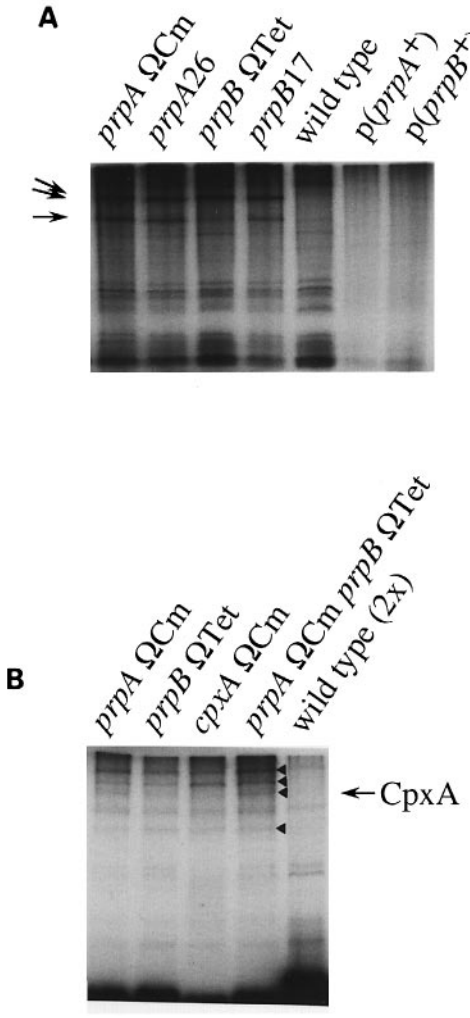

Fig. 9. *Escherichia coli* phosphorylated proteins as viewed from 1D gel electrophoreses. Bacterial cultures were incubated for 30 min at 30°C with [^{32}P]orthophosphate (200 μCi/ml). [^{32}P]orthophosphate-labeled extracts were from (**A**) *prpA*::ΩCm (SR2879), *prpA*26 (SR1505), *prpB*::ΩTet (SR2921), *prpB*17 (SR1599), isogenic wild-type MC4100, MC4100 carrying plasmid pDM1695 (*prpA*$^+$), MC4100 carrying plasmid pDM1755 (*prpB*$^+$); (**B**) *prpA*::ΩCm (SR2879), *prpB*::ΩTet (SR2921), *cpxA*::ΩCm, *prpA*::ΩCm *prpB*::ΩTet double-null mutant bacteria, isogenic wild-type MC4100 for this last sample twice the amount of material was loaded. Arrows show proteins for which synthesis is increased. The position of CpxA is indicated by an arrow in (B).

PrpA acting either as a phospho-histidine phosphatase on RcsC or as a phospho-aspartic phosphatase on RcsB. It is quite interesting that an independent phosphatase enzyme such as PrpA can also fine tune the regulation of other two component systems in the cell.

Identification of in vivo targets of PrpA and PrpB

It is well known that some proteins get phosphorylated in *E.coli* (Freestone *et al.*, 1995). The physiological importance of *E.coli* phosphatases in modulating the phosphorylation status of such proteins was addressed by examining the total protein profiles of wild-type *E.coli* versus strains lacking *prpA* or *prpB*, on regular SDS–PAGE as well as on 2D gels (Figures 9A and B, and 10). Consistent with our model for the involvement of Prp proteins in dephosphorylation of phosphoproteins, an increased number of phosphoproteins was seen in extracts prepared from strains carrying mutations in *prpA* or *prpB* as compared with the isogenic wild-type strain (Figure 9A). Approximately 20 different phosphoproteins were observed in both bacteria carrying null mutations of *prpA* or *prpB* and point mutants leading to a loss-of-function of the phosphatase activity (*prpA26* and *prpB17*, see Table VI). As shown in Figure 9A and B, at least four additional phosphoproteins accumulate in either *prpA* or *prpB* mutant bacteria. Moreover, when wild-type *E.coli* was transformed with a plasmid carrying either the *prpA*$^+$ gene (pDM1695) or the *prpB*$^+$ gene (pDM1755), most of the phosphorylated proteins seen in the wild-type extract no longer accumulated (Figure 9A).

Figure 9B shows evidence for the accumulation of phosphorylated CpxA (P~CpxA) in the *prpA* and *prpB* null mutant bacteria. The identification of the band corresponding to CpxA was achieved by running a sample extracted from *cpxA* null mutant strain. Bacteria deleted for both *prpA* and *prpB* show an increase in the accumulation of phosphorylated proteins, including P~CpxA, as compared with the wild type (Figure 9B).

In an attempt to identify some of the phosphorylated proteins that are the targets of PrpA and PrpB phosphatases, some of these cell extracts were also analyzed by 2D equilibrium gel electrophoresis (Figure 10). Not all phosphoproteins could be resolved on such gels since the rank of ampholines used allows a good separation only between pH 7 and 4.5. In addition, there might be some membrane proteins which may not enter the gel. Hence mostly six different phosphoproteins (out of 20) accumulating in either *prpA* or *prpB* null mutant bacteria can be resolved on these gels (Figure 10). As a control, we also labeled bacteria carrying mutations in the *cpxA* gene, either a null mutation (*cpxA*::ΩCm) or *cpxA** a chromosomal allele leading to a gain-of-function of CpxA. Then, to further substantiate that the Prp proteins act via the Cpx signal transduction pathway, extracts prepared from bacteria overexpressing the *cpxR* gene were also analyzed. Hence, the major spot corresponds to the P~CpxR protein whose position on the 2D gel was assigned by running a ^{32}P-labeled extract from bacteria carrying the *cpxR*$^+$ gene-containing plasmid (pDM1787). Finally, the P~CpxR protein was also found to accumulate in strains carrying the *cpxA** allele (a change of T252 to R, see Table VI) and to disappear in strains lacking the *cpxA* gene (*cpxA*::ΩCm). This *cpxA** allele corresponds to a mutation leading to a gain-of-function i.e. a hyper-kinase activity of the CpxA protein and corresponds to a similar mutation *env*Z11 (T247R; Aiba *et al.*, 1989). EnvZ is the histidine kinase most closely related to CpxA (Weber and Silverman, 1988).

Overexpression of PrpA induces the heat shock response

We then compared the global protein profiles as labeled with [^{35}S]methionine of wild-type and pDM1695-(*prpA*$^+$)-carrying strain, by 2D equilibrium gel electrophoresis (Figure 11A). While an increase in the accumulation of Eσ^{32}-transcribed heat shock proteins was found, as judged from the overall protein profiles of the 2D gels, some other proteins were found to be present in diminished amounts (Figure 11A). These results were further sup-

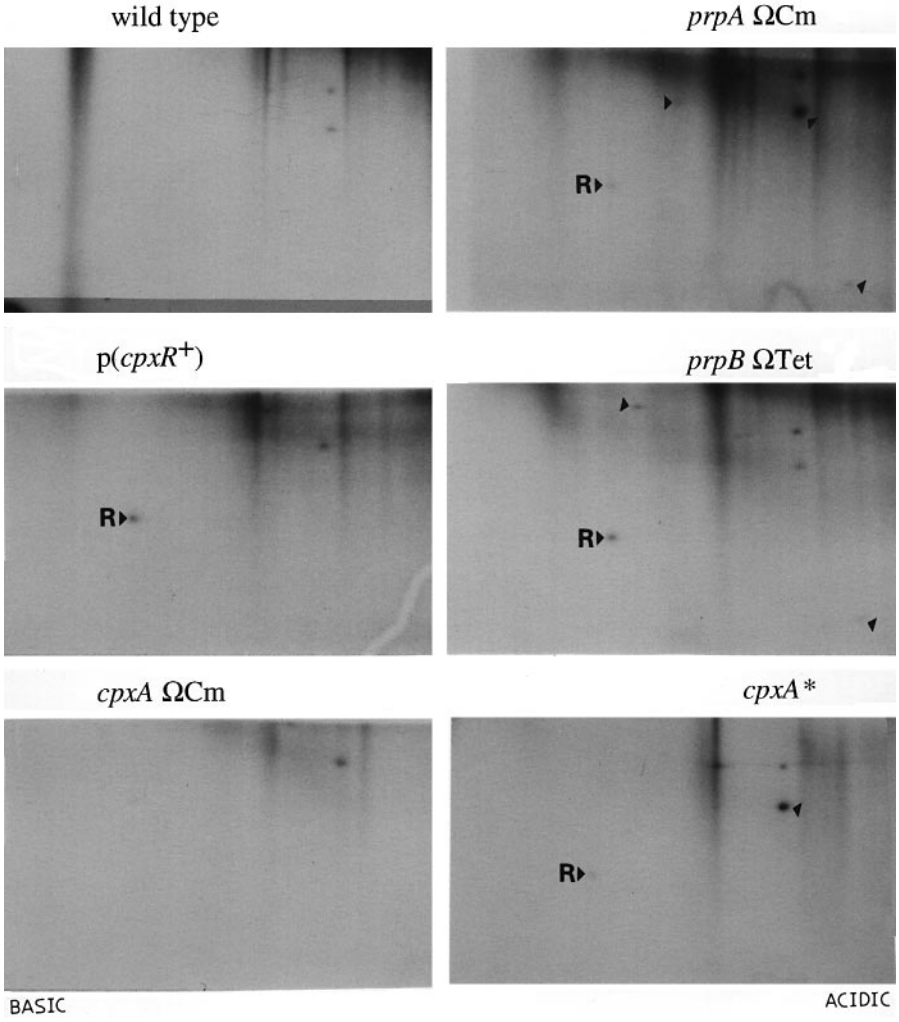

Fig. 10. PrpA and PrpB activities modulate the phosphorylated status of various proteins. *Escherichia coli* phosphorylated proteins as seen from 2D equilibrium gel electrophoreses. Extracts were from the following strains. Left column: isogenic wild-type strain (MC4100), wild-type strain carrying the *cpxR*$^{+}$-containing plasmid (pDM1787), and the *cpxA*::ΩCm mutation. Right column: MC4100 carrying the *prpA*::ΩCm mutation (SR2879), MC4100 carrying the *prpB*::ΩTet mutation (SR2921) and MC4100 carrying the *cpxA*:* mutation (SR3570), i.e. a mutation conferring a hyper-kinase activity to CpxA. Samples were prepared as described in the legend to Figure 9. The proteins were resolved in the first dimension on 1.6% (pH 5.0–7.0) and 0.4% (pH 3.5–10.0) ampholines (Pharmacia) and by 12.5% SDS–PAGE in the second dimension. Autoradiograms of the dried gels are shown. Arrow designated 'R' points to the position corresponding to the phosphorylated CpxR protein. The other differences are depicted with bold arrows.

ported by the observation of an increased transcription of Eσ^{32}-transcribed promoters as monitored from *lon–lacZ*, *htpG*–lacZ and *groELS–lacZ* promoter fusions (Table V). However, the increase in the activity of Eσ^{32}-dependent promoters is not as dramatic as with the *htrA* promoter (Table V). To confirm these results, we also examined *in vivo* the level of transcription of the major heat shock gene *dnaK*, by performing Northern blot analysis. Total RNA was extracted from isogenic bacteria carrying either the vector alone or the plasmid pDM1695 (*prpA*$^{+}$) and probed for the accumulation of *dnaK*-specific message. As shown in Figure 11B, at least a 2- to 3-fold increase in the accumulation of *dnaK*-specific message is observed even at normal temperatures (30°C) upon overexpression of *prpA*.

In addition to the heat shock proteins, the 2D gels revealed that accumulation of many other proteins was affected when *prpA* was present on a multicopy plasmid (Figure 11A, lower panel). As predicted from the calculated pI, the PrpA protein migrates on this 2D gel towards the basic end (Figure 11A, lower panel). Quite interesting is the separation of PrpA protein into two spots. This suggests that PrpA is modified *in vivo* and that this modification may possibly account for regulating its activity.

Discussion

This study demonstrates that accumulation of misfolded proteins in the extracytoplasm is sensed by multiple transcriptional systems. The primary response depends on the presence of HtrA, a periplasmic protease. The levels of HtrA are controlled at the transcriptional level and the Eσ^{E} polymerase is responsible for the synthesis of most, but not all, *htrA* transcripts. Mutations in the *rpoE* gene encoding the sigma factor σ^{E} lead to a severe, but partial, decrease in *htrA* transcription (Raina *et al.*, 1995). In order to understand how signaling for increased *htrA* transcription is transduced between the two cell compartments, we performed extensive mutagenesis and isolated three additional loci which affected *htrA* transcription.

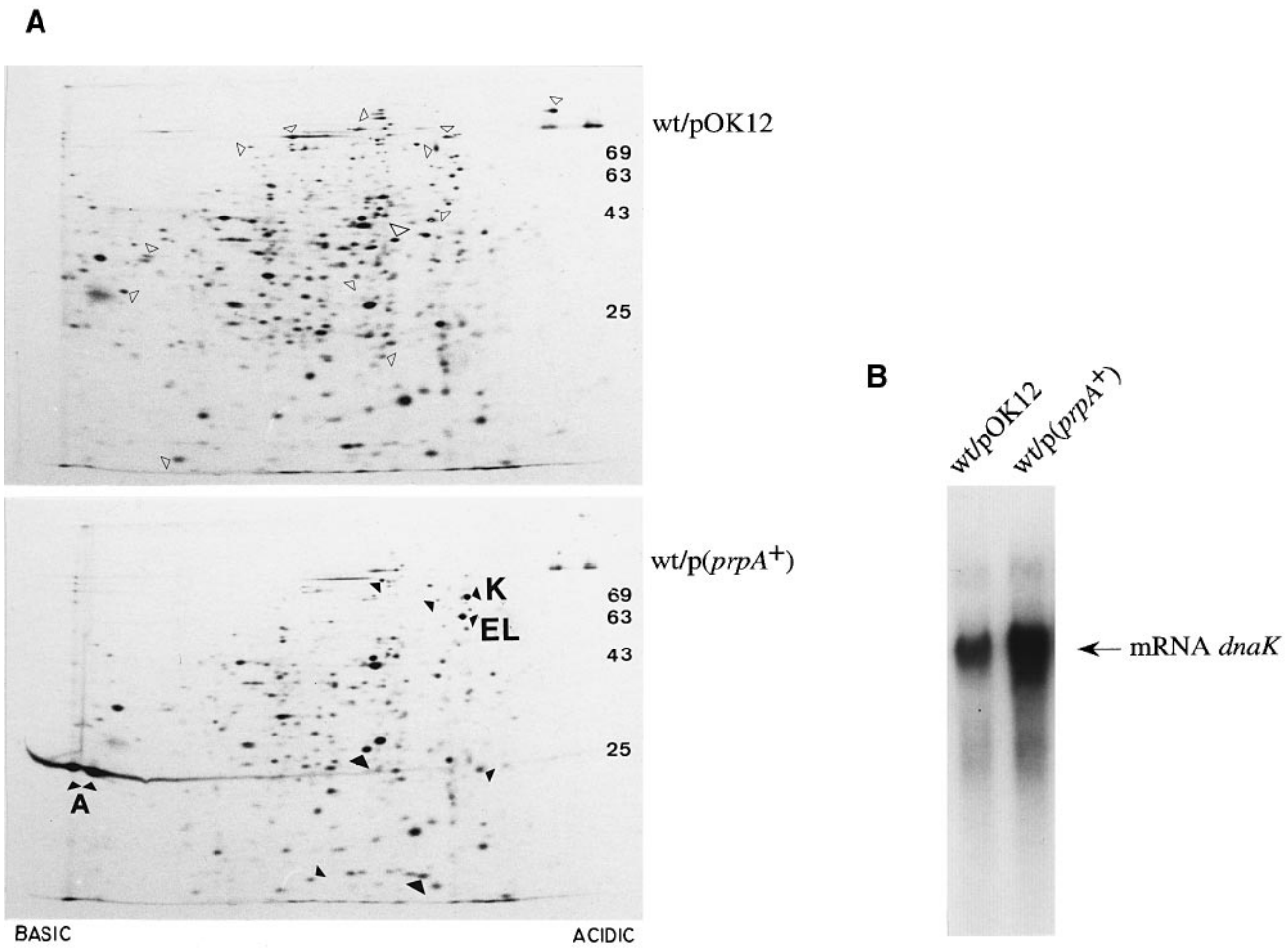

Fig. 11. (**A**) Global effects of *prpA* overexpression as viewed from 2D equilibrium gel electrophoreses. [^{35}S]methionine-labeled extracts from MC4100 carrying the vector alone, pOK12 (top panel) and MC4100 carrying plasmid pDM1695 (*prpA*$^{+}$) (lower panel), were prepared and analyzed as described in the legend to Figure 9. Open arrows in the top panel point toward proteins which are less abundant when *prpA* is overexpressed (see lower panel). Bold arrows in lower panel show proteins for which the synthesis is increased upon overexpression of *prpA*. K and EL designate the positions of the DnaK and GroEL proteins, respectively. The double arrows designated 'A' point towards the PrpA protein. Numbers in the margins indicate the molecular weight in kDa of molecular markers. (**B**) Northern RNA analysis of *dnaK* transcripts from isogenic bacteria carrying either vector alone or pDM1695 *prpA*$^{+}$. RNA was extracted from bacteria grown at 30°C. Approximately 5 μg of total RNA was loaded per lane and analyzed by the Northern blot technique and probed with ^{32}P-labeled random primed 300 ng of the 508 bp *Eco*RI–*Nru*I DNA fragment internal to the *dnaK* gene.

These mutations were mapped to the *cpxR cpxA* operon, the others mapped into two new genes *prpA* and *prpB*. We found that these mutations affected specifically *htrA* transcription in a manner independent of the Eσ^{E} transcription activity. Interestingly, such mutants had no effect on the transcriptional activity of the two other known genes of the *rpoE* regulon, namely *rpoH* or *rpoE* itself. The *prpA* gene was again isolated in a complementary genetic approach looking for gene products which in multicopy significantly induced *htrA* transcription.

PrpA and PrpB are prokaryotic type I-like phosphatases

PrpA and PrpB are ~50% identical at the amino acid level. They seem to be prototypes of classical eukaryotic type I serine/threonine phosphatases especially in their catalytic domains, based on significant sequence homology. Biochemical characterization proved that both PrpA and PrpB could hydrolyze pNPP, a typical substrate for phosphatase activity. In addition, PrpA and PrpB were both able to dephosphorylate protein substrates phosphorylated either at serine/threonine or tyrosine residues, with efficiencies comparable with that of λ-PP, a recently characterized phosphatase with catalytic properties similar to type I phosphatases (Barik, 1993; Zhuo *et al.*, 1993). Four point mutations leading to a loss of phosphatase activity were isolated in *prpA*. Of these, three mapped in residues D24 and H26. They correspond to residues D20 and H22 in λ-PP which have been shown, by site-directed mutagenesis, to be essential for the phosphatase activity of the λ protein (Zhuo *et al.*, 1994). From the 3D structures of human type I phosphatase (Egloff *et al.*, 1995; Goldberg *et al.*, 1995) and calcineurin (type II phosphatase) (Griffith *et al.*, 1995), these residues are also predicted to be part of the metal-binding pocket in PrpA. Similarly, one *prpB* loss-of-function mutant was found to carry a change for the highly conserved residue H78 to N which corresponds to H76 in λ-PP. The finding of phosphoprotein phosphatases in such a simple organism also raises the question about their *in vivo* role and function. If no requirement for a phosphatase activity in bacteriophage λ is yet known, *E.coli* phosphatases like PrpA and PrpB seem to play a

Table V. Effect of PrpA overexpression on the heat shock response

	β-galactosidase activity (Miller units) at 30°C
$E\sigma^{32}$-transcribed genes	
MC4100 *lon–lacZ*/pOK12	331 ± 27
MC4100 *lon–lacZ*/pDM1695 (*prpA*$^+$)	645 ± 41
MC4100 *htpG–lacZ*/pOK12	296 ± 29
MC4100 *htpG–lacZ*/pDM1695 (*prpA*$^+$)	591 ± 37
MC4100 *groELS–lacZ*/pOK12	712 ± 45
MC4100 *groELS–lacZ*/pDM1695 (*prpA*$^+$)	1502 ± 61
$E\sigma^{E}$-transcribed genes	
MC4100 *htrA–lacZ*/pOK12	148 ± 9
MC4100 *htrA–lacZ*/pDM1695 (prpA$^+$)	843 ± 47
$E\sigma^{70}$-transcribed genes	
MC4100 *htrP–lacZ*/pOK12	2080 ± 170
MC4100 *htrP–lacZ*/pDM1695 (*prpA*$^+$)	1910 ± 173

All the strains are isogenic and were made *recA*$^-$ prior to making the assays.

direct role in the signal transduction pathways coupled with activation of gene transcription. Finally, it appears from this study that there are still some unknown features within the catalytic domains of phosphatases which make large differences in their properties. PrpA and PrpB are clear examples of this. Both *in vivo* and *in vitro*, they behave quite differently (towards the protein substrates casein and MyBP, for example) despite a high degree of homology (50% identity).

It is interesting that neither *prpA* nor *prpB* genes are essential for bacterial growth under normal conditions (30°C). This is probably consistent with the fact that, so far, none of the known components, periplasmic folding catalysts or periplasmic chaperones, are essential for bacterial growth under normal conditions. For example none of the *dsb* genes is essential, neither are any of the peptidyl prolyl isomerases (RotA, FkpA and SurA), nor OmpH/Skp; even though, in the absence of most of them, folding of exported proteins is retarded or impaired and the σ^E regulon is induced (Missiakas *et al.*, 1996a; 1996b).

How do PrpA and PrpB influence htrA transcription?

Various lines of evidence are presented showing that the PrpA protein phosphatase activity, and to some extent that of PrpB, play an active role in the induction of *htrA* transcription. First, overexpression of PrpA and PrpB led to a 4- to 6-fold increase in *htrA* transcription. Second, some chromosomal point mutations were isolated which led to a decrease in *htrA* transcription and were shown to map to the *prpA* and *prpB* genes. These specific mutations were identified as changes such as D24 to V, H26 to N and H26 to L in the case of *prpA* and H78 to N in the case of *prpB*. These mutants behaved quite like *prpA* or *prpB* null mutations. Third, overexpression of these mutant proteins in the same cloning system as the wild-type *prpA* gene did not lead to the 4- to 6-fold induction of *htrA* transcription. Since positions D24 and H26 correspond to highly conserved residues in the active site of type I phosphatases, this directly implicates the phosphatase activity of PrpA as the key element of its *in vivo* physiological role. Similarly, mutation H78 to N in *prpB* leads to a loss-of-function of the protein *in vivo*. The corresponding position has been shown to be essential for λ-PP activity using site-directed mutagenesis (Zhuo *et al.*, 1994). Fourth, the phosphatase-dependent activation of *htrA* transcription by either PrpA or PrpB was completely dependent on the presence of functional CpxR and CpxA proteins. The main pieces of evidence for this are the findings that the multicopy effect of *prpA* or *prpB* on *htrA* transcription was abolished in a *cpxR cpxA* null mutant and that no synergistic effect was observed when combined with *cpxR cpxA* mutations. Fifth, *in vivo* experiments showed that active PrpA and PrpB modulate the phosphorylated status of the Cpx system as reflected by the increased amount of P~CpxR detectable in *prpA* or *prpB* null mutant backgrounds (Figures 8, 9 and 10) and a decrease in the accumulation of P~CpxR when *prpA*$^+$ is overexpressed (Figure 8). Since P~CpxA also accumulates in *prpA* or *prpB* null mutant backgrounds (Figure 9B), it is likely that, like λ-PP (Zhuo *et al.*, 1993), both *E.coli* phosphatases have a histidine phosphatase activity. Such a phosphatase activity will affect the phosphorylated status of both CpxA and CpxR *in vivo*.

The CpxR and CpxA proteins (Weber and Silverman, 1988; Dong *et al.*, 1993) are highly homologous to the OmpR EnvZ two component system. This implies that PrpA, which *in vitro* exhibits very good serine and tyrosine phosphatase activities, is behaving *in vivo* as a good histidine and/or aspartyl phosphatase. Interestingly, the homologue of PrpA in bacteriophage λ has been shown to be an efficient histidine phosphatase *in vitro* using P~NRII or P~CheA as the substrates (Zhuo *et al.*, 1993). In *Bacillus subtilis*, the RapA and RapB proteins have been shown to be aspartyl-phosphatases of the P~Spo0F response regulator (Perego *et al.*, 1996). Our observations show that decreasing the phosphorylated status of the Cpx system was important to obtain maximal transcription of *htrA*, in cases when the HtrA protease was highly required in the periplasm. Clearly, the phosphatase activity of PrpA and to some extent PrpB are important to fine-tune the efficient transcription at the *htrA* promoter, possibly in two ways: (i) by allowing a faster turnover at the promoter since the main transcription, will yet come from the $E\sigma^E$ transcriptional machinery; (ii) by preventing the binding of P~CpxR to low affinity repression sites which may block the transcriptional process. It is very well documented in the case of OmpR, the regulatory protein most related to CpxR, that excess of P~OmpR in the cell switches its function from activator to repressor (Rampersaud *et al.*, 1994; Harlocker *et al.*, 1995).

PrpA modulates the phosphorylated state of multiple two component systems as well as affecting the accumulation of major heat shock proteins

The *E.coli* protein phosphatases also affected other two component signal transduction systems. In the case of the RcsB RscC system, PrpA overexpression seemed to decrease the signal transduction process presumably by dephosphorylating P~RcsC or P~RcsB and thereby limiting DNA binding. It seems that like the λ-PP, PrpA has a very general phosphatase activity with no strict substrate specificity. This might be of some advantage for the

intruding bacteriophage. However, in *E.coli*, non-specific dephosphorylation by PrpA may need to be curtailed. It is therefore interesting that transcription of *prpA* is quite weak, leading to very little accumulation of the protein in the cell. Such a situation changes upon heat shock since *prpA* transcription is induced.

Based on the observation that *prpA* overexpression leads to an overall altered protein profile as compared with the isogenic wild type (Figure 11A), and induces the accumulation of heat shock proteins at elevated levels (Figure 11A and Table V), it is tempting to speculate that the phosphorylated species of DnaK and GroEL (MacCarty and Walker, 1991; Sherman and Goldberg, 1992) might be acting directly as cellular thermometers by sensing temperature upshifts. Our findings that overexpression of PrpA triggers a heat shock response are consistent with a model that postulates that the unphosphorylated forms of chaperones do not bind the substrates very well (Sherman and Goldberg, 1992). Indeed, such a situation will lead to the accumulation of misfolded proteins and thereby trigger the σ^{32}-dependent heat shock response. It is equally interesting that in the case of PrpA overproduction, many proteins accumulate at reduced rates as compared with the isogenic wild type (Figure 11A).

Conclusion

It remains to be understood why protein misfolding occurring in the extracytoplasmic compartments signals a requirement for more HtrA protein using at least two different transcriptional regulation pathways. What makes HtrA so important? And what in the periplasm exactly triggers this requirement for HtrA? One possibility could be that the levels of HtrA are sensed directly by CpxA and by the σ^E regulon via the RseA protein (Missiakas *et al.*, 1996a). RseA is located in the inner membrane and modulates σ^E activity by a direct protein–protein interaction (RseA–σ^E) which is presumably relieved upon extracytoplasmic stimuli (Missiakas *et al*., 1997). The only argument against sensing HtrA levels is that deleting the *htrA* gene alone induces neither the Cpx Prp nor the σ^E regulons (Raina *et al*., 1995), although this would mimic an extreme condition where HtrA is completely titrated out upon accumulation of too many misfolded proteins. Hence the 'trigger(s)' responsible for signaling extracytoplasmic stresses remain(s) to be identified. Interestingly, this type of intercompartmental signaling between periplasm and cytoplasm is also found in yeast. A membrane sensor protein, very similar to the bacterial histidine kinases such as CpxA, was found to play an important role in signaling protein misfolding occurring in the endoplasmic reticulum to the transcriptional machinery (Mori *et al.*, 1993; Cox *et al.*, 1993). The various levels of control which might modulate the activity of such a system are not known. It would be interesting to know if these other elements are similar to those found in *E.coli*.

Materials and methods

Bacterial strains and plasmids

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

Media and chemicals

Luria-Bertani (LB) broth, MacConkey medium and M9 minimal medium were prepared as described by Miller (1992). Labeling experiments using [^{35}S]methionine in the M9 high-sulfur medium were performed as previously described (Missiakas *et al.*, 1993). When necessary, the media were supplemented with ampicillin (100 μg/ml), tetracycline (15 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (20 μg/ml). The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used at a final concentration of 40 μg/ml in the agar medium.

Isolation of trans-acting mutations

Trans-acting mutations which reduce β-galactosidase expression from the *htrA–lacZ* transcriptional fusion exclusively, were isolated through two independent genetic approaches as described earlier (Raina *et al.*, 1995). Briefly, P1 bacteriophage lysates of strain MC4100 (Lac$^-$) carrying random insertions of either mini-Tn*10* (Tet^R) or mini-Tn*10* (Kan^R) transposons, were treated with the hydroxylamine mutagen (Miller, 1992). These mini-Tn*10* marked putative mutants were transduced into strains SR1458 and SR1710 carrying the single copy fusion *htrA–lacZ* and *rpoHP–lacZ* respectively. Alternatively, a *mutD* mutation was transduced into MC4100, and such a strain was used as a host to construct mutagenized mini-Tn*10* libraries. These mutagenized pools were transduced into SR1458 (*htrA–lacZ*) and SR1710 (*rpoH*P3–*lacZ*), and again screened on X-gal-containing plates for reduced β-galactosidase activity. Bona fide candidates were also assayed in culture for β-galactosidase activity, as described by Miller (1992). Two classes of mutations could be distinguished. The first ones affected transcription of both fusions *htrA–lacZ* and *rpoH*P3–*lacZ* and have already been described (Raina *et al.*, 1995). The second class affected transcription of *htrA–lacZ* and not *rpoH*P3–*lacZ*. Three such complementation groups were obtained and a representative of each group is reported here with strain numbers: SR1572, SR1599 and SR2522. To map these new classes of mutations, complementing cosmid clones (selected from a cosmid library described by Raina *et al.*, 1995) were identified because they rescued the Lac-down phenotype and were able to recombine the linked Tet^R or Kan^R markers. DNAs from these cosmid clones were prepared for further subcloning experiments and for mapping to the *E.coli* DNA library in bacteriophage λ (Kohara *et al.*, 1987) using ^{32}P-labeled random-priming techniques (Sambrook *et al.*, 1989).

Cloning of prp genes and ORF221 from λnin region

Chromosomal DNA isolated from the *E.coli* wild-type strain MC4100 was used to construct a library in the p15A-based vector pOK12 (Vieira and Messing, 1991), as described previously (Raina *et al.*, 1995). This library was used to transform strain SR1458 carrying the single copy fusion *htrA–lacZ*. Transformants with an increased LacZ activity were selected on plates containing X-gal. DNA was extracted from ~50 such clones and transformed again in SR1458 (*htrA–lacZ*) and SR1710 (*rpoH*P3–*lacZ*). Clones breeding true were retained. Among them, 12 were found to induce the *lacZ* expression of *htrA–lacZ* but not of *rpoH*P3–*lacZ*. These clones were also found to revert the decreased β-galactosidase activity from *htrA–lacZ* observed with one of the *trans*-acting mutations isolated in strain SR1572. These clones were used for mapping experiments and ^{32}P-labeled nick-translation (Sambrook *et al.*, 1989). They hybridized to bacteriophage λ336 of the ordered *E.coli* genomic library (Kohara *et al.*, 1987) and were shown to carry the wild-type *prpA* gene.

The cosmid DNA complementing the mutation carried in SR1599 was shown to hybridize to bacteriophage λ449 and λ450 of the ordered *E.coli* genomic library (Kohara *et al.*, 1987). Partial digestion with *Sau*3A and ligation in the *Bam*HI site of the pOK12 vector (pDM1754) led to the identification of a 2.2 kb DNA fragment able to complement SR1599 mutant bacteria. A minimal clone (pDM1755) carrying a 1.3 kb DNA fragment was generated using exonuclease III which was sufficient to complement *prpB* mutant bacteria and used for sequencing. It was found to contain an ORF which corresponds to the gene designated as *prpB*.

Overexpression of PrpA was achieved by amplifying the minimal coding region of the *prpA* gene by PCR, using primers 5′-AGGAAAATACATATGAAACAGGCT-3′ and 5′-GCGGTTGGATCCGCATTGAGG-3′. The resulting amplified DNA product was cloned into the T7 promoter expression vector pAED-4 (pDM1574 *prpA*$^+$). The pAED-4 vector was a kind gift of Dr S.Doering. The minimal *prpB* coding sequence was amplified by PCR, using the primers 5′-gtaaaaccatggcatcta-3′ and 5′-taacaccggatccctcatgct-3′. The PCR product was digested with *Nco*I and *Bam*HI and cloned into pSE420 Invitrogen vector (pDM1757 *prpB*$^+$) which contains the *trc* promoter and the *lacI*^{q} repressor. The λ-PP gene was amplified from DNA prepared from wild-type bacteriophage λ, using 5′-GTGAAACATATGCGCTAT-3′ and 5′-CGCTTTGGATCCTCATGCGCC-3′ as primers. The resulting amplified DNA was cloned

Table VI. Bacterial strains and plasmids

	Relevant characteristics	Reference or source
Strains		
MC4100	F^- *araD139* Δ(*argF–lac*)U169 our collection	
CA8000	HFr	our collection
CAG118486	*eda*::Tn*10*	Singer *et al.* (1989)
SR1208	MC4100 ϕ (*groE–lacZ*)	Missiakas *et al.* (1993)
SR1458	MC4100 ϕ (*htrA–lacZ*)	Raina *et al.* (1995)
SR1588	MC4100 ϕ (*htrL–lacZ*)	Missiakas *et al.* (1993)
SR1710	MC4100 ϕ (*rpoH*P3–*lacZ*)	Raina *et al.* (1995)
SR2195	MC4100 ϕ (*rpoE*P2–*lacZ*)	Raina *et al.* (1995)
SR1572	MC4100 *prpA*24 (D24V)	this work
SR1505	MC4100 *prpA*26 (H26N)	this work
SR1603	MC4100 *prpA*107 (L107Q)	this work
SR1599	MC4100 *prpB*17 (H78N)	this work
SR2879	MC4100 *prpA*::ΩCm^R	this work
SR2921	MC4100 *prpB*::ΩTet^R	this work
SR3025	MC4100 *prpA*::ΩCm^R*prpB*::ΩTet^R	this work
SR2393	CA8000 Δ*rpoE*(*Hin*dIII–*Hpa*I):: ΩTet^R	Raina *et al.* (1995)
SR2522	MC4100 *cpxA*22	Raina *et al.* (1995)
SR2609	CA8000 *dsbD*::ΩCm^R	Missiakas *et al.* (1995)
SR3570	MC4100 *cpxA** (T252R)	this work
PND242	*cpxA*::ΩCm^R	Danese *et al.* (1995)
SR2970	*cpxA*::ΩCm^R transduced to SR1458	this work
PND325	*cpxR* ::ΩSpec^R	Danese *et al.* (1995)
SR2969	*cpxR* ::ΩSpec^R transduced to SR1458	this work
WBS1106	MC4100 Φ (*lamB–lacZ–phoA*) Hyb1-1 [λ p1(209)]-Δ*phoA*532	Cosma *et al.* (1995)
DM1674	MC4100 *clp*Q::ΩCm^R	Missiakas *et al.* (1996c)
Plasmids		
pSR1665	cosmid clone *prpA*$^+$	this work
pSR1669	cosmid clone *prpB*$^+$	this work
pDM506	pOK12 carrying a 2.8 kb *prpA*$^+$–*Sau*3A fragment	this work
pDM1695	pOK12 carrying a 900 bp *prpA*$^+$–*Pvu*II–*Acc*I fragment	this work
pSR2573	pDM506 (*prpA*24 D24V)	this work
pSR2575	pDM506 (*prpA*26 H26N)	this work
pSR2591	pDM506 (*prpA*107 L26Q)	this work
pDM1754	pOK12 carrying a 2.2 kb *prpB*$^+$–*Sau*3A fragment	this work
pDM1755	pOK12 carrying a 1.3 kb *prpB*$^+$–*Exo*III generated clone	this work
pDM1574	pAED-4 carrying 721 bp *prpA*$^+$–*Nde*I–*Bam*HI fragment	this work
pDM1757	pSE420 carrying 688 bp *prpB*$^+$–*Nco*I–*Bam*HI fragment	this work
pDM1786	pWSK30 carrying a 2.4 kb *cpxA*$^+$ *cpxR*$^+$ *Nde*I–*Stu*I fragment	this work
pDM1787	pWSK30 carrying the *cpxR*$^+$ *Exo*III generated clone	this work
pSR2828	pOK12 carrying *htrA*$^+$ cloned from Kohara phage λ119	this work
pSR3040	pAED-4 carrying 671 bp λ*nin* ORF221 (λ-PP) *Nde*I–*Bam*HI fragment	this work

into vector pAED-4 (pSR3040 λ*nin* ORF221). All the PCR-generated clones coding for PrpA, PrpB and λ-PP were sequenced and shown not to carry any mutation. The *cpxR cpxA* operon was subcloned from the DNA of bacteriophage λ541 of the Kohara library (Kohara *et al.*, 1986). A 2.4 kb *Nde*I–*Stu*I DNA fragment carrying the whole operon was blunted and ligated into the *Eco*RV site of the pWSK30 vector (Wang and Kushner, 1991). The resulting plasmid pDM1787 was retained since it carried the operon in frame with the T7 RNA polymerase-dependent promoter.

Construction of the chromosomal cpxA allele*

Site-directed mutagenesis was used to replace Thr252 by an Arg using plasmid pDM1786. This change was based on the known allele of *env*Z11 which is known to confer a hyper-kinase activity to the histidine kinase due to a loss of the autophosphatase activity (Aiba *et al.*, 1989). Mutagenesis was performed using Quick change site-directed mutagenesis kit from Stratagene and mutagenic primers 5′-CACGAGCT-GCGCCGCCCGCTGACGCGT-3′ and 5′-ACGCGTCAGCGGGCGG-CGCAGCTCGTG-3′. Replacement of the wild-type *cpxA* gene for this mutant allele was performed by subcloning the mutant *cpxA* gene into the phagemid vector pBIP (Slater and Maurer, 1993) and as described earlier (Raina *et al.*, 1995). Sucrose-resistant and ampicillin-sensitive colonies were retained. Loss of the wild-type *cpxA* gene and replacement by the *cpxA** allele was verified by scoring for resistance to 15 μg/ml amikacin (Weber and Silverman, 1988) and linkage to the marker *clpQ*::ΩCm (Missiakas *et al.*, 1996c).

Disruption of the prpA and prpB genes

To construct a null allele of the *prpA* gene, an ΩCm cassette (Fellay *et al.*, 1987) previously digested at *Bam*HI and blunted, was introduced into the unique *Pst*I site (also blunted using T4 DNA polymerase prior to ligation) of *prpA* coding region as shown in Figure 2. This site is located before the active site of the mature protein. An ΩTet cassette (Fellay *et al.*, 1987) previously digested at *Sma*I, was introduced into the unique *Bst*BI site of *prpB* coding region using plasmid pDM1754 (Figure 2). The *Bst*BI site in *prpB* was blunted using T4 DNA polymerase prior to ligation. Transfer of these null alleles onto the chromosome was performed as described previously (Raina and Georgopoulos, 1990).

RNA isolation, Northern blot analysis and mapping of 5′ termini

Total cellular RNA was isolated by using the hot SDS–phenol extraction procedure (Sambrook *et al.*, 1989). To define the transcriptional start site(s) of the *prpA* gene, ~10 ng of an oligonucleotide probe 5′-CCCGCAATTCTCTGATAAACG-3′, which is complementary to nt positions 17–38 of the *prpA* sequence, was annealed to 10 μg of total cellular RNA. The annealed primer was extended by AMV reverse transcriptase (Promega), essentially as previously described (Raina and

Georgopoulos, 1990). The primer extension products were electrophoresed on the same gel as the dideoxy sequencing reactions, using the same primer.

Purifications of RNAP core, σ^{70} and σ^{32} have been described previously. Run-off transcription experiments were performed as described earlier (Raina *et al.*, 1995). The template used was a linear DNA *Sau*3A–*Pst*I DNA fragment of 659 bp from pDM1695 which contains the promoter region of *prpA*. For the S1 nuclease protection experiments, the same DNA fragment was used as a probe.

For Northern blot analysis of *dnaK*-specific message, probes were made by the random priming technique (Sambrook *et al.*, 1989), using a 508 bp *Eco*RI–*Nru*I fragment prepared from plasmid pDM38 (*dnaK*$^+$ *dnaJ*$^+$; Missiakas *et al.*, 1993), and by radiolabeling with [^{32}P]dCTP (3000 Ci/mmol). Aliquots of 5 μg of RNA isolated from isogenic bacteria carrying either vector pOK12 alone or plasmid pDM1695 (*prpA*$^+$) at 30°C were used for the Northern blot analyses.

Purification of proteins and immunoprecipitation

Escherichia coli bacteria carrying plasmid pDM1574 (*prpA*$^+$) or pDM1757 (*prpB*$^+$) or pSR3040 (λ*nin* ORF221) were induced with 5 mM IPTG at an OD of 0.2 at 600 nm for 5 h. All purification steps were performed at 4°C. Cells were resuspended in buffer A [50 mM Tris–HCl, pH 7.8, 5 mM $MnCl_2$, 5 mM DTT, 0.05 M NaCl, 20% (v/v) glycerol] and lysed by sonication.

PrpA protein pelleted with the membrane fraction

This pellet was resuspended in buffer A with 0.1% Triton X-100 and spun at 15 000 *g* for 45 min, at 4°C. Proteins from the soluble fraction were discarded and the pellet containing aggregated PrpA (~95% of the proteins) was dissolved in buffer A containing 3 M guanidium hydrochloride. Renaturation was done by dilution (20-fold) in buffer A containing 50% glycerol. This solution was spun at 15 000 *g* for 45 min at 4°C to remove the insoluble particles and loaded onto a Q-Sepharose column. Native PrpA protein was eluted with a linear NaCl gradient (0.1–0.6 M) at a concentration of ~0.15 M NaCl.

PrpB and λ-PP proteins were recovered from the soluble fraction of proteins in buffer A (after sonication of cells and centrifugation 15 000 *g*, 45 min, 4°C) and loaded onto Q-Sepharose. Pools containing each of the proteins were further purified onto phenyl–Sepharose column equilibrated in buffer A containing 0.6 M NaCl (without $MnCl_2$). The column was washed and proteins were eluted using a linear gradient of NaCl (0.6–0.01 M NaCl).

Fractions containing purified proteins, as judged by Coomassie Brilliant Blue-stained SDS–PAGE, were pooled, dialyzed against buffer A and used directly for biochemical assays. 100% activity as depicted in Table II refers to specific pNPP phosphatase activities for λ-PP, PrpA and PrpB after purification, i.e. 3500, 3900 and 3800 units/mg, respectively. Protein concentrations were estimated by using the Bradford assay (Bio-Rad). In the case of PrpA, this measured value correlated well with the calculated extinction molar coefficient of 61 500/M/cm as determined from the absorbance spectrum of the fully unfolded protein.

For the immunoprecipitation experiment, after labeling cells were harvested (0.5 units at $OD_{595\ nm}$) and resuspended in 10 mM Tris–HCl pH 8, containing 1 mM EDTA and 2% Triton X-100. After lysis, the supernatants were recovered by centrifugation (18 000 *g*, 30 min, 4°C) and incubated with 50 ml of Affi-gel 10 beads (Bio-Rad) coupled to IgGs purified from an anti-CpxR serum using DEAE–TrysacrylM chromatography.

Phosphatase assay

Standard pNPPase assays were performed in 50 mM Tris buffer pH 7.8 containing 2 mM $MnCl_2$, 2 mM DTT and 10% glycerol, at 25°C for 10 min (or otherwise indicated). pNPP was used as a substrate at a concentration of 20 mM in a 1 ml reaction and the increase of *p*-nitrophenol upon addition of phosphatases was monitored at 405 nm on an Uvikon 940 spectrophotometer.

Dephosphorylation of protein substrates was assayed using either phosphorylated casein or myelin basic protein (MyBP)

Phosphorylated [^{32}P]Ser/Thr-casein and [^{32}P]Tyr-casein were prepared by phosphorylation of α-casein (Sigma) using respectively the catalytic subunit of protein kinase A (Sigma) and pp60$^{c\text{-}src}$ tyrosine kinase (Oncogene Science). In each case, a 1 ml reaction mixture was prepared using 3.2 mg/ml casein. Preparation of [^{32}P]Ser/Thr-casein was performed using 0.5 mM [γ-^{32}P]ATP with 20 mM DTT, 20 mM Mg-acetate in Tris buffer 50 mM pH 7.4. Preparation of [^{32}P]Tyr-casein was performed using 1.5 mM [γ-^{32}P] ATP, 15 mM DTT, 20 mM $MgCl_2$, 0.015% Brij 35, 0.1 mM EDTA in HEPES buffer 50 mM pH 7.5. Either 7 μg of the catalytic subunit of protein kinase A or 20 units of pp60$^{c\text{-}src}$ were added and each independent reaction was incubated at 30°C for 4 h. Phosphorylated casein was recovered by TCA precipitation and extensively dialyzed against a 50 mM Tris buffer pH 7.8, at 4°C.

Dephosphorylation assays of [^{32}P]casein were performed using 0.1–0.6 mg/ml of protein phosphatases in a 1 ml reaction. 100 μl of the mixture were withdrawn at different times and the reaction was quenched by adding TCA (20%). The pellets were washed thoroughly with 10% TCA, and all the soluble materials were pooled and counted for ^{32}P released in the supernatant. The precipitated TCA material was resuspended in a neutralizing buffer (final pH 7) and counted separately.

Phosphorylated [^{32}P]Ser/Thr-MyBP and [^{32}P]Tyr-MyBP were prepared according to the manufacturer's instructions (New England Biolabs) using the catalytic subunit of protein kinase A and Abl protein tyrosine kinase, respectively. Dephosphorylation assays of [^{32}P]MyBP were performed using 0.1–0.6 μg/ml of protein phosphatases in a 0.5 ml reaction. Aliquots of 60 μl of the mixture were withdrawn at different times and the reaction was quenched by adding TCA (20%). Soluble materials were counted for ^{32}P released in the supernatant.

Accession numbers

The accession numbers for the sequences reported in this paper are U51991 and U51682 for PrpA and PrpB proteins, respectively.

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