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A new heat-shock gene, *ppiD*, encodes a peptidyl–prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*

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We have identified a new folding catalyst, PpiD, in the periplasm of *Escherichia coli*. The gene encoding PpiD was isolated as a multicopy suppressor of *surA*, a mutation which severely impairs the folding of outer membrane proteins (OMPs). The *ppiD* gene was also identified based on its ability to be transcribed by the two-component system CpxR–CpxA. PpiD was purified to homogeneity and shown to have peptidyl–prolyl isomerase (PPIase) activity *in vitro*. The protein is anchored to the inner membrane via a single transmembrane segment, and its catalytic domain faces the periplasm. In addition, we have identified by site-directed mutagenesis some of the residues essential for its PPIase activity. A null mutation in *ppiD* leads to an overall reduction in the level and folding of OMPs and to the induction of the periplasmic stress response. The combination of *ppiD* and *surA* null mutations is lethal. This is the first time two periplasmic folding catalysts have been shown to be essential. Another unique aspect of PpiD is that its gene is regulated by both the Cpx two-component system and the σ^{32} heat shock factor, known to regulate the expression of cytoplasmic chaperones.

Keywords: CpxA–CpxR/folding/heat shock/outer membrane/PPIase

Introduction

Bacteria have evolved many adaptive systems in order to cope with various environmental stresses. Among these, heat shock produces a highly conserved response which in *Escherichia coli* is regulated by two alternative sigma factors, σ^{32} and σ^{E}. These two factors govern the transcription of two heat-shock regulons which, respectively, are specialized in coping with protein misfolding in the cytoplasm and the extra-cytoplasm, i.e. periplasm and outer membrane. Protein misfolding in the extra-cytoplasm induces an additional stress response (other than the σ^{E}-dependent stress response), which is regulated by the two component system CpxR–CpxA. The periplasmic protease HtrA, involved in the degradation of misfolded polypeptides, is encoded by a gene which is transcribed both by the $E\sigma^{E}$ polymerase and the CpxR activator (Danese *et al*., 1995; Raina *et al*., 1995). However, *fkpA* which encodes a peptidyl–prolyl isomerase of the FKBP family in the periplasm (Missiakas *et al.*, 1996), belongs to the σ^{E} regulon (Danese and Silhavy, 1997), whereas *dsbA* and *ppiA*, which encode periplasmic thiol:disulfide oxido-reductase and peptidyl–prolyl isomerase respectively, are under the control of the Cpx system (Danese and Silhavy, 1997; Pogliano *et al*., 1997).

Unlike the cytoplasm which contains many ATP-dependent chaperones with wide substrate specificity, the periplasm appears to contain two defined types of folding catalysts. Folding of most translocated proteins encounters two types of rate-limiting steps, which are overcome by two classes of catalysts: protein disulfide isomerase (PDI) and peptidyl–prolyl *cis–trans* isomerase (PPIase). In *E.coli*, there are at least six known Dsb proteins (reviewed by Missiakas and Raina, 1997a). These proteins are involved in the oxidation of disulfide bonds or the rearrangement of wrongly paired disulfides (Bardwell *et al*., 1991; Missiakas *et al.*, 1994; Zapun *et al.*, 1995; Rietsch *et al*., 1996). PPIases are found in both the cytoplasm and the periplasm. They catalyse the rapid interconversion between the *cis* and *trans* forms of the peptide bond Xaa–Pro. Three distinct families of PPIases have been identified so far; these include the cyclophilins (PpiA in the periplasm), FKBP-like proteins (FkpA in the periplasm) and the newly discovered parvulin family. The first member of the parvulin family was identified as a cytosolic *E.coli* protein (Rahfeld *et al.*, 1994), whose gene was later designated *ppiC*. Interestingly, the yeast PpiC homologue, ESS1, is the only essential PPIase for yeast. Surprisingly, yeast mutants lacking all the other 12 PPIases are viable, although some of them are heat-shock inducible (Dolinski *et al*., 1997). In *E.coli*, a second member of the parvulin family, known as SurA, was identified in the periplasm. SurA is involved in the correct folding of outer membrane protein (OMP) monomers. We isolated this gene as a multicopy suppressor of *htrM* (Missiakas *et al*., 1996). *htrM* mutants synthesize altered lipopolysaccharide (LPS), and as a consequence lack most of the OMPs in their outer membrane. This suppression effect is due specifically to correction of the proper outer membrane profile. Also, the accumulation of misfolded OMPs was shown to induce the σ^{E}-dependent response to protein misfolding in the extra-cytoplasm. Overexpression of *surA* was shown to dampen this response in many cases where misfolded polypeptides accumulate, suggesting a general chaperone-like function for SurA. We and others have shown that mutations in *surA* confer severe defects in the amounts or maturation of OMPs (Lazar and Kolter, 1996; Missiakas *et al*., 1996; Rouvière and Gross, 1996). Bacteria lacking *surA* turn on the σ^{E} stress response constitutively (Missiakas *et al.*, 1996; Rouvière and Gross, 1996). Finally, *surA* mutants are extremely sensitive to detergents and hydrophobic drugs such as novobiocin, phenotypes reminiscent to the leakiness of the outer membrane.

In the present study, we took advantage of the hypersensitivity of *surA* mutants to novobiocin and selected for

multicopy suppressors. We identified a new gene and based on its sequence homology to members of the parvulin family, we have designated this gene *ppiD*. *ppiD* was also identified in an independent genetic screen aimed at identifying new members of the CpxR–CpxA regulon.

We show here that *ppiD* encodes a membrane-anchored polypeptide of 623 amino acids, of which the last C-terminal 589 residues are located in the periplasm. Transcriptional analysis of *ppiD* revealed that the gene belongs to two stress regulons: the CpxR–CpxA regulon and the σ^{32} regulon. This is the first member of a periplasmic folding catalyst to be regulated by the classical heat-shock sigma factor σ^{32}.

Results

ppiD is a multicopy suppressor of surA

We have shown previously that a null mutation in *surA* leads to a highly pleiotropic phenotype. One of the defects is hypersensitivity to antibiotics such as novobiocin, due to a higher permeability of the outer membrane. *surA* mutant bacteria do not form colonies in the presence of novobiocin concentrations >10 μg/ml, while isogenic wild-type bacteria grow in the presence of 40 μg/ml of novobiocin (Missiakas *et al*., 1996). We constructed a chromosomal DNA library lacking *surA* and used it to transform the *surA* null mutant. Candidates resistant to 30 μg/ml of novobiocin were selected.

Plasmid DNA from seven such candidates was isolated and shown to breed true by retransforming a *surA* null mutant. The DNA was used to probe the ordered *E.coli* DNA library (Kohara *et al*., 1987). All seven plasmids hybridized to bacteriophage λ 148(3B6) and λ 149(7E2) clones. This corresponds to a genetic map position in the 9.5–10 minute region on the *E.coli* chromosome. This area includes the known serine protease, Lon, and the DNA-binding protein, HupB. DNA from one such plasmid, pSR3239, was used to construct a minimal subclone 2.4 kbp *Bsa*BI–*Sma*I DNA fragment in the p15A-based vector, pOK12. This subclone, pCD51, was found to be sufficient to restore novobiocin resistance to the *surA* null mutant (Figure 1; Table I). Since our initial clone pSR3239 also carries the intact *hupB* gene, we subcloned the 1 kbp *Eco*NI–*Kpn*I DNA fragment which carries the intact *hupB* gene (pCD56), and found that it does not complement a *surA* null mutant (Figure 1). Sequence analyses of pCD51 revealed the presence of a single complete open reading frame (ORF) which encodes a polypeptide of 623 amino acids. Sequence examination also revealed homology to PPIase. Hence, this gene was designated *ppiD*. It carries a putative parvulin-like domain located between amino acids 227 and 357. This predicted amino acid sequence shares 34% identity with an ORF of unknown function from *Haemophilus influenza* (SwissProt accession No. P44092). The next closest homologue is a very recently sequenced ORF of unknown function from *Acinetobacter* (PID entry e1173385), with a sequence identity of 29% over its entire length. PpiD also has sequence similarity to the SurA protein. More precisely, residues 86–121 of PpiD align with residues 65–100 of SurA (44% identity). A second region lying between residues 334 and 357 of PpiD seems to correspond to the parvulin-like domain of SurA (residues 251–274). It is known that SurA has two

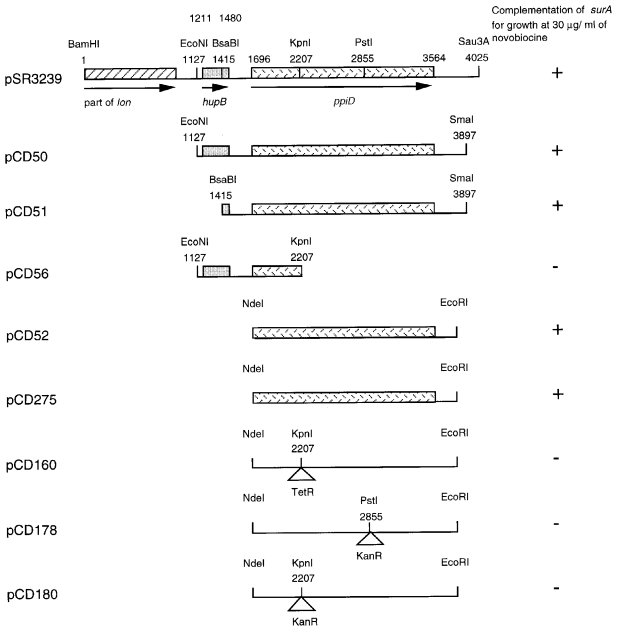

Fig. 1. Restriction map of the *ppiD* gene and surrounding DNA sequences. The different plasmids constructed with either wild-type or the disrupted *ppiD* gene are also shown. Columns on the right indicate the ability of the different clones to confer novobiocin resistance to the *surA* mutant.

very similar parvulin-like domains at its C-terminus. These residues include the motif VGFHIL and are highly conserved among the members of the PpiC (parvulin) family. However, it should be pointed out that the highly conserved residues H and L are replaced by L and V, respectively, in the PpiD amino acid sequence. Also, the predicted amino acid sequence of PpiD shows the presence of a single parvulin-like domain, whereas SurA carries two such domains.

Identification of ppiD as a new member of the CpxR–CpxA regulon

In our effort to understand the nature of the Cpx-dependent stress response, we have constructed a library of transcriptional fusions using the single copy promoter probe vector pFZY (see Materials and methods). We have shown previously that overexpression of the *prpA* gene leads to the constitutive induction of the Cpx pathway (Missiakas and Raina, 1997b). Hence, we looked for promoter fusions, which are highly induced (deeper blue colonies) on X-gal-containing plates, in the presence of a plasmid carrying the *prpA* gene under control of the inducible *lac* promoter (pDM1574). We isolated ~70 such clones and transduced a genetically well characterized *cpxR* null allele (Danese *et al*., 1995) into them. Only those clones which showed a decline in β-galactosidase activity were retained (Figure 2). Fifteen such isolates were retained and plasmid DNA prepared from them was sequenced. Among them, 12 clones were found to contain sequences from the promoter region of the *ppiD* gene.

ppiD is also a bona fide heat shock gene under the control of σ^{32}

To analyse the transcriptional activity and promoter usage of *ppiD*, we constructed a single copy *ppiD–lacZ* fusion

Table I. Complementation of *surA* and comparative resistance to various agents reflecting membrane defects of the *ppiD* mutant as compared with other folding agents

	Colony-forming units/ml						
	LBA	Nov			SDS		
		10	15	20	0.2%	1%	2%
MC4100	10^9	10^9	10^9	10^9	10^9	10^9	10^9
MC4100 *ppiD*::ΩKan	8×10^7	7×10^5	5×10^3	0	8×10^6	5×0^3	0
MC4100 *skp*$^-$	2×10^9	7×10^8	6×10^5	3×10^5	10^9	7×10^7	6×10^5
MC4100 *ppiD*::ΩKan *skp*$^-$	5×10^8	5×10^7	2×10^2	0	3×10^8	2×10^2	0
htrM$^-$	2×10^7	0	0	0	0	0	0
htrM$^-$ (pCD275 *ppiD*$^+$)	3×10^9	5×10^8	5×10^7	2×10^6	5×10^9	2×10^8	2×10^9
surA::ΩKan	3×10^8	10^2	0	0	5×10^3	4×10^3	0
surA::ΩKan (pCD275*ppiD*$^+$)	10^9	9×10^8	6×10^6	5×10^5	8×10^7	8×10^7	5×10^5
surA::ΩKan (pCD273 A350)	5×10^8	10^2	0	0	9×10^3	5×10^3	0

Note that *htrM* mutants do not form colonies on MacConkey agar but *htrM*::Tn*5* transformed with *ppiD*$^+$ (pCD275) fully restored the growth on MacConkey agar plates, particularly at 37°C, and suppressed the mucoid phenotype.

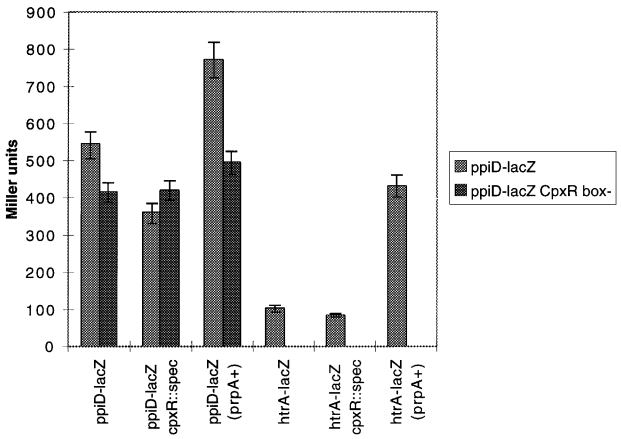

Fig. 2. The transcription of the *ppiD* gene is under the control of the CpxR–CpxA two-component system. The β-galactosidase activity was measured in the wild-type *ppiD–lacZ* fusion or when transduced with a null mutation of *cpxR*. Induction of *htrA* transcription serves as an internal control. The shaded bars show the β-galactosidase activity from the *ppiD–lacZ* fusion deleted for the region containing the three potential CpxR-binding boxes.

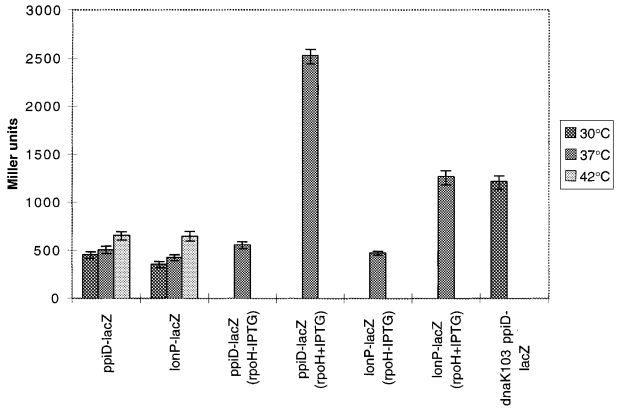

Fig. 3. The *ppiD* is also a heat-shock gene under the control of σ^{32}. Cultures of isogenic bacteria carrying single copy fusions to either the promoter of *ppiD* or the *lon* gene were tested for β-galactosidase activity at different temperatures. These fusions were transformed with *rpoH*$^+$ plasmid with the inducible *lac* promoter (pSR1332) grown in minimal medium supplemented with 0.2% glucose up to an OD of 0.2; aliquots were treated with or without the *lac* inducer IPTG (1 mM) for 20 min. The β-galactosidase activity was measured as described above. The last lane represents, as a further control, the β-galactosidase activity from a *ppiD–lacZ* fusion in a *dnaK*103 background. Each sample was assayed for β-galactosidase activity four times, and the data presented are the average of three independent experiments.

using the promoter region of *ppiD* and the λRS45 and pRS550 vectors (Simons *et al*., 1987). Using this fusion, we observed that *ppiD* transcription is heat-shock inducible (Figure 3), which is reminiscent of classical heat-shock gene induction. The single copy *ppiD–lacZ*-carrying strain (CD212) was transformed with a vector carrying either the *rpoH* gene under the control of an inducible promoter (pSR1332; Missiakas *et al.*, 1993a) or the *rpoE* gene (pSR1628; Raina *et al.*, 1995). As shown in Figure 3, induction of the *rpoH* gene product from pSR1332 produces a 10-fold increase in β-galactosidase activity using the *ppiD–lacZ* fusion, whereas no induction is observed with the *rpoE*$^+$ plasmid (pSR1628). Furthermore, no reduction is observed in the presence of the *rseA*$^+$-carrying plasmid (pSR2661; Missiakas *et al.*, 1997) which encodes the specific anti-σ^E factor, which in high dosage represses the transcription from σ^E-dependent promoters (Table II).

It is known that mutation in *dnaK*, *dnaJ*, *grpE* or *htrC* leads to a constitutive elevated induction of the σ^{32} heat-shock response. The *ppiD–lacZ*-carrying strain was transduced into the *dnaK*103 mutant. As can be seen in Figure 3, the β-galactosidase expression is highly induced.

Table II. *ppiD* transcription is not affected by overexpression of either *rpoE* or its specific anti-sigma factor *rseA*

	β-galactosidase activity (Miller units)
ppiD–lacZ	515 ± 25
ppiD–lacZ (p*rpoE*$^+$)	595 ± 33
ppiD–lacZ (p*rseA*$^+$)	505 ± 23
htrA–lacZ	123 ± 10
htrA–lacZ (p*rpoE*$^+$)	945 ± 72
htrA–lacZ (p*rseA*$^+$)	23 ± 7

Taken together, the data clearly show that *ppiD* has another promoter in addition to its CpxR–CpxA-dependent promoter, and that this promoter is under σ^{32} control. This is the first example so far of a periplasmic folding catalyst placed under the control of σ^{32}.

Identification of the heat-shock protein PpiD on two-dimensional gels

The PpiD protein has not up to now been identified as a heat-shock protein on two-dimensional gel isoelectric focusing gels. The reasons could be that its molecular weight coincides with that of HtpG and DnaK (all are ~70 kDa in size). In addition, the predicted pI of HtpG is 5.09 and that of PpiD is 4.95. PpiD, being an inner membrane protein, may migrate differently somehow, perhaps overlapping HtpG on these gels. In fact, in many of our earlier two-dimensional heat-shock gels, we did observe a protein induced by a 50°C heat shock which runs close to HtpG and DnaK. Hence, we constructed two isogenic strains (*ppiD*::ΩTet *htpG*$^+$ and *ppiD*::ΩTet Δ*htpG*) and compared the global heat-shock profiles of cultures of these strains after a sudden 50°C heat shock for 5 min. Samples were analysed by two-dimensional gel electrophoresis. As can be seen in Figure 4, there is a clear induction of an ~70 kDa polypeptide in the *ppiD*$^+$ *htpG*$^+$ sample (Figure 4A) but not in that of *ppiD*::ΩTet Δ*htpG* (Figure 4C). As a control for the spot identified as PpiD, we also added [^{35}S]methionine-labelled PpiD to heat-shock extracts from *ppiD*::ΩTet Δ*htpG* (Figure 4B). This clearly indicates that PpiD is a heat-shock protein and accumulates at a temperature of 50°C. The positions of other heat-shock proteins such as GroEL and DnaK are also shown in Figure 4B.

Acetyl phosphate and the cpxA mutations influence the transcription of ppiD*

We further examined the CpxR–CpxA transcriptional dependence of the *ppiD* gene. It is known that the level of acetyl phosphate modulates the activation or repression of two-component systems such as CpxR–CpxA. Thus we determined the β-galactosidase activity of *ppiD–lacZ* under different growth conditions which affect the level of acetyl phosphate *in vivo*. It is known that the use of pyruvate as a carbon source results in the maximum level of intracellular acetyl phosphate. Indeed, a 3-fold activation of the *ppiD* promoter can be obtained when cultures are shifted from glycerol to pyruvate medium (Figure 5).

We have also used the SR3570 strain carrying a *cpxA** mutation in which T252 is changed to R252 (Missiakas and Raina, 1997b). This chromosomal allele leads to a gain of function of CpxA, presumably a hyperkinase activity (Missiakas and Raina, 1997b). This mutation is similar to a gain-of-function mutation in *envZ*12 (Aiba *et al*., 1989). We transduced the *ppiD–lacZ* fusion into *cpxA** mutant SR3570, resulting in CD220. This strain again showed a 2- to 3-fold induction of *ppiD–lacZ* activity (Table III). In control experiments we transduced a *htrA–lacZ* fusion into the same *cpxA** mutant (Table III). The increased *ppiD–lacZ* expression is in fact more than that observed for the *htrA–lacZ* fusion. These results further confirm that the transcription of *ppiD* is in part regulated by the two-component system, CpxR–CpxA.

Mapping of ppiD transcriptional start sites

In order to understand the transcriptional regulation of *ppiD*, we determined the transcriptional initiation site(s) under different growth conditions and in different genetic backgrounds. RNA was extracted from bacterial cultures grown either at 30°C or following a brief shift to 50°C. Using the primer extension technique, the transcriptional start sites were mapped. As can be seen in Figure 6, lane 2, using RNA from those cultures subjected to the 50°C

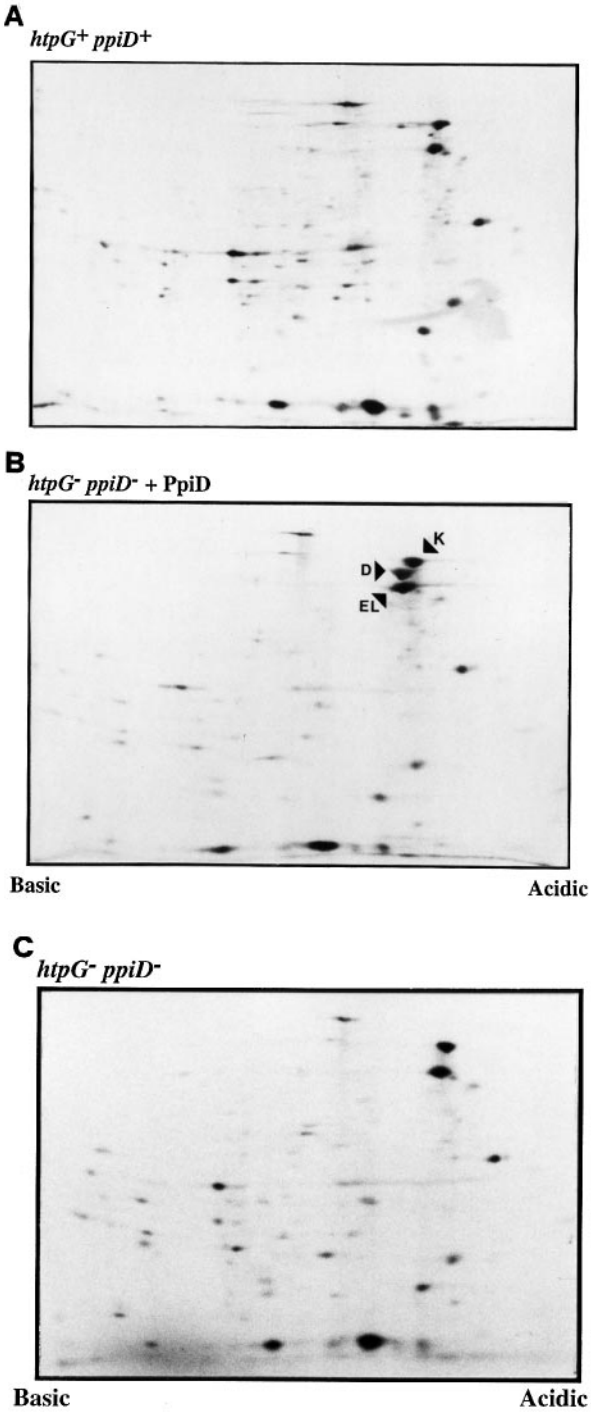

Fig. 4. Identification of the PpiD protein as a heat-shock protein by two-dimensional electrophoresis. Cultures of isogenic strains wild-type (**A**), *ppiD*::ΩTet *htpG*$^-$ + *PpiD* (**B**) and *ppiD*::ΩTet Δ*htpG* (**C**) were grown at 30°C, shifted to 50°C for 5 min, then labelled for another 5 min with [^{35}S]methionine (50 μCi/ml). 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. For the second dimension, samples were electrophoresed on a 12.5% SDS gel. Autoradiograms of the relevant portions of dried gels are shown. The arrow marked D point to the position of PpiD, and K and EL indicate the positions of 70 kDa DnaK and 63 kDa GroEL heat-shock proteins.

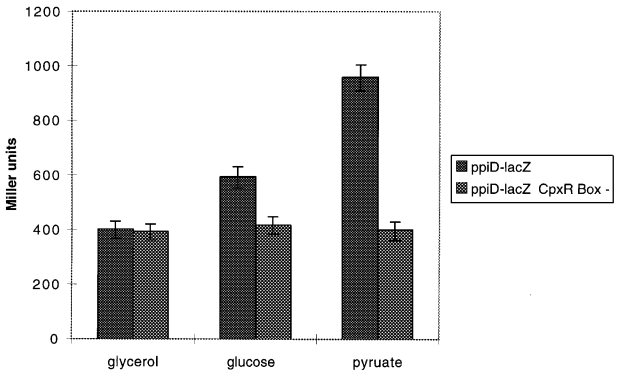

Fig. 5. Critical role of the CpxR-binding domain and the levels of acetyl phosphate in the transcription of the *ppiD* gene. Cultures of isogenic strains CD212, carrying the wild-type full-length *ppiD–lacZ* fusion, and CD221, carrying *ppiD–lacZ* fusion with deletion of the three putative CpxR boxes, were used for these experiments. Cultures were grown in regular LB medium up to an OD of 0.5 at 595 nm. Cultures were spun, washed, diluted 1:100 and transferred to minimal medium supplemented with different carbon sources such as glycerol, glucose or pyruvate and allowed to reach an OD of 0.2 at 595 nm.

Table III. CpxR–CpxA-dependent regulation of the *ppiD* gene[a]

	β-galactosidase activity (Miller units)
CD212 = MC4100 *ppiD–lacZ*	510 ± 23
CD215 = CD212*cpxA**	1420 ± 72
SR1458 = MC4100 p*htrA–lacZ*	110 ± 7
CD220= p*htrA–lacZ cpxA**	177 ± 12

[a]The hyperkinase CpxA* induces its transcription.
All measurements were performed in triplicate and the averages are presented.

shift, two transcriptional start sites designated as Phs1 and P* could be mapped 75 and 83 nucleotides, respectively, upstream of the translational initiation start site. No corresponding signal is seen from RNA extracted at 30°C, which clearly indicates that transcription from the start site designated as Phs1 is heat-shock inducible. The –10 and –35 regions upstream of this start site show a perfect match with the σ^{32}-regulated promoters (for the –10 box CCCC and for the –35 box CTTGTG, to be compared with the consensus CTTGAA). In addition, the spacing between the two canonical boxes is 15 nucleotides, a feature common to most of the σ^{32}-regulated promoters. We do not know at present which sigma factor is responsible for the transcription initiating at nucleotide 83 (P*). The two additional start sites located at nucleotides 85 and 93 appear only in the RNA extracted at 30°C (lane 3) and not at 50°C. Thus, they are not heat-shock induced and may correspond to σ^{70}-coupled CpxR–CpxA-dependent transcription. Examination of the sequence shows at least three conserved CpxR boxes at positions –261 (GGTAAAGAG), –221 (GGTAAGC) and –209 (GGTAACT) (Figure 7) upstream of the translational initiation codon.

Deletion of the putative CpxR boxes leads to a decline in ppiD transcription

As mentioned above, there are three putative CpxR-binding boxes (Figure 7) similar to what has been described

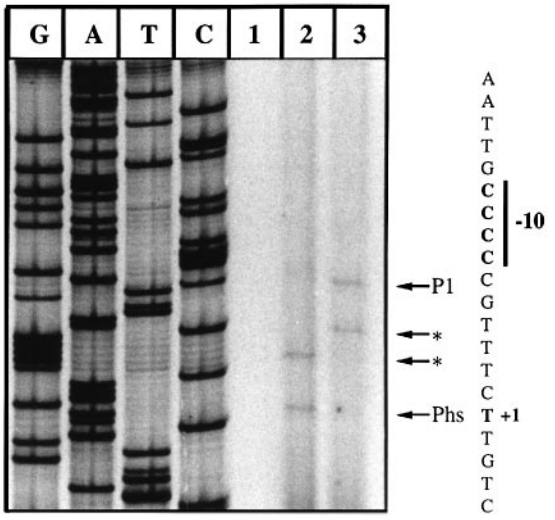

Fig. 6. Mapping of *ppiD* transcriptional start sites. Primer extension reactions of total cellular RNA hybridized to a ^{32}P end-labelled oligonucleotide probe, complementary to nucleotides –5 to 17 of the *ppiD* sense strand. The annealed primer was extended by AMV reverse transcriptase. RNA was extracted from wild-type MC4100 bacteria grown at 30°C (lane 3), or shifted to 50°C for 10 min (lane 2). Lane 1 represents a control annealed RNA plus primer without addition of AMV reverse transcriptase. Lanes labelled G, A, T and C correspond to the dideoxy sequencing reactions carried out using the same oligonucleotide as the primer.

for *dsbA* (Pogliano *et al.*, 1997). To assay their role *in vivo*, we removed the first 147 nucleotides from plasmid pCD189 by digestion with *Bam*HI and *Fok*I, resulting in plasmid pCD191 (Table IV). This removes all of the three putative CpxR-binding boxes. This promoter fusion still carries the σ^{32}-regulated promoter Phs1, as well as 43 nucleotides upstream of it. This promoter fusion was analysed and shows an overall decline in promoter strength (Figure 2). It no longer responded to changes in the level of acetyl phosphate (compare *ppiD–lacZ* and *ppiD–lacZ* CpxR boxes$^-$ in Figure 2). Also, introduction of the *cpxR* null allele did not change the *ppiD* promoter activity. Furthermore, this promoter fusion is not induced by overexpression of the *prpA* gene product, but is still subject to regulation by σ^{32}, like the original fusion construct CD212. This further confirms that *ppiD* transcription is in part regulated by the two-component system CpxR–CpxA, and that the whole nucleotide region deleted contains the CpxR-binding boxes which are important in its regulation.

Identification, purification and localization of the PpiD protein

We constructed a minimal subclone of *ppiD*, pCD52, by PCR in the pET24a expression vector with a T7 promoter. This plasmid was first sequenced and then shown to complement *ppiD* fully and to suppress *surA* mutant bacteria. Expression of the protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and, consistent with the predicted mass of the deduced amino acid sequence, was shown to be a protein of ~69 kDa (Figure 8). Examination of the PpiD amino acid sequence predicted it to be an inner membrane protein, with a single transmembrane domain spanning amino acid residues 16–34 with its N-terminus in the cytoplasmic membrane. The rest of

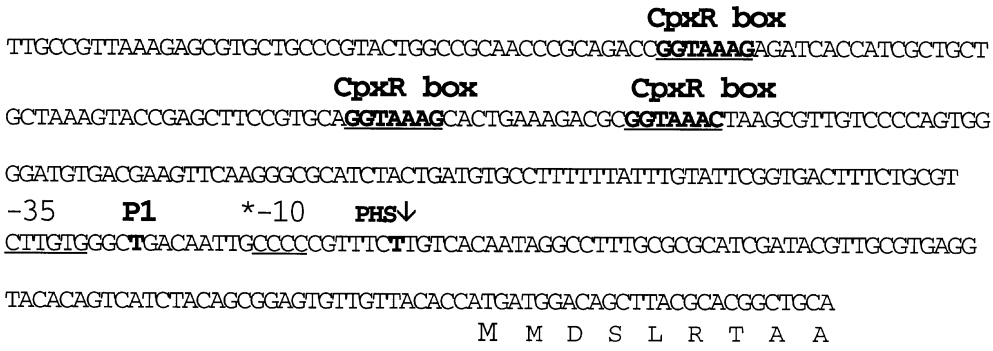

Fig. 7. Nucleotide sequence of the promoter region of the *ppiD* gene. The initiation start designated as PHS corresponds to the promoter under σ^{32} control. The corresponding –10 and –35 regions are underlined. The other initiation site designated P1 corresponds to one of the start sites resulting from primer extension of RNA isolated at 30°C. The three putative CpxR-binding boxes are highlighted in bold and underlined.

Table IV. Bacterial strains and plasmids

	Relevant characteristics	Reference or source
Strains		
CA8000	HfrH *thi*	our collection
JB23	Δ*htpG*	Bardwell and Craig (1988)
MC4100	F$^-$ *araD*139 Δ(*argF-lac*) U169	our collection
SR21	CA8000 *htrM* :: Tn*5*	Raina and Georgopoulos (1991)
SR3205	MC4100 *surA*::ΩKan^r	Missiakas *et al.* (1996)
SR3570	MC4100 *cpxA** (T252R)	Missiakas and Raina (1997b)
CD63	SR3205 (pCD51, *ppiD*$^{+)}$	this study
CD162	MC4100 *ppiD*::ΩTet at the *Kpn*I site	this study
CD212	MC4100 ϕ(*ppiD-lacZ*)	this study
CD213	CD212 transduced into SR1458	this study
CD214	CD212 transduced into SR2195	this study
CD215	MC4100 ϕ(*ppiD-lacZ*) *cpxA**	this study
CD220	SR1458 *cpxA**	this study
CD221	CD212 deleted for the three CpxR boxes	this study
CD247	MC4100 *ppiD*::ΩKan at the *Kpn*I site	this study
CD249	MC4100 *ppiD*:ΩKan at the *Pst*I site	this study
CD259	CD247 transduced into SR1421	this study
CD261	CD247 transduced into SR1359	this study
CD269	SR3205 *surA*::ΩKan^r (pDM154 *surA*$^+$)	this study
CD270	CD162 *ppiD*::ΩTet (pCD275)	this study
Plasmids		
pKO3	allelic replacement vector	Link *et al.* (1997)
pFZY1	promoter probe vector	Koop *et al.* (1987)
pDM1554	pSK *surA*+	Missiakas *et al.* (1996)
pSR1232	pTTQ *rpoH*$^+$ (1.3 kbp *Eco*RV)	Missiakas *et al.* (1993a)
pSR1574	pSE420 *prpA*$^+$ (721 bp *Nde*I–*Bam*HI)	Missiakas and Raina (1997b)
pSR1628	pOK12 *rpoE*$^+$ (1.4 kbp *Sau3*A)	Raina *et al.* (1995)
pSR1865	pOK12 *dsbA*$^+$ (1.8 kpb *Sau3*A)	Missiakas *et al.* (1993b)
pSR2661	pKO12 *rseA*$^+$ (1210 bp *Hind*III–*Msc*I)	Missiakas *et al.* (1997)
pSR3239	pOK12 carrying a 4 kbp *ppi*D$^+$ DNA fragment	this study
pCD50	pOK12 carrying the 2.8 kbp *Eco*NI–*Sma*I *ppiD*$^+$	this study
pCD51	pOK12 carrying the 2.5 kbp *Bsa*BI–*Sma*I *ppiD*$^+$	this study
pCD52	pET24-a carrying the minimal *ppiD*$^+$coding sequence (1.9 kbp *Nde*I–*Eco*RI)	this study
pCD56	pOK12 carrying the 1.1 kbp *Eco*NI–*Kpn*I *hupB*$^+$	this study
pCD57	pAED-4 carrying the minimal *ppiD*$^+$coding sequence	this study
pCD160	pCD57 with an ΩTet inserted at the *Kpn*I site	this study
pCD174	pCD57 with an ΩKan inserted at the *Kpn*I site	this study
pCD175	pKO3 with an ΩKan inserted at the *Pst*I site	this study
pCD176	pKO3 with an ΩTet inserted at the*Kpn*I site	this study
pCD189	pRS550 (p*ppiDlacZ*) carrying 360 nucleotides of the *ppiD* promoter	this study
pCD191	pCD189 first 147 nucleotides containing the CpxR boxes deleted	this study
pCD271	pCD57 carrying mutation G347 to A	this study
pCD273	pCD57 carrying mutation I350 to A	this study
pCD275	pSE420 carrying the minimal *ppiD*$^+$coding sequence	this study

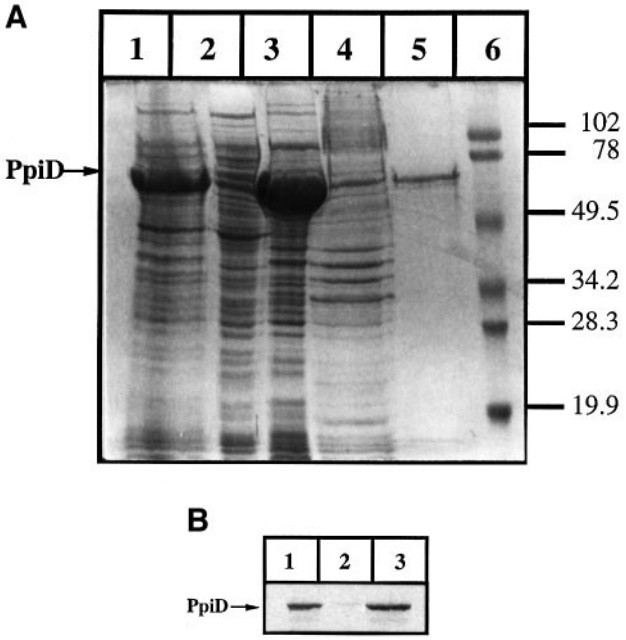

Fig. 8. (**A**) Purification of PpiD. Cultures of strains carrying plasmid pCD51 in which the *ppiD* gene is under the transcriptional control of the T7 polymerase promoter were grown at 37°C in M9 minimal medium. Expression of the T7 RNA polymerase was induced by addition of IPTG (1 mM) for 2 h. Lane 1 is the total extract from induced bacteria carrying plasmid pCD51, lane 2 represents the soluble fraction, lane 3 represents the inner membrane fraction after extraction with sarkosyl, lane 4 contains the aggregated and outer membrane proteins not released by sarkosyl, and lane 5 contains purified PpiD protein after gel filtration and further purification on MonoQ (FPLC). Proteins were resolved by SDS–PAGE (12.5% acrylamide), and a Coommasie Blue-stained dried gel is shown. (**B**) For the immunoprecipitation experiments, a 10 ml culture of wild-type bacteria was labelled with [^{35}S]methionine (100 μCi/ml) for 10 min and fractionated as above. Lane 1 is PpiD immunoprecipitated from total cell extracts, lane 2 represents soluble fraction and lane 3 corresponds to the inner membrane fraction.

the polypeptide (amino acids 35–623) is predicted to be in the periplasm. The overexpressed PpiD protein was indeed found to localize to the inner membrane (Figure 8A) and could be released either upon Triton X-100 (1%) or sarkosyl (0.2%) treatment. The solubilized membrane fraction containing PpiD was first purified by gel filtration followed by an anion exchange chromatography. The purified protein was eluted with a linear gradient of KCl (0.05–1 M) containing 0.2% NP-40.

The topology was confirmed further by constructing random Tn*phoA* fusions (Manoil and Bailey, 1997). It is known that alkaline phosphatase is active in the periplasm only under exponential growth conditions in wild-type bacteria (Derman and Beckwith, 1991). We sequenced the junctions between six randomly chosen AP$^+$ PhoA fusions and found them to be located at residues 115, 223, 229, 309, 437 and 453. As further proof, we inserted the AP$^+$ cassette (Gutierrez and Devedjian, 1989) at the unique *Pst*I site in the *ppiD* gene. This site is located in the vicinity of the PpiC-like domain present in PpiD. Like the other six AP$^+$ fusions tested, this one was also found to be AP$^+$. In order to verify that the N-terminal part is located in the cytoplasm, we inserted an AP$^+$ cassette after the sixth amino residue. This fusion was found to be AP$^-$. Quantitative measurement of alkaline phosphatase activity in this fusion was 55 U versus >2000 U for periplasmic PhoA fusions. Taken together, all these data confirm that PpiD is located in the inner membrane, with the bulk of it facing the periplasm, including its parvulin-like domain.

More conclusively, the membrane localization of PpiD from wild-type bacteria was finally confirmed by immunoprecipitation, using total cell extracts, or the soluble or membrane fraction. As can be seen, most of the PpiD could be immunoprecipitated from either the inner membrane fractions or the total cell extracts only (Figure 8B). Thus, PpiD is clearly an inner membrane protein, since these experiments were carried out at the single copy level without any overproducing plasmid.

Construction of null alleles of ppiD

To study the *in vivo* role of *ppiD*, we constructed two null alleles, using the ΩKan cassette. One allele was constructed using plasmid pCD57 by inserting either the ΩKan or ΩTet cassette at the unique *Kpn*I site (this site is located within the 5′ region of the *ppiD* coding sequence). The second allele was constructed by inserting the ΩKan cassette at the *Pst*I site (Figure 1). These alleles were transferred to the chromosome as described by Link *et al*. (1997), and transferred to various *E.coli* backgrounds using bacteriophage P1 transduction.

As expected, all these null alleles were found to be >90% linked to the *hupB* gene which is located just upstream of *ppiD*. Since *ppiD* is heat-shock regulated, we tested whether its product is required for bacterial growth at high temperatures. No noticeable growth difference was observed between the *ppiD* null mutant and the isogenic wild-type for temperatures ranging from 30 to 43°C. A weak temperature-sensitive phenotype was obtained when bacteria were plated at >43.5°C.

Phenotypic defects associated with ppiD null bacteria

Since the *ppiD* gene was cloned originally as a multicopy suppressor of the *surA* null mutation and the predicted amino acid sequence analyses show that they share some sequence homology, we examined the possibility that these two genes encode overlapping activities. We found that similarly to *surA* mutants, *ppiD* null mutants are hypersensitive to hydrophobic antibiotics such as novobiocin (>20 μg/ml), and to detergents such as SDS (>2%) (Table I). These phenotypes are often associated with membrane defects. The sensitivity to novobiocin and SDS is less drastic than that observed for bacteria carrying a null mutation in *surA* (Missiakas *et al*., 1996), but more pronounced than that observed for isogenic bacteria carrying a null mutation in the *skp* gene. The *skp* gene product is also needed for membrane integrity and maturation of OMPs (Chen and Henning, 1996; Missiakas *et al*., 1996). Interestingly, growth of a double *skp ppiD* null mutant was inhibited in the presence of 15 μg/ml of novobiocin and exhibited an increased sensitivity towards SDS (Table I). Also, all the three null alleles of *ppiD* accumulated extragenic suppressors at a very high frequency (>10^{-2}).

Synthetic lethality of ppiD and surA null mutations

Because *ppiD* in multicopy suppresses the phenotypes associated with a *surA* mutant, and since mutations in

either of the genes have defects in the outer membrane, we decided to examine the phenotypes of the double *ppiD surA* null mutant. However, repeated attempts to construct such a double null mutant failed. Hence, we reasoned that such a combination must be synthetically lethal. To confirm this, we showed that we could only cross the *ppiD* null allele into a strain CD269 which carries the *surA* gene cloned onto a plasmid. A converse set of experiments gave the same results, i.e. a *surA* null allele can be transduced into strain CD270 since it carries *ppiD* on a plasmid (Table IV). In this plasmid, the *ppiD* gene is under P*lac* control and carries the *lac* repressor gene *lacIQ* on the plasmid. The *surA*::ΩKan transductants could be obtained only in the presence of IPTG. Hence, these genetic experiments proved convincingly that the *ppiD* and *surA* mutant combination exhibits a synthetic lethal phenotype. These results are interesting when compared with the requirement for PPIases in other organisms. Yeast has 12 PPIases and none of them is essential for growth; in fact yeast remains viable even when all these 12 genes are deleted (Dolinski *et al*., 1997). However, there is an additional PPIase which is the only member of the PpiC family, ESS1, and its gene is an essential gene in yeast. In *E.coli*, the presence of two such homologues in the same compartment, SurA and PpiD, would explain why neither of them is essential for growth, although they have individual pleiotropic phenotype defects. These results may also explain the high occurrence of extragenic suppressors, which probably dampens the phenotypic defects of *ppiD* null mutant bacteria.

The synthetic lethality due to loss of both *surA* and *ppiD* function is unique. *Escherichia coli* has two more PPIases in the periplasm, namely FkpA and RotA. Double null mutants of *fkpA*::ΩTet *ppiD*::ΩKan as well as *rotA*::ΩKan *ppiD*::ΩTet are easily obtained (Table I). Thus, *surA* and *ppiD* gene products must have many overlapping functions as well as complementary functions, and may have some common essential substrate(s) which can account for the synthetic lethal phenotype.

Lack of ppiD leads to a constitutive induction of the σ^E-dependent heat-shock response

It is now well established that the absence of various folding catalysts in the periplasm induces the σ^E stress regulon, due to the accumulation of misfolded transported proteins (Missiakas *et al*., 1996; Missiakas and Raina, 1997a,b). If the *ppiD* gene product is indeed needed for folding of some periplasmic or outer membrane proteins, we should expect a null mutation to have increased Eσ^E activity. The *ppiD* null alleles were transduced into different Eσ^E promoter fusion-carrying strains. As can be seen (Figure 9), there is an ~3-fold induction of Eσ^E activity as reflected by the induction of β-galactosidase activity from different promoters tested (*htrA–lacZ*, *rpoE*P2–*lacZ* and *rpoH*P3–*lacZ*) as compared with isogenic wild-type strains. Also, a further additive effect is observed in a double *skp* and *ppiD* null combination. Furthermore, a combination of a *ppiD* null allele with a *htrA* mutation also leads to a further additive effect (Figure 9). This is quite similar to what we observed earlier by combining mutations of different catalysts of periplasmic folding with a *htrA* mutation (Raina *et al*., 1995). Since *ppiD* is a heat-shock gene with one of its promoters under control of the *rpoH*, we also transduced *ppiD* null alleles into different promoter fusion backgrounds which are under σ^{32} transcriptional control, such as *groEL–lacZ* (CD261) and *htpG–lacZ* (CD259). The results clearly show that neither of the two Eσ^{32} promoters are induced (Table V). Thus, lack of *ppiD* gene product induces only the Eσ^E-dependent periplasmic unfolding response but not that of Eσ^{32}-transcribed genes. Thus, protein misfolding associated with the *ppiD* null mutant bacteria should be specific for extra-cytoplasmic proteins.

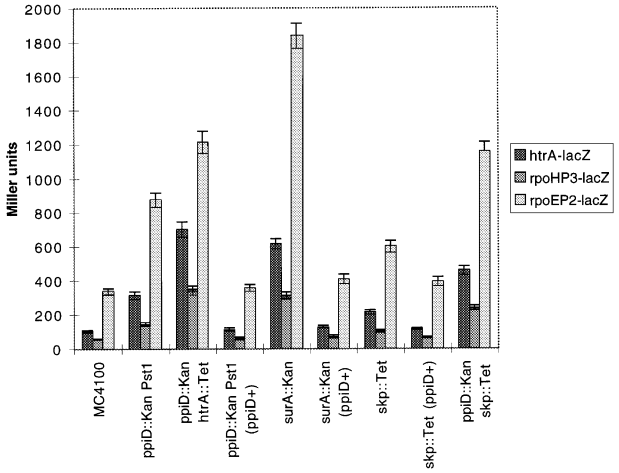

Fig. 9. The periplasmic unfolding stress response is induced in *ppiD* mutants, and overexpression of *ppiD* can suppress such a response in the absence of the other catalyst of folding in the periplasm. Isogenic cultures of bacteria carrying fusions to different *rpoE*-regulated promoters in the presence of a null mutation in either the *ppiD*, *surA* or *skp* genes were analysed for β-galactosidase activity.

Table V. Null mutations in *ppiD* do not affect classical heat-shock regulation

	β-Galactosidase activity (Miller units)
groEL–lacZ	915 ± 77
groEL–lacZ ppiD::ΩKan	877 ± 84
htpG–lacZ	363 ± 42
htpG–lacZ ppiD::ΩKan	343 ± 45

The β-galactosidase activities of *lacZ* operon fusions to the the *groEL* and *htpG* promoters in different backgrounds are given.

ppiD null mutants exhibit altered OMPs profiles

The increased detergent sensitivity, high basal Eσ^E activity and synthetic lethality observed with *surA* suggested that there must be a requirement for PpiD for the outer membrane biogenesis or maturation of OMPs. Hence, we analysed the total membrane profiles of wild-type as compared with isogenic *ppiD* null mutant bacteria (CD247). The inner and outer membranes proteins were separated using sucrose gradients, as previously described (Missiakas *et al*., 1996). The protein profiles obtained were quite different (Figure 10). The amounts of all three major OMPs were reduced, i.e. OmpF, OmpC and OmpA. As a control, we also used a *surA* null mutant under similar experimental conditions. Although the amount of OMPs is reduced in both *surA* and *ppiD* mutant bacteria, there are some differences. For example, as shown in

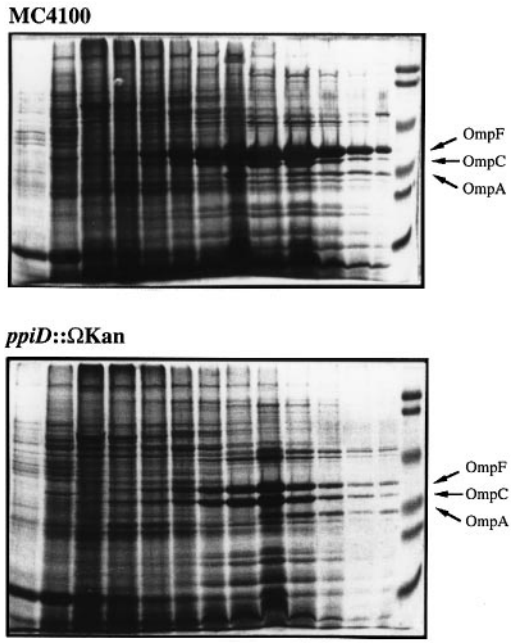

Fig. 10. The outer membrane is altered in the *ppiD* null mutant. Profiles of inner and outer membrane proteins from sucrose gradients (15–50%) analysed from isogenic wild-type strain MC4100 (upper panel) and a *ppiD*::ΩKan (lower panel). The major porins OmpF, OmpC and OmpA are indicated by arrows.

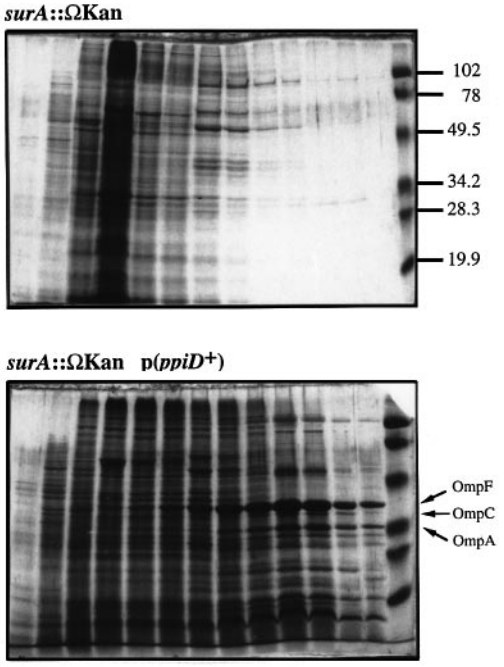

Fig. 11. Complementation of outer membrane defects in *surA* by multicopy *ppiD*. Profiles of inner and outer membrane proteins were analysed as described in Figure 10. Profiles from a *surA* mutant were compared with mutants transformed with a plasmid carrying *ppiD*$^+$ (lower panel). The major porins OmpF, OmpC and OmpA are indicated.

Figure 11, the OMP pattern is affected much more severely in a *surA* null mutant than in a *ppiD* null, although the overall amount of OMPs is significantly reduced in the *ppiD* null mutant.

We also examined the state of maturation of LamB (Figure 12, lanes 1–3). There was an overall reduction in the amounts of LamB in the *ppiD* mutant. As a control, we used a known periplasmic folding catalyst DsbC whose maturation is unaltered in a *ppiD* mutant (Figure 12).

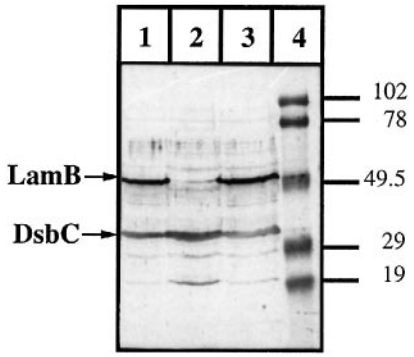

Fig. 12. Defective folding status of LamB porin in a *ppiD* mutant. Lane 1, samples from wild-type MC4100 bacteria; lane 2, bacteria carrying *ppiD*::ΩKan; and lane 3, a *ppiD*::ΩKan-carrying *ppiD*$^+$ plasmid. The positions of LamB and DsbC are indicated. Proteins were transferred to a nitrocellulose filter and probed with antibodies raised against LamB and DsbC.

Complementation of outer membrane defects in surA and htrM mutants by multicopy ppiD

We also analysed the effect of overproduction of PpiD in a *surA* mutant background. As shown in Figure 11, there is a near complete restoration of normal levels of OMPs upon overexpression of *ppiD*. This further supports our earlier finding that overproduction of PpiD compensates for the membrane leakiness of *surA* mutants and restores resistance to novobiocin and SDS.

Also, overexpression of *ppiD* leads to a decrease in Eσ^E activity which is induced abnormally in *surA* mutants (Figure 9). The same is true for the *ppiD*-induced Eσ^E response: overexpression of *surA* restores the normal level of Eσ^E activity in a *ppiD* null mutant. Again, these data suggest that both *surA* and *ppiD* perform similar functions in terms of protein folding. Nevertheless, they must have some specific substrates as well, which would account for the synthetic lethality observed.

We previously identified the *surA* gene as a multicopy suppressor of *htrM* (*rfaD)* mutant bacteria (Missiakas *et al*., 1996). *htrM* (*rfaD)* encodes an ADP-l-glycol-D-mannoheptose-6-epimerase (Raina and Georgopoulos, 1991) and participates in LPS biogenesis. LPS is needed for folding of OMPs (Schnaitman and Klena, 1993; Sen and Nikaido, 1993), and a *htrM* mutant which synthesizes an altered LPS exhibits extremely low amounts of OMPs and, as a consequence, has an altered outer membrane (Missiakas *et al*., 1996). Because of their leaky outer membrane, *htrM* mutants are unable to grow on MacConkey agar containing bile salts and are resistant to a variety of bacteriophages such as λ or P1. Overproducing SurA was found to restore both the growth on MacConkey agar, and the normal levels and folding of OMPs including LamB, which serves as a receptor for bacteriophage λ.

In the present study, we examined whether overexpression of *ppiD* could also complement *htrM* mutant bacteria. Interestingly, we found that multicopy *ppiD* (using *ppiD* under the control of an inducible promoter pCD275 or its own promoter pCD51) allowed the growth on MacConkey agar and restored the sensitivity to different bacteriophages

Table VI. Multicopy suppression of bacteriophage adsorption defects of *htrM* mutant bacteria by overexpression of *ppiD*

	iλ h $^{\lambda}$	P1
CA8000	100	100
CA8000 *htrM*	3×10^{-2}	7×10^{-2}
CA8000 *htrM* (pCD275 *ppiD*$^{+}$)	66	72
CA8000 *htrM* (pDM1554 *surA*$^{+}$)	37	45

Table VII. The substrate specificity of PpiD and PpiD active site mutant variants in comparison with SurA

Xaa	PpiD	PpiDG347A	PpiDI350A	SurA
Ala	1060	1.1	0.7	7.8
Gly	630	0.9	1.0	3.1
Leu	2310	7.0	4.0	15.7
Val	740	2.1	1.6	9.2
Ile	636	1.7	1.4	7.8
Trp	430	0.9	0.9	3.6
Phe	730	1.1	0.6	6.1
His	930	0.9	0.7	7.3
Glu	3400	1.2	0.9	12.3
Lys	520	0.9	0.7	6.3

The values presented are k_{cat}/K_m (/μM/s).
The PPIase assay was carried out at 4°C using the substrate Suc-Ala-Xaa-Pro-Phe-p-Na dissolved in 460 mM LiCl. The measurements were taken in 35 mM HEPES pH 7.8.

such as λ and P1 (Tables I and VI). This complementation was best observed at 37°C or above. Our previous attempts at looking for multicopy suppressors for *htrM* mutants were all performed at 30°C using low copy number plasmids and, therefore, did not allow the identification of *ppiD*. We now know that *ppiD* is a heat-shock gene and that its transcription is controlled by Eσ^{32}.

PpiD has peptidyl–prolyl isomerase activity in vitro, and active site mutagenesis

Preliminary examination of the amino acid sequence of PpiD revealed the presence of a single parvulin (PpiC)-like domain located between amino acid residues 227 and 357. This parvulin-like domain is repeated twice in SurA. The presence of such a domain in PpiD could explain why *ppiD* could be isolated as a multicopy suppressor of *surA*. SurA has been shown to have a very weak PPIase activity *in vitro* (Missiakas *et al*., 1996; Rouvière and Gross, 1996). We examined the activity of purified PpiD using various Suc-Ala-Xaa-Pro-Phe-p-Na substrates. These experiments provided good evidence that PpiD can catalyse the rotation around the peptidyl–prolyl bond Ala–Pro of the tetrapeptide with greater efficiency than SurA. The estimated K_{cat}/K_m value varied from 0.4 to 3.4/μM/s, which is quite high compared with the value estimated using purified SurA. The maximal activity was obtained for Glu, followed by Leu and Ala residues N-terminal to the Pro residue (Table VII).

In order to identify some of the residues critical for PpiD activity, we isolated point mutations linked to *hupB*, based on their ability to induce the σ^{E} response (increased *htrA–lacZ* activity) as well as hypersensitivity to novobiocin (see Materials and methods). *hupB* is >95% linked to *ppiD*. Twenty three such mutants were obtained and sequenced. Two mutations were found to be changes of G312 (GGC to CGC) to R and G313 (GGC to CGC) to R. Two other mutations were changes of L298 to either S (TTA to TCA) or F (TTA to TTT). Another mutation was found to change I350 (ATT to TAT) to F. After completion of this work, the three-dimensional structure of Pin1, a human PpiC homologue, was solved (Ranganathan *et al*., 1997). By comparison, it is possible to conclude that G312, G313, G347 and I350 are expected to be part of the active site of PpiD. Since a G to R substitution is rather drastic, we decided to change G312, G313, G347 and I350 to A using site-directed mutagenesis. All these new mutations led to a loss of PpiD activity in terms of their ability to either complement a *surA* null mutant or reduce the *htrA–lacZ* activity induced by periplasmic stresses (Table I). Among these mutant PpiD proteins, we chose to purify the two mutants carrying the G347 to A and I350 to A substitutions, since they were more severely affected in their *in vivo* activity. These two mutant proteins appeared to lack any detectable PPIase activity (Table VII). This further confirms that these residues are part of the catalytic site, similarly to Pin1, although there is not a complete conservation of the amino acid sequence around this region.

Discussion

In this work, we have identified a novel heat-shock protein which belongs to the classical heat-shock σ^{32} regulon. This protein is a catalyst which is required for the folding of exported proteins. This is the first σ^{32} heat-shock protein which has been shown to participate in the folding of non-cytoplasmic proteins. The gene encoding this protein was identified while looking for additional components of protein folding in the extra-cytoplasm. Most of the known periplasmic folding catalysts so far have been found to be under the control of either the alternative sigma factor σ^{E} or the two-component system CpxR–CpxA (Danese and Silhavy, 1997; Pogliano *et al*., 1997). Both regulons have evolved to respond to stresses occurring in the extra-cytoplasm, and globally in the cell envelope, and we have used this knowledge as a tool to determine the presence of additional periplasmic folding catalysts.

The folding of OMPs has been shown to be dependent on LPS, the SurA and Skp proteins (reviewed by Missiakas and Raina, 1997a). Among the folding agents, SurA requirement has been found to be acute, since the maturation of many OMPs is affected in *surA* mutant bacteria. Consequently, a *surA* deletion leads to the induction of the periplasmic heat-shock response, which is Eσ^{E} dependent. In this work, we tried to determine whether other proteins are also required for OMP folding. To do so, we directly selected for genes which can bypass the requirement for the *surA* gene product, *in vivo*.

In parallel, we also looked for genes transcribed by the Cpx two-component system. The CpxR–CpxA system is induced as a parallel pathway which monitors misfolding events occurring in the envelope. We took advantage of our recent finding that CpxR–CpxA-dependent promoters such as *htrA* (a periplasmic protease) are constitutively induced upon overexpression of *prpA* in a strictly CpxR–CpxA-dependent manner (Missiakas and Raina, 1997b).

Hence, an *E.coli* promoter fusion library was transformed with a *prpA* plasmid and promoters strongly induced upon PrpA overproduction were selected. However, we retained only those promoters which were turned down in a *cpxR* null background. All these genetic approaches led to the identification of a new gene, *ppiD*. Based on the predicted amino acid sequence, *ppiD* encodes a membrane-anchored PPIase with resemblance to members of the PpiC family, such as surA.

Why did these approaches identify only the ppiD gene?

First, our data clearly demonstrate that overexpression of *ppiD* can functionally compensate for lack of SurA. Secondly, we selected those promoters specifically induced upon overexpression of *prpA*. We had shown earlier that overexpression of *prpA* leads to the induction of the CpxR–CpxA regulon as well as the classical heat-shock response regulon under the control of σ^{32}. As a consequence, only a promoter region carrying both CpxR-binding boxes and a σ^{32}-dependent promoter has been selected. In addition, none of the other known catalysts of folding can bypass the requirement for *surA*. For example, neither *skp* nor *fkpA* in multicopy restore normal OMP profiles in *surA* mutant bacteria (our unpublished data). Also, our earlier attempts to target putative periplasmic chaperones by searching for mutants which induce the periplasmic heat-shock response ($E\sigma^E$-dependent) were all performed at 30°C or below. A null mutation in *ppiD* induces only a marginal induction of the σ^E response at 30°C (2- to 3-fold), as compared with 37°C (5-fold), meaning that the misfolding defect of a *ppiD* null mutant can be screened far more easily at 37°C than at 30°C.

PpiD is a member of the PpiC family

It has been shown that members of the PpiC family (parvulin-like) are also well conserved across evolution. First, the sequence examination showed that PpiD contains a parvulin-like domain between amino acids 277 and 357. Consistent with this, we clearly demonstrated that PpiD has a PPIase activity *in vitro*. This activity is in fact much higher than the one measured for SurA, another member of the PpiC family. Residues G347 and I350 of PpiD were found to be essential for the PPIase activity. By sequence homology, these residues can be placed in the active site of the structure of PinI, the parvulin human homologue (Ranganathan *et al*., 1997). These are the first residues shown to be essential for PPIase activity, based on site-directed mutagenesis. Thus, the PPIase activity seems to be essential for its *in vivo* role as a folding catalyst.

Synthetic lethality of the surA ppiD null combination

One of the most important findings of this study is that a double null combination of *surA* and *ppiD* genes is lethal under all the growth conditions tested. This is unique for *surA* and *ppiD*, since a *ppiD* null mutation can be combined with either *ppiA* (*rotA*), *fkpA* or even *skp* null mutations. Such a lethality can be explained by an overlap of function between *surA* and *ppiD* gene products and independent and different transcriptional regulation of each gene. This may also explain why neither of these genes is essential. The overlap in their functions is based on the following observations: (i) overexpression of *ppiD* can restore the normal membrane profile in *surA* mutant bacteria as well as suppress other membrane defects such as sensitivity to novobiocin; (ii) PpiD overproduction restores the elevated σ^E activity observed in *surA* mutant bacteria; (iii) overexpression of *ppiD* can restore membrane defects of *htrM* mutant bacteria which lack proper LPS; and (iv) phenotypes exhibited by *ppiD* mutants can be corrected by overexpressing the *surA* gene.

PpiD so far is the only folding catalyst for extracytoplasmic function which is rpoH regulated

Until now, it seemed that protein folding in the cytoplasm of *E.coli* was assisted exclusively by the classical heat-shock regulon placed under the control of *rpoH* ($E\sigma^{32}$), whereas genes responsible for folding in the extracytoplasm belonged to either the $E\sigma^E$ regulon or the CpxR–CpxA regulon. Here we report a unique observation that a folding catalyst encoded by the *ppiD* gene and needed for the folding of OMPs is placed under the control of *rpoH*, the sigma factor transcribing cytosolic heat-shock proteins. This is probably quite important for bacterial physiology. It is known that elevated temperatures not only lead to the aggregation of proteins in general, but also to changes in the composition of the outer membrane. At high temperatures, some OMPs are more abundant, e.g. OmpC (reviewed by Pratt *et al.*, 1996), and the lipid composition also changes. This in turn affects the structure of LPS. This may be the reason why *E.coli* has chosen one of the important catalysts of folding needed for membrane biogenesis to be regulated by the classical heat-shock sigma factor σ^{32}. It should be pointed out that unlike *rpoH*, *rpoE* (encoding the alternate heat-shock factor σ^E) transcription is not induced at high temperatures. *rpoE* transcription is governed by two different promoters which respond differently to temperature changes; however, the global amount of RNA synthesized remains constant under any conditions (Raina *et al.*, 1995; Rouvière *et al.*, 1995). Our unpublished data also show that *surA* transcription is not heat-shock induced and is neither *rpoE* (σ^E) nor CpxR–CpxA dependent. Consistent with these observations, we found that the best suppression of both *htrM* and *surA* mutants by *ppiD* overexpression occurred at or above 37°C.

Materials and methods

Bacterial strains and plasmids

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

Selection strategies and cloning of the ppiD gene

The *ppiD* gene was cloned from a DNA library as a multicopy suppressor of a *surA* mutant using the *in vivo* mini-Mu plasmid cloning system (Groisman and Casadaban, 1986). The mini-Mu lysate was prepared on a MudII 4042-carrying strain, into which a *surA*::Tn*10* Tet mutation previously was introduced, to prevent the cloning of the $surA^+$ gene during subsequent selection. Multicopy suppressors were selected based on their ability to allow *surA* mutant bacteria to form colonies at concentrations of novobiocin >30 μg/ml. It should be pointed out that *surA* mutant bacteria do not form colonies at >10 μg/ml of novobiocin due to defective membranes. Only those candidates were retained which also suppressed the sensitivity of the *surA* mutant to detergents such as SDS and sodium deoxycholate. The plasmid DNA prepared from 10 such mini-Mu plasmids was subjected to partial *Sau*3A digestion to produce DNA fragments of ~4 kbp in length. These DNA fragments were gel purified and ligated into the *Bam*HI site of the p15A-based

vector pOK12 (Vieira and Messing, 1991). The ligation reaction was used to transform *surA* mutant bacteria plated on agar plates supplemented with 30 μg/ml novobiocin.

The restriction enzyme digestion pattern of seven such randomly chosen plasmids showed that they all carried the same chromosomal insert with a unique *Kpn*I site. ^{32}P-labelled nick-translated DNA from these plasmids was hybridized to the ordered *E.coli* genomic library (Kohara *et al.*, 1987). All of them were found to map to the same region of the *E.coli* chromosome. One of these plasmids, pSR3239, was chosen for further subclonings and resulted in the construction of pCD50 which contains a 3 kbp *Eco*NI–*Sma*I DNA fragment. A further minimal subclone, pCD51, containing 2.4 kbp *Bsa*BI–*Sma*I was constructed. Both plasmids were able to suppress *surA* mutant bacteria for resistance to novobiocin or SDS, to the same extent as the original plasmid pSR3239.

The overexpression of PpiD was achieved by first amplifying the *ppiD* gene by PCR of the minimal coding region, using primers 5′-GTTGTTACCATATGGACAGC-3′ and 5′-TTGCGAGGCGAATT-CAGGATTG-3′, and the product was digested with *Nde*I and *Eco*RI, and cloned into the T7 promoter expression vectors pET24-a (pCD52). To produce controlled expression, we also cloned the *ppiD* gene in a tightly regulated expression system under control of Ptrc, having a *lac*O operator as well as a *lac*I^q repressor pSE420 (pCD275). This plasmid, pCD275, was also used in many complementation studies.

Screening for CpxR–CpxA-dependent promoters

We were interested first in constructing a complete promoter library. To do this, chromosomal DNA isolated from *E.coli* wild-type strain MC4100 was subjected to *Sau*3A partial digestion. DNA fragments in the range 400–800 bp were gel purified and cloned into the single copy F-based promoter probe-based vector pFZY (Koop *et al.*, 1987) at its unique *Bam*HI site. Lac$^+$ clones were pooled *en masse* and transformed into a *dsbD* mutant background (Missiakas *et al.*, 1995). We have shown previously that the induction of *htrA* transcription is fully optimized only when functional CpxR–CpxA and Prp proteins are present (Missiakas and Raina, 1997b). The *htrA* transcription is partly dependent on CpxR–CpxA. Hence, we pooled all Lac$^+$ deeper blue Amp^R colonies from the *dsbD* mutant background and transformed them further with a *prpA*$^+$ plasmid wherein *prpA* expression is inducible with IPTG from the *lac* promoter. Approximately 30 000 colonies were plated on LB-agar + glucose to repress *prpA* induction, and ~500 colonies per plate were taken and replica plated on LB-agar + IPTG to induce the expression of *prpA*. Approximately 70 transformants were selected which were deep blue, and were later transduced with the *cpxR* null allele (Danese *et al.*, 1995). Fifteen such plasmids which exhibited less β-galactosidase activity were selected for further analyses and sequenced. Twelve of them were found to have a promoter sequence region corresponding to *ppiD*.

We also constructed a new single copy promoter fusion to the promoter region of *ppiD*. The DNA of the promoter region of *ppiD*, as predicted from the above experiments, was PCR amplified using primers 5′-GTTAACGAGCGTGGATCCCGTACT-3′ and 5′-GATACCGAATT-CAATCTTGAG-3′. This amplified DNA was digested with *Bam*HI and *Eco*RI and cloned into the corresponding sites of the promoter probing vector pRS550 (Simons *et al.*, 1987), resulting in plasmid pCD189. A single copy lysogen was made using λ RS45 (Simons *et al.*, 1987) for further use. To compare our data with known heat-shock-dependent promoters or *rpoE*-regulated promoters, we used our previously constructed *groEL–lacZ*, *htpG–lacZ* (SR1353), *rpoEP2–lacZ* (SR2195) and *htrA–lacZ* (SR1458) fusions (Raina *et al.*, 1995; Missiakas *et al.*, 1996). Also, the already described *rpoH*$^+$-, *rpoE*$^+$- and *prpA*$^+$-carrying plasmids were used to verify further the transcriptional regulation of *ppiD*.

Disruption of the ppiD gene

In order to construct a null allele of *ppiD*, we inserted an ΩTet cassette (Fellay *et al.*, 1987), previously digested at its *Bam*HI restriction sites and made blunt, into the unique *Kpn*I site (also made blunt using T4 DNA polymerase prior to ligation) of the *ppiD* coding region, as shown for plasmid pCD57 (Figure 1). Similarly, an ΩKan cassette from plasmid pUCK4 was inserted at the *Kpn*I site as well as at the *Pst*I site into plasmid pCD57. The resulting DNA fragments carrying the disrupted *ppiD* gene were digested appropriately from plasmids pCD160 and pCD174, respectively, and were introduced into the unique *Sma*I site of the vector pKO3 which carries a temperature-sensitive replicon as well as the *sacB* gene for counter selection (Link *et al.*, 1997). The resulting plasmids pCD180 and pCD178 were transformed into wild-type strain MC4100 at 42°C to select for co-integrate formation, and subsequently streaked at 30°C on LB-Tet or LB-Kan plates supplemented with 5% sucrose. Bacteria which had lost plasmid (loss of Cm^R) and were either Tet^R or Kan^R were retained.

RNA isolation and mapping of 5′ termini

Total cellular RNA was isolated by using the RNeasy kit from Qiagen. To prepare RNA produced at different temperatures, cultures were grown at 30°C and aliquots were shifted to 50°C for a period of either 5 or 10 min. Immediately after heat shock, cultures were lysed by guanidinium isothiocyanate, and RNA was purified as per the manufacturer's instructions. To define the transcriptional start site(s) of the *ppiD* gene, ~10 ng of the oligonucleotide probe 5′-CGTAAGCTGTTCCATCATGGTG-3′, which is complementary to nucleotide positions –5 to 17 of the *ppiD* coding sequence, was annealed to 10 μg of total cellular RNA. The annealed primer was extended by avian myeloblastosis virus (AMV) reverse transcriptase. The primer extension products were electrophoresed on the same gel as the dideoxy sequencing reactions, using the same primer.

Determination of topology of PpiD

The membrane topology of PpiD was determined by the method developed recently by Manoil and Bailey (1997). We used both λ Tn*lacZiln* and λ Tn*phoAlin* to place these fusions on the plasmid pCD57. We obtained mostly XP$^+$ colonies with λ Tn*phoAlin*. The exact junctions of fusions were sequenced as suggested by Manoil and Bailey (1997). We also used the PhoA$^+$ cassette described by Gutierrez and Devedjian (1989) and inserted it at the unique *Pst*I site of the *ppiD* coding sequence. This site is located next to the parvulin-coding domain facing the periplasm. Another PhoA fusion was constructed by first creating an *Att*II restriction site after the sixth amino acid predicted to be part of domain residing in the cytoplasm and then inserted the PhoA$^+$ cassette.

Site-specific mutagenesis

Since *ppiD* is >90% linked to *hupB*, we used this as a linked marker to obtain point mutations. Briefly, a P1 bacteriophage lysate was grown on the *hupB*::Kan strain and the lysate was mutagenized with hydroxylamine as described earlier (Raina *et al*., 1995). This mutagenized lysate was transduced into a *htrA–lacZ* fusion, and Lac colonies were picked. These transductants were tested further for sensitivity to 20 μg/ml of novobiocin and 2% SDS. Twenty three independent isolates were retained, as they exhibited a phenotype close to that of *ppiD* null mutants. In the present study, we were more interested in the determination of active site residues of the putative parvulin domain. We PCR amplified the minimal parvulin domain from the chromosomal DNA prepared from these mutants. These PCR products were sequenced directly using standard procedures.

Based on the above experiments and results obtained therein, we also carried out site-directed mutagenesis by changing G312, G313, G316, G347 and I350 to A residues. This was achieved by oligonucleotide-directed mutagenesis, following the procedure described in the Quick Change kit from Stratagene, using *Pfu* DNA polymerase. For PCR amplification, 50 ng of template pCD51 DNA was used. The resulting plasmids carrying the mutated *ppiD* gene were verified by sequencing and by their inability to complement *ppiD* null mutants. Out of these, pCD271 A347 and pCD273 A350 mutant plasmids were used for purification of mutant proteins.

PpiD purification and peptidyl–prolyl isomerase activity

Escherichia coli carrying plasmid pCD51 (*ppiD*$^+$) were induced with 1 mM IPTG, at an OD of 0.4 at 595 nm for 4 h. Cells were resuspended in buffer A (50 mM Tris–HCl pH 7.2, EDTA 1 mM, KCl 50 mM, glycerol 10% v/v) and lysed by sonication. The lysate was centrifuged at 35 000 r.p.m. for 45 min at 4°C. Protein recovered in the membrane fraction was resuspended in buffer A containing 0.2% sarkosyl to solubilize the inner membrane and then spun again. The soluble inner membrane fraction was dialysed against buffer A containing the non-polar detergent NP-40 (0.2%) and loaded onto a gel filtration column (G75). The PpiD-containing fractions were loaded on a MonoQ column (Pharmacia). The protein of >95% purity eluted with a KCl gradient of 0.05–1 M.

The PPIase activity was determined by a protease-coupled assay using chymotrypsin and succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide as the substrates (Fischer *et al.*, 1992). The reaction mixture contained chymotrypsin in HEPES buffer 35 mM, pH 7.8 and the reaction was started by adding sequentially the substrate dissolved in 460 mM LiCl in trifluoroethanol and one of the purified proteins. A pre-incubation of chymotrypsin and PpiD was avoided to minimize protein degradation. The time course of the reaction was monitored at 390 nm on a Uvikon

940 spectrophotometer. The relative specific constants k_{cat}/K_m were calculated as described by Rahfeld *et al.* (1994) and Fischer *et al.* (1992).

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