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Periplasmic Peptidyl Prolyl cis-trans Isomerases Are Not Essential for Viability, but SurA Is Required for Pilus Biogenesis in Escherichia coli

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In Escherichia coli, FkpA, PpiA, PpiD, and SurA are the four known periplasmic cis-trans prolyl isomerases. These isomerases facilitate proper protein folding by increasing the rate of transition of proline residues between the cis and trans states. Genetic inactivation of all four periplasmic isomerases resulted in a viable strain that exhibited a decreased growth rate and increased susceptibility to certain antibiotics. Levels of the outer membrane proteins LamB and OmpA in the quadruple mutant were indistinguishable from those in the surA single mutant. In addition, expression of P and type 1 pili (adhesive organelles produced by uropathogenic strains of E. coli and assembled by the chaperone/usher pathway) were severely diminished in the absence of the four periplasmic isomerases. Maturation of the usher was significantly impaired in the outer membranes of strains devoid of all four periplasmic isomerases, resulting in a defect in pilus assembly. Moreover, this defect in pilus assembly and usher stability could be attributed to the absence of SurA. The data presented here suggest that the four periplasmic isomerases are not essential for growth under laboratory conditions but may have significant roles in survival in environmental and pathogenic niches, as indicated by the effect on pilus production.

Protein folding within the extracytoplasmic compartments of gram-negative bacteria occurs in a unique milieu that is acutely susceptible to changes due to direct contact with the environment. The periplasm is devoid of ATP (53) and is an oxidizing environment. The lack of ATP in the periplasm precludes the use of the prototypic GroEL/ES class of chaperones to aid in protein folding (54). However, the periplasm of Escherichia coli contains a variety of different classes of protein folding factors, including disulfide bond catalysts and peptidyl-prolyl cis-trans isomerases (PPIases) (for reviews, see references 15 and 34). PPIases have been shown to facilitate the cis-trans isomerization of proline residues both in vitro and in vivo (16, 32, 50). Isomerization of proline residues is known to be a rate-limiting step in protein folding (1, 25, 30, 37). The four known periplasmic PPIases in E. coli are FkpA, PpiA, PpiD, and SurA.

With the exception of surA, inactivation of the periplasmic PPIases has not resulted in observable defects. surA mutants are sensitive to various detergents, hydrophobic dyes, and antibiotics (5, 28, 39). In addition, outer membrane porins exhibit a reduced rate of trimerization in surA mutants (28, 39). The three-dimensional structure of SurA (7) revealed a groove within the putative isomerase domain (5) that appears to bind the peptide substrate. Recently, multiple groups have demonstrated the ability of SurA to bind peptide motifs that are characteristic of outer membrane β-barrel proteins (6, 8, 18, 51). While the current evidence suggests SurA increases the rate of folding of β-barrel proteins, no proteins have been identified that require the PPIases for proper folding and/or maturation.

The specialized chaperone/usher pathway is involved in the assembly of more than 160 different types of adhesive pili on the surface of gram-negative bacteria. P and type 1 pili are two highly similar organelles assembled via this pathway and are composed of multiple subunits that are assembled in a hierarchical manner from the tip to the base. The ultimate apical subunit of P pili, PapG, serves as the adhesin that binds specifically to globoside (GbO4) on kidney epithelial cells (13). Assembly of the tip continues with the addition of an adaptor protein, PapF, followed by multiple subunits of PapE to form the tip fibrillum (22, 29). PapF is an adaptor molecule that connects the tip fibrillum to the pilus rod, which comprises a helical polymer of PapA subunits. PapD and PapC serve as the dedicated periplasmic chaperone and outer membrane usher, respectively. The periplasmic chaperone is comprised of two immunoglobulin-like domains and interacts with each immunoglobulin-like pilin via donor-strand complementation (3, 41). PapD binds the pilin subunits in the periplasm as they emerge from the general secretion machinery at the inner membrane. The folding of the pilus subunit is then catalyzed to occur directly on the chaperone template (2). The folded subunit remains bound to the chaperone in a mechanism that results in the stabilization of the subunit and prevention of
premature subunit aggregation in the periplasm (42). PapD also directs the pilus subunits to the PapC usher at the outer membrane (40). PapC forms a pore in the outer membrane and serves as the site for assembly and extrusion of the surface organelle (48). In the type 1 pilus system, a similar hierarchy begins with the tip adhesin FimH (42), which binds to mannosylated proteins within the uroplakin plaques of bladder epithelial cells (52). The additional type 1 structural subunits are assembled similarly to P pili, employing the chaperone FimC and the usher FimD.

Although the pilus systems have dedicated periplasmic chaperones that are required to stabilize pilin subunits and facilitate pilus assembly, additional, more general periplasmic protein folding factors may also have critical roles in pilus biogenesis. For example, the periplasmic disulfide bonding catalyst DsbA is required for proper folding of PapD (23). In addition, PapD is high in proline content, and two of these residues are in the cis configuration (41), suggesting that PPIases may act upon PapD. In this report, the four known periplasmic PPIases in E. coli were inactivation inactivated, resulting in a viable strain with some growth defect. The periplasmic PPIases, particularly SurA, were shown to be involved in the assembly of pili.

### MATERIALS AND METHODS

**Strains and plasmids.** All strains used in this study are indicated in Table 1. P1 transduction and transformation have been described previously (45). Plasmid pPAP1 contains the surA gene under arabinose control (38). Plasmid pPap5 carries the entire pap operon under control of the native promoter. Plasmid pPap37 is a derivative of pPap5, where papD has been insertionally inactivated (46). Plasmid pETS9 carries the entire fim operon under control of Ptrc, while pETS4 contains Sim under Ptrc (44).

**Media and growth.** Media were prepared as described previously (45). Antibiotics (Sigma, St. Louis, MO) were used at the following concentrations: 125 μg/ml ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 25 μg/ml tetracycline.

For growth studies, bacteria were grown overnight to saturation in Luria-Bertani (LB) media containing the appropriate antibiotics. Cultures were washed twice with LB media and subcultured at a dilution of 1:500 in LB media. After approximately five cell generations, cells were subcultured again at a dilution of 1:50 in fresh LB media with the appropriate antibiotics. Growth experiments were performed at 37°C.

For antibiotic sensitivity assays, 100 μl of a saturated culture was added to 3 ml of molten LB top agar and plated to LB agar. Amikacin (30 μg), bacitracin (10 units), novobiocin (30 μg), and vancomycin (30 μg) disks (Difco Laboratories, Detroit, MI) were placed on the solidified top agar. The diameter of the zone of inhibition around each antibiotic disk was measured after overnight incubation at 37°C.

**Electron microscopy.** Bacteria were allowed to adsorb onto formvar/carbon-coated copper grids for 1 min. Grids were washed in distilled H2O and stained with 1% aqueous uranyl acetate (Ted Pella, Inc., Redding, CA) for 1 min. Excess liquid was gently wicked off, and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV.

**Immunoblot analysis.** For total extracellular proteins, saturated cultures were normalized for total number of cells, and whole cells were lysed by the addition of Laemmli buffer and immersion in a boiling water bath for 10 min (26). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose (Schleicher and Schuell, Keene, NH), and subjected to immunoblot analysis using antisera raised against LamB, OmpA, and MBA (33).

For analysis of P pilus components, the pap operon was induced by growth on tryptic soy agar plates for 16 h at 37°C. For type 1 components, bacteria were grown statically overnight at 37°C (with 20 μM isopropyl-β-thiogalactopyranoside for pETS9 and pETS4 and 0.2% arabinose for pAER1). Bacteria were harvested by resuspension in phosphate-buffered saline and normalized by optical density at 600 nm. Outer membranes or periplasmic were harvested as described previously (12, 48). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and visualized by immunoblot analysis using antibody directed against PapA, PapC, PapD, or FimD (4, 12, 49).

**RESULTS**

Although four proteins with apparently similar enzymatic activities are present within the periplasm of E. coli, little is known about their specific roles in the maturation of extracytoplasmic proteins. To define the requirement for each isomerase, the genes were inactivated by insertion of an antibiotic resistance cassette. All possible combinations of mutant alleles of the periplasmic isomerases were evaluated to determine specific roles in cell viability, membrane integrity, and steady-state levels of periplasmic and outer membrane proteins.

**Growth rates.** Growth rates of each single and mutant combination were compared to that of the wild-type parental strain (MC4100) in shaking LB broth at 37°C (Fig. 1). There were no defects in the growth rates of any of the single and double mutants compared to the wild type. In contrast to a previous report (11), insertional inactivation of both ppiD and surA did not result in a synthetic lethal phenotype in this system. Three
of the triple mutant combinations (fkpA ppiD surA, fkpA ppiA ppiD, and ppiA ppiD surA) displayed growth defects during the mid- to late logarithmic phase (Fig. 1), while the other triple mutant (fkpA ppiA surA) was indistinguishable from the wild type (data not shown). It is interesting that the three triple mutants that exhibited a growth defect all carry a mutation in ppiD. This phenotype was not observed in any double mutant including ppiD, suggesting that multiple PPIases share some functions with PpiD. The quadruple PPIase mutant strain exhibited the most pronounced growth defect (Fig. 1). This defect was not due to the presence of multiple antibiotics in the growth medium, since the fkpA ppiA surA triple mutant grew normally in media with the same antibiotic composition.

Antibiotic sensitivity. Certain antibiotics (novobiocin, bacitracin, and vancomycin) are ineffective against E. coli because they are unable to traverse the outer membrane (36). It has been previously reported that mutations in surA lead to defects in the integrity of the outer membrane, resulting in increased sensitivity to these antibiotics (28, 39). The PPIase mutants were evaluated for their antibiotic sensitivities by determining the zone of inhibition of growth around a filter disk saturated with the indicated antibiotic. The surA mutant zones of inhibition were 8, 10, and 14 mm for novobiocin, bacitracin, and vancomycin, respectively (Fig. 2). In contrast, none of the other single mutants exhibited any sensitivity to these antibiotics (data not shown). In fact, resistance to all three antibiotics (novobiocin, bacitracin, and vancomycin) was present in the quadruple mutant and augmented in the quadruple mutant. WT, wild type.

Protein levels in the outer membrane. Inactivation of fkpA, ppiA, or ppiD individually or in combination had no discernible effect on the levels of outer membrane proteins visualized by SDS-PAGE and Coomassie blue staining (data not shown). In contrast, inactivation of surA led to decreased levels of LamB and OmpA (Fig. 3), as well as OmpF and OmpC (data not shown). The decrease in levels of LamB and OmpA with the quadruple mutant was equivalent to that observed with the surA single mutant (Fig. 3).

Pilus biogenesis. One of the most abundant classes of proteins produced by E. coli are pilins assembled by the chaperone/usher pathway. Pathogenic strains of E. coli contain as many as 12 chaperone/usher operons in their genomes. Pili are comprised of thousands of repeating subunits, and there are typically hundreds of pili produced by a bacterial cell. Thus, >10^6 subunits may be assembled per generation via the chaperone/usher pathway under a given environmental condition. The usher shares biochemical characteristics of an outer membrane β-barrel pore. However, the usher represents only a minor component of the outer membrane and is not readily detectable by Coomassie blue staining. Therefore, the effect of PPIase activity on stability of the usher would likely not have been observed in previous studies. Given the abundance of pilins in the usher and in the chaperone (Table 2) and that the ushers PapC and FimD contain predicted SurA binding motifs (34), we hypothesized that periplasmic PPIases may play an important role in pilus biogenesis.

Since K12 strains of E. coli do not carry the genes required for P pilus biogenesis, the mutant strains described above were

![FIG. 2. Sensitivity of PPIase mutants to antibiotics. Zones of inhibition around filter disks saturated with amikacin (black bars), novobiocin (gray bars), bacitracin (crosshatched bars), and vancomycin (hatched bars) were measured after overnight incubation. Susceptibilities similar to that observed with the surA single mutant, the quadruple mutant strain exhibited a growth defect all carry a mutation in ppiD. This phenotype was not observed in any double mutant including ppiD, suggesting that multiple PPIases share some functions with PpiD. The quadruple PPIase mutant strain exhibited the most pronounced growth defect (Fig. 1). This defect was not due to the presence of multiple antibiotics in the growth medium, since the fkpA ppiA surA triple mutant grew normally in media with the same antibiotic composition.

![FIG. 3. Effect of PPIase mutations on extracytoplasmic protein stability. The levels of LamB, maltose binding protein (MBP), and OmpA were evaluated in the mutant strains by immunoblot analysis. Outer membrane protein levels are decreased in mutants that include disruption of surA. WT, wild type.]

### Table 2. Proline content of selected E. coli proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of prolines</th>
<th>No. of amino acids</th>
<th>% Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>PapA</td>
<td>5</td>
<td>185</td>
<td>2.7</td>
</tr>
<tr>
<td>PapC</td>
<td>34</td>
<td>836</td>
<td>4.1</td>
</tr>
<tr>
<td>PapD</td>
<td>17</td>
<td>240</td>
<td>7.1</td>
</tr>
<tr>
<td>PapE</td>
<td>12</td>
<td>173</td>
<td>6.9</td>
</tr>
<tr>
<td>MalE</td>
<td>21</td>
<td>396</td>
<td>5.3</td>
</tr>
<tr>
<td>LamB</td>
<td>9</td>
<td>446</td>
<td>2.0</td>
</tr>
<tr>
<td>OmpA</td>
<td>19</td>
<td>346</td>
<td>5.5</td>
</tr>
</tbody>
</table>
transformed with a plasmid (pPap5) that contained the complete pap (pyelonephritis-associated pilus) operon under control of the native promoter. The extent of P piliation of the wild-type and mutant cells was evaluated by negative-stain electron microscopy and the ability to agglutinate human red blood cells (46). Under the induction conditions used here, approximately 85% of the wild-type bacteria were heavily piliated, while only about 15% had low levels of pili on the surface (Fig. 4). Wild-type levels of piliation were observed on all strains except those carrying the surA mutation. Piliation was reduced in all strains carrying surA mutations, as determined by electron microscopy, agglutination of human red blood cells, and purification of pili (Fig. 4).

The molecular basis of the pilus biogenesis defect in the absence of SurA was investigated by analyzing the effect of the surA mutation on the PapC outer membrane usher and PapD periplasmic chaperone. The steady-state level of PapC was determined by immunoblot analysis of outer membranes isolated from the mutant strains. There was a significant decrease in the amount of PapC in the outer membranes of mutants that lacked SurA activity (Fig. 5A). PapC was not observed in any other compartment in these strains (data not shown). To investigate the effect of PPIases on assembly of type 1 pili, a plasmid carrying the intact fim operon was transformed into the panel of laboratory PPIase mutants. As observed with PapC, overproduction of the type 1 pilus usher FimD was significantly reduced in all cases where surA was inactivated (Fig. 5B).

To eliminate the artifact of protein overproduction from a plasmid system, the role of SurA in pilus usher maturation was evaluated using an intact chromosomal fim operon. surA was inactivated in our prototypic cystitis-derived E. coli strain, UTI89. Agglutination of guinea pig red blood cells, an in vitro measure of type 1 function (27, 47), was reduced for the surA mutant. The hemagglutination (HA) titer was 512 and 64 for UTI89 and the surA mutant, respectively, and was restored to

FIG. 4. P pilus biogenesis in PPIase mutants. Representative negatively stained electron micrographs depicting the extent of piliation of the mutant strains are shown (bar = 0.5 μm). The table insert indicates the proportions of cells that are highly piliated, paired with human red blood cell HA titer. Strains used: (A) MC4100/pPap37, (B) MC4100/pPap5, (C) surA pPap5 mutant, (D) ppiA ppiD fkpA pPap5 mutant, (E) ppiA ppiD fkpA surA pPap5 mutant.
256 when surA was complemented on the plasmid pAER1. The surA mutation reduced FimD to barely detectable levels in the outer membrane fraction (Fig. 5C), and its presence was restored when surA was supplied in trans (Fig. 5C).

The ability of the periplasmic chaperones to fold into stable conformations was also evaluated with all of the PPIase mutant combinations. The steady-state level of PapD (Fig. 6) or FimC (data not shown) was unaffected by the presence or absence of any of the PPIases, except in the quadruple mutant, where chaperone levels were slightly reduced (Fig. 6). Previous reports have shown that pilin subunits misfold and are degraded in the absence of a functional chaperone (24) (Fig. 6, MC4100/pPap37). The ability of PapA to accumulate in the periplasm was used as an indicator of the functionality of PapD. Except in the case of the quadruple mutant, the steady-state levels of PapA (Fig. 6) and FimA (data not shown) were unchanged in the periplasm regardless of the presence of PPIases in the periplasm, suggesting that the chaperone is folded into the proper functional conformation to participate in the folding and stabilization of the structural subunits. The decreased levels of PapA may reflect decreased amounts of all of the subunits, thus accounting for the decrease in the HA titer of the quadruple mutant (Fig. 2), suggesting that proper folding of PapD and/or PapA requires at least one functional PPIase. Alternatively, the altered growth rate of the quadruple mutant may be an indicator of other defects, as in the rate of protein synthesis.

**DISCUSSION**

There are four unique *cis-trans* prolyl isomerases within the periplasm of *E. coli*: the cyclophilin PpiA (31), the FKB-like isomerase FkpA (20), and two parvulin domain-containing isomerases, SurA and PpiD (11, 28). SurA and FkpA appear to function as both isomerases and chaperones in protein folding. The maturation of outer membrane proteins is unaffected when the SurA PPIase activity is specifically inactivated, and the chaperone domain alone complements surA mutation (5), suggesting that the chaperone activity is more important in vivo. More recently, separable chaperone and isomerase domains were similarly described for FkpA (43). Thus far, chaperone domains not have been described for either PpiA or PpiD. The spectrum of substrates for each of the PPIases is not known, though SurA binds peptide motifs characteristic of β-barrel proteins (6, 8, 18, 51). Redundancy of function among the PPIases has not been investigated, although *ppiD* is reported to be a multicopy suppressor of surA (11).

Contrary to a previous report (11), the present study showed the *ppiD surA* double mutant to be viable. In fact, all possible
mutant combinations of the four PPIases were viable, including the quadruple mutant. This argues that the known periplasmic PPIases are not essential for growth of *E. coli* under laboratory conditions. However, our studies suggest there may be pleiotropic effects for the quadruple mutant. For instance, the increased susceptibility of the quadruple mutant to certain antibiotics suggests that membrane integrity is more compromised in this strain than in the *surA* single mutant. It has been shown that *Saccharomyces cerevisiae* mutants lacking all 12 cyclophilin and FKBP-like PPIases are still viable, and the phenotype of the dodecuple mutant is only the sum of those of the single mutants (14). However, the single parvulin homolog in *S. cerevisiae* is essential for growth (17). In the case of *E. coli*, multiple protein folding catalysts are present in the periplasm, including PPIases, disulfide bond catalysts, and molecular chaperones (for a review, see reference 15). As with *SurA* and FkpA, some of these catalysts are also bifunctional. Therefore, redundancy in function may cross catalyst classes, and phenotypes may not be revealed without inactivation of enzymes from multiple classes. This hypothesis is supported by the observation that *surA skp* and *surA degP* double mutants exhibit synthetic lethality in *E. coli* (38).

Evidence regarding the molecular mechanisms of the PPIase activity and chaperone function is mounting, yet very little is known about the substrates processed by these unique enzymes. Should the absence of PPIases alter only the rate of protein folding, identification of specific substrates might require techniques that monitor folding kinetics (e.g., pulse-labeling of nascent peptides or in vitro refolding). These types of techniques are prohibitive when evaluating the complex milieu of the periplasm and outer membrane. Further, analysis of protein levels at steady state may not be informative if these substrates utilize other protein folding catalysts in the periplasm. For instance, the abundance of prolines in the P pilus chaperone PapD (7% of the amino acids) might suggest PapD as a prime candidate as a substrate for PPIase activity. In fact, there was minimal effect on the steady-state levels of PapD even in the quadruple mutant, suggesting that PapD folding does not require PPIases. However, PapD is known to require the activity of DsbA for proper disulfide bond formation and pilus biogenesis (23). Due to the nature of protein folding, one could imagine that if multiple folding catalysts were required for a single polypeptide to properly fold, the order of engagement with the catalysts would have dramatic effects on the outcome. Therefore, consecutive interaction with multiple folding catalysts would be most efficient if the various catalysts were in a heteropolymERIC complex; thus far, there is no evidence to suggest a multicatalytic protein-folding complex within the periplasm of *E. coli*. Using PapD as an example, then, each polypeptide may utilize only one protein folding catalyst to achieve its final conformation.

While it is clear that FkpA, PpiA, and PpiD exhibit in vitro PPIase activity, it is unclear what role these enzymes play in the folding and maturation of proteins in the extracytoplasmic compartments. Inactivation of these three enzymes under laboratory conditions had no discernible phenotype in the present assays. The lack of a phenotype for the *ppiA, ppiD*, and *fkpA* mutants significantly hinders the ability to identify specific protein substrates of these isomerases. Yet the substrates for *SurA* are becoming more evident. Previous reports have implicated a role for *SurA* in the folding of β-barrel proteins (6, 8, 51) and, more specifically, in the maturation of LamB and OmpA in the outer membrane (28). We have confirmed that the assembly of these two outer membrane proteins relies on *SurA* alone and does not involve the other *E. coli* periplasmic PPIases. In addition, our data show that usher proteins from the chaperone/usher pilus biogenesis systems also require *SurA*.

In this study, inactivation of all four known periplasmic cis-trans peptidyl prolyl isomerases had minimal effects on the growth rate, although more substantial effects on antibiotic sensitivity, in *E. coli*. *SurA* was shown to be critical for biogenesis of both P and type 1 pili. *SurA* and possibly the other PPIases may act on important protein substrates encoded only in pathogenic strains or produced in specific environments, raising difficulties in determining roles for these proteins in laboratory strains of *E. coli*. In this report, the strongest phenotype for *SurA* was observed in a pathogenic strain of *E. coli*, one whose genome encodes approximately 1,000 proteins not present in laboratory strains of *E. coli*. The effect of *surA* mutation on pilus biogenesis implies a role for *SurA* in urinary tract infection pathogenesis. In addition, although the *surA* mutant is viable under laboratory conditions, the *surA* mutant in the uropathogenic *E. coli* strain UTI89 is not viable within the murine bladder epithelium (D. A. Hunstad, S. S. Justice, and S. J. Hultgren, unpublished). Furthermore, *SurA* is required for suppression of cytokines produced by bladder epithelial cells in response to uropathogenic *E. coli* (21). In a parallel vein, MIP (FkpA homolog) in *Legionella pneumophila* and *Salmonella enterica* serovar Typhimurium var. *Copenhagen* is not essential for laboratory growth but is required for survival within macrophages (10, 19). We conclude that elicitation of the substrates and requirements for the periplasmic cis-trans prolyl isomerases may be revealed by examination of growth and survival in pathogenic strains of bacteria and by utilizing animal models.

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