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Role for Skp in LptD Assembly in *Escherichia coli*

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The periplasmic chaperone Skp has long been implicated in the assembly of outer membrane proteins (OMPs) in *Escherichia coli*. It has been shown to interact with unfolded OMPs, and the simultaneous loss of Skp and the main periplasmic chaperone in *E. coli*, SurA, results in synthetic lethality. However, a Δ*skp* mutant displays only minor OMP assembly defects, and no OMPs have been shown to require Skp for their assembly. Here, we report a role for Skp in the assembly of the essential OMP LptD. This role may be compensated for by other OMP assembly proteins; in the absence of both Skp and FkpA or Skp and BamB, LptD assembly is impaired. Overexpression of SurA does not restore LptD levels in a Δ*skp* Δ*fkpA* double mutant, nor does the overexpression of Skp or FkpA restore LptD levels in the Δ*surA* mutant, suggesting that Skp acts in concert with SurA to efficiently assemble LptD in *E. coli*. Other OMPs, including LamB, are less affected in the Δ*skp* Δ*fkpA* and Δ*skp bamB*::*kan* double mutants, suggesting that Skp is specifically necessary for the assembly of certain OMPs. Analysis of an OMP with a domain structure similar to that of LptD, FhuA, suggests that common structural features may determine which OMPs require Skp for their assembly.

The cell envelope of the Gram-negative bacterium *Escherichia coli* includes three subcellular compartments: the inner membrane (IM), the outer membrane (OM), and the aqueous space between them, known as the periplasm. The outer membrane is unique, as it is an asymmetric bilayer with an outer leaflet composed of lipopolysaccharide (LPS) and an inner leaflet of phospholipids. The OM also contains two major classes of proteins: lipoproteins, most of which are essentially soluble periplasmic proteins that are attached to the inner leaflet of the OM by a lipidated N terminus, and integral β-barrel proteins known as outer membrane proteins (OMPs) (1).

There are two essential OMPs in the outer membrane (2–4). One is BamA, which along with its four associated lipoproteins, BamBCDE, is responsible for inserting itself and other β-barrel proteins into the OM (2, 5, 6). The other is LptD, which, along with its associated lipoprotein LptE, is responsible for inserting LPS into the outer leaflet of the OM (7, 8). LptD is an especially complicated substrate of the OMP assembly machinery. The C terminus of this protein forms its integral β-barrel, while the N terminus is a soluble periplasmic domain that is homologous to the periplasmic LPS transport protein LptA (4, 7, 8). The N- and C-terminal domains of LptD possess two cysteines each, which form two nonconsecutive disulfide bonds, each of which joins the N terminus to the barrel domain (9). In order for these disulfide bonds to be correctly formed and, thus, for the cell to be viable, LptD must first be inserted into the OM by the Bam complex, which also requires an interaction with its accessory lipoprotein, LptE (9, 10). The periplasmic oxidase DsbA has also been shown to play an important, although not essential, role in the formation of LptD's disulfide bonds (9).

Because the periplasm is an aqueous, oxidizing environment, LptD and other OMPs must be escorted by chaperones as they travel from the Sec translocon in the IM to the Bam complex in the OM (11). The main periplasmic chaperone in *E. coli* is SurA, a protein that also possesses peptidyl-prolyl *cis-trans* isomerase activity (11, 12). This protein is responsible for the assembly of the bulk mass of OMPs and is especially important for the biogenesis of LptD (9, 12). Although *lptD* transcription increases in the absence of *surA* (13), due to the induction of the σ^E stress response, the levels of LptD in this mutant are decreased dramatically, because the misassembled LptD that accumulates is rapidly degraded in the periplasm (9, 12).

Although the SurA pathway is the principal periplasmic chaperone pathway for OMPs in *E. coli*, there is also a secondary pathway made up of two proteins: the chaperone Skp and the chaperone and protease DegP (11, 14). As long as one of these two pathways remains intact, the cell is viable. However, if both the main SurA pathway and the backup Skp/DegP pathway are compromised (as in either an *surA skp* or *surA degP* double mutant), this synthetic interaction results in cell death (11, 14). It has also been demonstrated that the levels of virtually all OMPs decrease in the absence of both of these pathways (15).

Skp was initially reported to be a histone-like protein that binds DNA (16); it was later shown to bind OMPs and periplasmic proteins (17, 18). It exists in solution as a trimer and is structurally similar to the eukaryotic cytoplasmic chaperone prefoldin (19). Skp has been shown to interact with phospholipid membranes (20) and to facilitate the release of OMPs from spheroplasts (21) and, along with LPS, to insert unfolded OmpA into phospholipid membranes (22). More recently, experiments have provided evidence that β-barrels may be protected from aggregation within the cavity of the Skp trimer (23). Skp has also been shown to interact with the passenger domain of the autotransporter EspP at a different, earlier assembly step than SurA (24, 25). Despite this wealth of evidence supporting a role in OM biogenesis, an *skp* mutant displays only minor OM permeability and OMP assembly phenotypes *in vivo* (11), and no OMPs appear to depend on the Skp/DegP pathway for their assembly in *E. coli* (11, 15). However, in other organisms, Skp appears to play a more important physiological role (26). For example, it has been reported that Skp is

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essential for the folding and surface presentation of the α-domain (although not the barrel) of the autotransporter IcsA in *Shigella flexneri* (27).

In addition to SurA, the *E. coli* periplasm contains three additional peptidyl-prolyl *cis-trans* isomerases, FkpA, PpiA, and PpiD (which is anchored to the IM) (28), as well as additional chaperones. Mutants lacking any of these three proteins, or even all three simultaneously, exhibit only minor defects *in vivo* with respect to OM biogenesis and OMP assembly (28).

FkpA has long been known to act as a chaperone for nonnative or mutant *E. coli* proteins (29–31), but until recently, its physiological role was largely unclear. It has been shown that FkpA is necessary for colicin M toxicity *in vivo* (32, 33) and that its PPIase activity is required for this function (34). It has also been shown that FkpA interacts with high affinity with the passenger domain of the autotransporter EspP (35), and, along with DsbC, FkpA has been implicated in the folding of the nonnative passenger domain of a hybrid autotransporter protein (36).

Here, we show that Skp does play a role in OMP assembly, but this role can be performed by FkpA and is thus masked when FkpA is present in the cell. This role appears to be important only in the assembly of certain OMPs and can be compensated for by the overexpression of an additional periplasmic chaperone, Spy.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains were constructed by P1 transduction and plasmid transformation as previously described (37). Strains and plasmids used in this work are described in Table 1. The deletion alleles used are from the Keio collection (38). Alleles from the Keio collection were cured using the pCP20 plasmid as previously described (39). The *bamB*::*kan* insertion allele is from the Blattner collection from the *E. coli* Genome Project at the University of Wisconsin-Madison. The *degP*^{S210A} allele (11) was transduced using a linked *yadC*::Tn*10* allele (40), and the presence of the *degP*^{S210A} mutation was confirmed by sequencing. The pTrc99A::*cam* plasmid was constructed by amplifying the *cat* gene using primers with PvuI restriction sites (underlined) (5′-TTCGATCGTTGTAGGCTGGAGCTGCT-3′ and 5′-TTCGATCGGCATATGAATATCCTCCT-3′). This PCR product and the pTrc99A vector (41) were digested with PvuI and ligated together. The resulting pTrc99A::*cam* plasmid was used as the vector control, and candidate genes were cloned into it. The *skp* gene was amplified by PCR to contain EcoRI and HindIII sites (underlined) (primers 5′-GGAGAATTCGGTAAGGAGTTTATTATGAAAAAGTGG-3′ and 5′-CATGCAAAGCTTATCCAACTGCTGCGCTAAAT-3′) and cloned into pTrc99A::*cam*. The forward primer changed the naturally occurring GTG start codon to ATG (underlined). The *fkpA* gene was cloned using the PstI and HindIII sites (primers 5′-CATGCACTGCAGGTTAACCCTGGGGTGAGATG-3′ and 5′-CATGCAAAGCTTTTCCGCTTTCCAGCACTAAT-3′). The *dsbA* overexpression plasmids were constructed by amplifying the *dsbA* gene to contain EcoRI and XbaI sites (underlined) (primers 5′-CATGCTGAATTCCCCTTTGCAATTAACACCTATG-3′ and 5′-GAGCATTCTAGATTCACG GGCTTTATGTAATTT-3′). The pAER1 (14) (p*dsbA*$_{low}$) and pBAD18 (p*dsbA*$_{high}$) plasmids were digested with EcoRI and XbaI enzymes and ligated with the *dsbA* PCR product. Construction of the p*surA* plasmid (pAER1) has been previously described (14). The *pspy* plasmid was reconstructed as previously described (42).

Growth conditions. Strains were grown in Luria-Bertani (LB) medium at 37°C unless otherwise noted. Where appropriate, the LB medium was supplemented with antibiotics at the following concentrations: ampicillin, 125 μg/ml; kanamycin, 25 μg/ml; tetracycline, 25 μg/ml; and chloramphenicol, 20 μg/ml. Also, where indicated, L-arabinose was added to the medium at a concentration of 0.2% to induce the p*surA* and p*dsbA*

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference/ source
Escherichia coli strains		
MC4100	F$^-$ *araD139* Δ(*arg-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 thi*	53
JAS412	MC4100 Ara$^{+/r}$ Δ*fkpA*	This study
JAS416	MC4100 Ara$^{+/r}$	This study
JAS417	MC4100 Ara$^{+/r}$ Δ*skp*	This study
JAS420	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA*	This study
JAS431	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA yadC*::Tn*10 degP*^{S210A}	This study
JAS458	MC4100 Ara$^{+/r}$ Δ*fkpA yadC*::Tn*10 degP*^{S210A}	This study
JAS459	MC4100 Ara$^{+/r}$ *yadC*::Tn*10 degP*^{S210A}	This study
JAS460	MC4100 Ara$^{+/r}$ Δ*skp yadC*::Tn*10 degP*^{S210A}	This study
JAS80	MC4100 Δ*skp*	This study
JAS195	MC4100 Δ*fkpA*	This study
JAS475	MC4100 Δ*skp* Δ*fkpA*	This study
JAS16	MC4100 Δ*surA*	54
JAS502	MC4100 Ara$^{+/r}$ p*surA*	This study
JAS501	MC4100 Ara$^{+/r}$ pACYC177	This study
JAS505	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA* pACYC177	This study
JAS506	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA* p*surA*	This study
JAS497	MC4100 Δ*skp* Δ*fkpA* Δ*lamB*	This study
JAS523	MC4100 Δ*skp* Δ*fkpA* Δ*lamB* Δ*ompA*	This study
JAS535	MC4100 Δ*skp* Δ*fkpA* Δ*lamB* Δ*ompA* Δ*ompC*::*kan*	This study
JAS215	MC4100 Δ*surA* p*fkpA*	This study
JAS509	MC4100 Δ*surA* pTrc99A::*cam*	This study
JAS510	MC4100 Δ*surA* p*skp*^{ATG}	This study
JAS866	MC4100 Ara$^{+/r}$ p*dsbA*$_{low}$	This study
JAS867	MC4100 Ara$^{+/r}$ pBAD18	This study
JAS868	MC4100 Ara$^{+/r}$ p*dsbA*$_{high}$	This study
JAS874	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA* p*dsbA*$_{low}$	This study
JAS875	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA* pBAD18	This study
JAS876	MC4100 Ara$^{+/r}$ Δ*skp* Δ*fkpA* p*dsbA*$_{high}$	This study
JAS185	MC4100 *bamB*::*kan*	54
JAS205	MC4100 Δ*fkpA bamB*::*kan*	This study
JAS187	MC4100 Δ*skp bamB*::*kan*	This study
GS67	MC4100 Δ*skp* Δ*fkpA baeS*^{P255L}	This study
GS95	MC4100 Δ*skp* Δ*fkpA baeS*^{P255L} Δ*spy*::*kan ynjB*::Tn*10*	This study
GS100	MC4100 Δ*skp* Δ*fkpA* pTrc99A	This study
GS99	MC4100 Δ*skp* Δ*fkpA* p*spy*	This study
GS108	MC4100 Δ*surA* pTrc99A	This study
GS109	MC4100 Δ*surA* p*spy*	This study
GS321	MC4100 *baeS*^{P255L} Δ*yegL*::*kan*	This study
Plasmids		
p*surA*	*surA* cloned into pBAD18, then *araC*-P$_{BAD}$-*surA* fragment subcloned into pACYC177	14
pTrc99A::*cam*	pTrc99A with *cam* cassette cloned into *bla* gene	This study
p*fkpA*	*fkpA* cloned into pTrc99A::*cam*	This study
p*skp*^{ATG}	*skp* with start codon mutated to ATG cloned into pTrc99A::*cam*	This study
p*dsbA*$_{low}$	*dsbA* subcloned into the p*surA* plasmid	This study
p*dsbA*$_{high}$	*dsbA* cloned into pBAD18	This study
pTrc99A	High-copy-number vector with inducible *lac* promoter and *amp* resistance	41
p*spy*	*spy* cloned into pTrc99A	This study

plasmids and isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 50 μM to induce the p*spy* plasmid.

Efficiency-of-plating assays. Efficiency-of-plating assays were performed by growing an overnight culture in the appropriate medium and at the permissive temperature. The overnight cultures were added to the first row of wells of a 96-well plate (200 μl/well) and serially diluted 10-fold in the subsequent wells (20 μl of the preceding well into 180 μl of fresh LB medium). The overnight culture and serial dilutions were spotted onto LB agar plates containing the indicated antibiotics using a 48-pin replicator. Spots were allowed to dry, and the plates were incubated overnight at 30°C and 37°C unless otherwise indicated.

Western blot analysis. One-milliliter samples of strains were pelleted (16,000 × *g*, 1 min) and resuspended at a volume equal to the optical density at 600 nm (OD_{600})/14 (for LptD and FhuA blots), OD_{600}/40 (for Spy blots), or OD_{600}/7 (all other blots). Reducing and nonreducing LptD blots were performed as previously described (9). FhuA blots were performed using a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), and all others were performed with a nitrocellulose (Whatman GmbH, Dassel, Germany) membrane. Immunoblotting was performed using the following antibodies at the indicated dilutions: anti-LamB antibody (which cross-reacts with OmpA), 1:30,000; anti-BamA antibody, 1:20,000; anti-LptD antibody, 1:5,000; anti-FhuA antibody, 1:2,500; anti-TolC antibody, 1:30,000; anti-LptE antibody, 1:20,000; anti-BamB antibody, 1:7,000; anti-BamD antibody, 1:5,000; anti-BamC antibody, 1:20,000; anti-BamE antibody, 1:20,000; anti-SurA antibody, 1:8,000; anti-Skp antibody, 1:10,000; and anti-Spy antibody, 1:25,000. For FhuA blots, goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) was used at a dilution of 1:5,000 (Bio-Rad). For all other blots, donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at a dilution of 1:8,000 (GE Healthcare). Immunoblots were visualized using Luminata Classico Western HRP substrate (EMD Millipore Corporation, Massachusetts). The anti-TolC antibody was a gift from R. Misra; the anti-BamB, anti-BamD, and anti-LptE antibodies were a gift from D. Kahne; the anti-SurA antibody was a gift from R. Kolter; and the anti-FhuA antibody was a gift from J. Coulton.

Coomassie staining. Samples resuspended in SDS-PAGE sample buffer at a volume equal to OD_{600}/14 were boiled for 10 min and analyzed by SDS-PAGE. Gels were then incubated overnight in Coomassie brilliant blue staining solution (43) and destained the next day with Coomassie destaining solution (43).

Suppressor selection. Suppressor mutants were selected by plating 10 μl of an overnight culture of the Δ*skp* Δ*fkpA* strain on agar plates containing 55 mg/liter vancomycin. After overnight incubation at 37°C, colonies were isolated and analyzed.

RESULTS

Genetic interactions exist between *skp* and *fkpA*. The existence of two OMP chaperone pathways in *E. coli*, SurA and Skp/DegP, was first established by the discovery of synthetic phenotypes in double-mutant strains. The simultaneous loss of *surA* and *skp* or *surA* and *degP* causes synthetic lethality (11, 14). In order to further investigate the chaperone network in the *E. coli* periplasm, we constructed strains lacking combinations of the known periplasmic chaperones and *fkpA* (38). We then assayed the growth of these mutant strains. None of the single-mutant strains exhibited a growth defect at 37°C, although the Δ*skp* Δ*degP* double mutant did exhibit slightly slowed growth at this increased temperature. Strikingly, we found that although the Δ*skp* Δ*degP* Δ*fkpA*::*kan* triple mutant grows normally at 30°C, this strain grows poorly at 37°C. This temperature sensitivity suggests that in the absence of *skp* and *degP*, FkpA becomes critically important.

DegP possesses both chaperone and protease functions, and we investigated which of these is more important in the absence of *skp* and *fkpA*. It is known that the loss of *degP* results in temperature sensitivity at 42°C (44). Strains expressing a proteolytically inactive allele known as $degP^{S210A}$ are temperature sensitive at 42°C unless the $DegP^{S210A}$ protein is overexpressed; this suggests that the loss of the protease function of DegP is primarily responsible for the temperature-sensitive phenotype of the *degP* mutant (45). We hypothesized that the loss of *fkpA* and *skp* lowers the temperature at which the *degP* mutation becomes lethal, resulting in the observed temperature sensitivity of the triple mutant at 37°C. In order to examine this more closely, we constructed strains lacking *skp*, *fkpA*, or both in the $degP^{S210A}$ mutant background and assayed their growth by an efficiency-of-plating assay. None of the strains examined exhibit a growth defect at the permissive temperature, 30°C (Fig. 1A). However, the Δ*skp* Δ*fkpA* $degP^{S210A}$ triple mutant is temperature sensitive; like the Δ*skp* Δ*degP* Δ*fkpA*::*kan* strain, it grows very poorly at 37°C (Fig. 1A). The temperature sensitivity of this strain can be complemented by the expression of wild-type *degP* in *trans*, which provides further evidence of protein misfolding in the Δ*skp* Δ*fkpA* double mutant. In the absence of both *skp* and *fkpA*, the protease activity of DegP is required to degrade misfolded proteins that accumulate in the periplasm.

Loss of Skp and FkpA causes defects in OM biogenesis. Mutant strains that are defective in OM biogenesis also exhibit increased permeability to antibiotics and other small molecules (3). Thus, if the Δ*skp* Δ*fkpA* mutant is defective in the assembly of OMPs, it should exhibit increased OM permeability. We tested this possibility by performing efficiency-of-plating assays with the single- and double-mutant strains at 37°C on LB agar supplemented with 65 mg/liter vancomycin. Indeed, the Δ*skp* Δ*fkpA* double mutant is much more sensitive to this concentration of vancomycin than either single mutant (Fig. 1B).

To further investigate the potential OMP assembly defect in the Δ*skp* Δ*fkpA* strain, we examined OMP levels in this strain by Western blotting. The OMPs LamB, OmpA, and TolC have been previously used as model proteins. Because the LamB and OmpA proteins are rapidly degraded in the periplasm when they fall off-pathway (11), their levels in whole-cell lysates are representative of their levels in the OM, and decreased levels of these proteins are indicative of a general OMP assembly defect (11, 12). Conversely, TolC levels are known to increase in certain strains that exhibit an OMP assembly defect (46). Neither LamB nor OmpA is present at reduced levels in the Δ*skp* Δ*fkpA* mutant strain, and levels of TolC are not increased (Fig. 2A). This suggests that the OMP assembly defect in the Δ*skp* Δ*fkpA* mutant strain is not general. The Δ*surA* strain, which exhibits clear defects in the assembly of LamB, is shown here for comparison (Fig. 2A).

Loss of Skp and FkpA affects LptD levels. In the absence of a general OMP assembly defect in the Δ*skp* Δ*fkpA* strain, we investigated whether the observed OM permeability of this strain could be due to an LPS assembly defect resulting from decreased levels of LptD in the OM. Thus, we examined the levels of LptD by Western blotting. Because LptD is not fully oxidized until it has interacted with its accessory lipoprotein, LptE, and been inserted into the OM by the Bam complex, nonreducing SDS-PAGE provides us with an assay to determine the levels of correctly assembled LptD in the cell (8, 9). While the Δ*skp* and Δ*fkpA* single mutants exhibited no apparent reduction in oxidized or reduced LptD levels, a Δ*skp* Δ*fkpA* double mutant showed a considerable reduction in the levels of both oxidized and reduced LptD (Fig. 2B). These results suggest that in the absence of Skp and FkpA, there is a defect in the assembly of LptD.

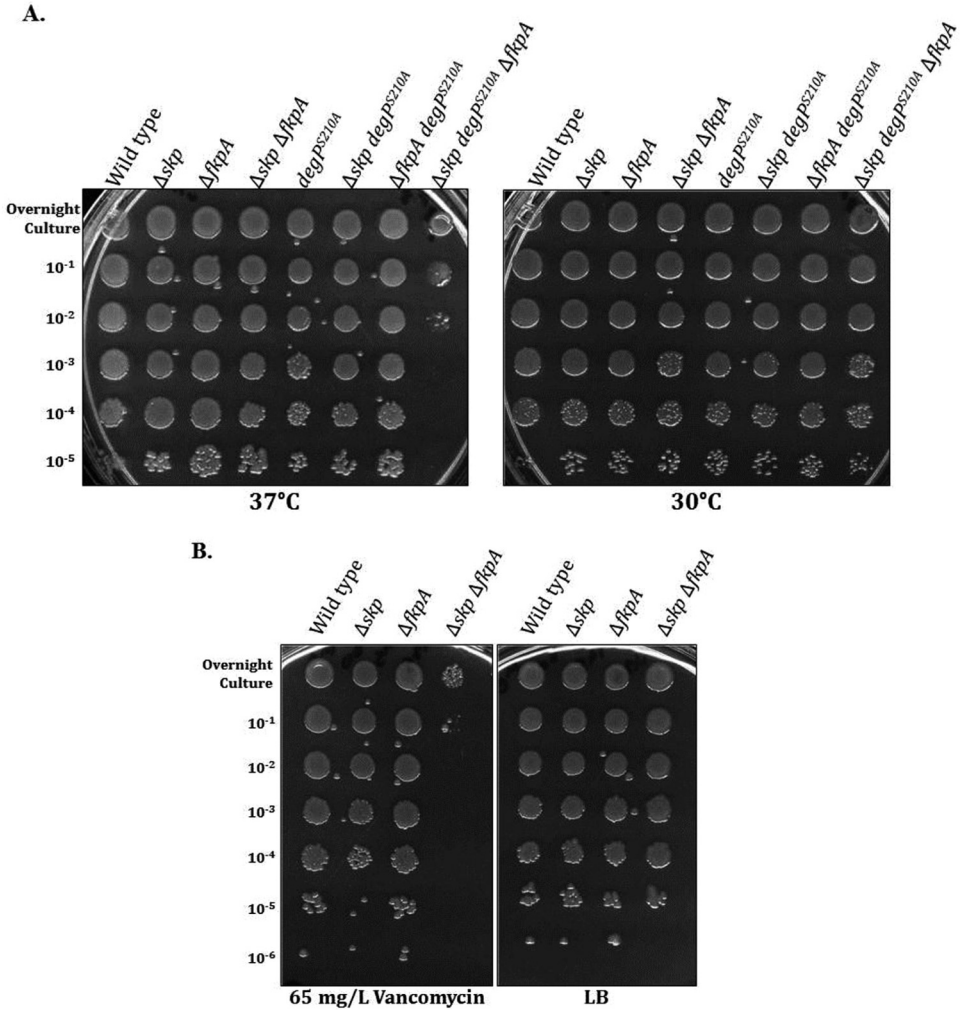

FIG 1 The Δ*skp* Δ*fkpA* double mutant exhibits phenotypes consistent with OMP assembly defects. (A) Overnight cultures were grown at the permissive temperature of 30°C. These cultures were then serially diluted in fresh LB medium, spotted onto LB agar, and incubated overnight at 30°C and 37°C. The $degP^{S210A}$ allele was moved using the linked *yadC*::Tn*10* marker, so this allele is also present in all of the $degP^{S210A}$ strains, and all of the strains shown are $Ara^{+/r}$. The Δ*skp* $degP^{S210A}$ Δ*fkpA* strain exhibits a defect of approximately 3 to 4 logs of growth at 37°C compared to that of the single and double mutants. (B) Growth of the wild-type, Δ*skp*, Δ*fkpA*, and Δ*skp* Δ*fkpA* strains was assayed on LB agar supplemented with 65 mg/liter vancomycin by efficiency-of-plating assay. The Δ*skp* Δ*fkpA* double mutant exhibits a defect of approximately 5 to 6 logs of growth on 65 mg/liter vancomycin.

If the loss of Skp and FkpA affects the levels of the Bam complex members, SurA, or the accessory lipoprotein LptE, it could cause the LptD assembly defect that we observed, because these proteins are necessary for the correct assembly of LptD. However, the levels of all of these proteins remained unchanged in the Δ*skp* Δ*fkpA* double mutant (Fig. 2C), leading us to conclude that Skp and FkpA participate directly in the assembly of LptD.

The role of Skp and FkpA in LptD assembly is unique. We considered the possibility that the decreased LptD levels in the Δ*skp* Δ*fkpA* double mutant might be caused by overloading the SurA pathway with substrate. If Skp and FkpA function as OMP chaperones, it is possible that the loss of both might saturate the main OMP assembly pathway, titrating SurA and causing some LptD to fall off-pathway and be degraded by periplasmic proteases (such as DegP). If this were indeed the case, increasing the levels of SurA in the Δ*skp* Δ*fkpA* double-mutant strain should restore LptD levels. Accordingly, we overexpressed SurA in a Δ*skp* Δ*fkpA* double mutant and examined the LptD levels in this strain by Western blotting. Overexpression of SurA did not increase the levels of LptD in the Δ*skp* Δ*fkpA* double mutant (Fig. 3A). Further, the deletion of a number of the most highly expressed OMPs (LamB, OmpA, and OmpC), which should decrease the load on the SurA pathway, did not restore levels of LptD in the Δ*skp* Δ*fkpA* double mutant (Fig. 3B).

We then wondered whether either Skp or FkpA could substitute for SurA in the assembly of LptD. In order to test this, we overexpressed Skp and FkpA in a Δ*surA* background and examined the levels of LptD in these strains by Western blotting. Overexpression of Skp or FkpA did not restore LptD levels (Fig. 3C). It is clear that SurA cannot perform the role of Skp and FkpA in LptD assembly, nor can Skp or FkpA compensate for the role of SurA in this process.

Another protein folding factor that is known to be required for the proper and efficient assembly of LptD is DsbA, which catalyzes the formation of the disulfide bonds in LptD (9). If the loss of Skp and FkpA leads to a defect in the formation of these disulfide

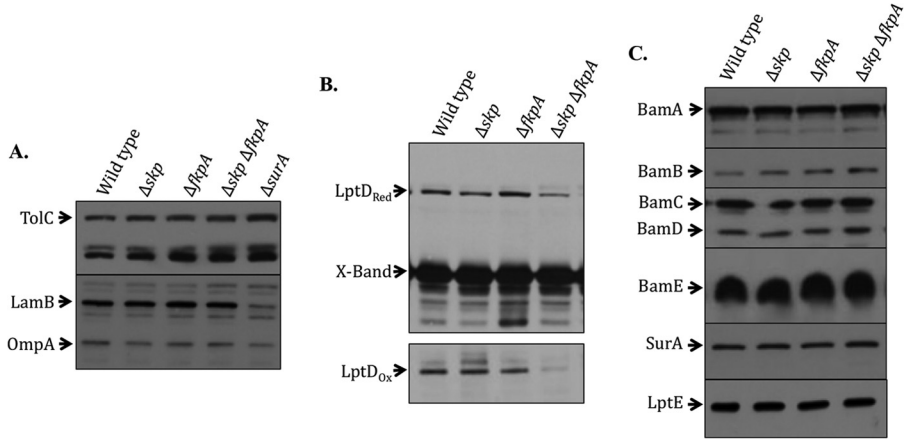

FIG 2 The Δ*skp* Δ*fkpA* double mutant exhibits an LptD assembly defect that is not due to loss of other LptD assembly factors. Cultures were grown to an OD_{600} of approximately 0.7 to 0.8. Whole-cell lysates were analyzed by SDS-PAGE and immunoblotted with the appropriate antibody. (A) A Δ*surA* strain is included for comparison. Levels of TolC, LamB, and OmpA are unaffected by the loss of *skp* and *fkpA*. (B) Samples were analyzed by SDS-PAGE in sample buffer with ($LptD_{Red}$) and without ($LptD_{Ox}$) 2-mercaptoethanol. "X-Band" is a cross-reacting band of approximately 55 kDa, shown here as a loading control. Levels of both oxidized and reduced LptD are decreased in the absence of Skp and FkpA. (C) The levels of proteins known to be important for LptD assembly are unchanged in the absence of Skp and FkpA.

bonds, LptD assembly would be impaired in a way that could possibly be compensated for by the overexpression of DsbA. However, the overexpression of DsbA from both low- and high-copy-number plasmids failed to restore LptD levels in the Δ*skp* Δ*fkpA* double mutant (Fig. 3D). Therefore, none of the periplasmic protein folding factors that are known to be important for LptD assembly can substitute for Skp and FkpA.

Skp/FkpA and BamB exhibit a functional relationship. The nonessential Bam complex lipoprotein BamB contributes to the assembly of a large number of OMPs. In most cases, OMPs that require SurA also require BamB (2, 6, 11, 46, 47). LptD, however, is an exception. In the absence of BamB, LptD levels remain relatively unaffected (2). However, when LptD assembly is impaired, as is the case in an *lptD4213* mutant, the loss of *bamB* causes synthetic lethality (2). Thus, *bamB* and *skp* mutants exhibit similar phenotypes; each has little effect on LptD assembly unless an additional defect exists.

We hypothesized that LptD is assembled normally in a *bamB* mutant strain because of the role played by Skp and FkpA. In order to address this possibility, we examined LptD levels in Δ*skp bamB*::*kan* and Δ*fkpA bamB*::*kan* double-mutant strains by Western blotting. In the Δ*skp bamB*::*kan* double-mutant strain, LptD levels are much lower than in either of the single mutants, and the Δ*fkpA bamB*::*kan* strain exhibits a slight LptD assembly defect (Fig. 4). LptD levels are largely unaffected by the loss of any of these individual proteins, but the loss of two of these proteins in combination impairs LptD assembly.

We also examined the assembly of LamB in these mutants. LamB represents an OMP that is strongly affected by the loss of BamB and SurA, but its assembly is unaffected by the loss of *skp* and *fkpA* (Fig. 2A). Although the Δ*skp bamB*::*kan* double-mutant strain exhibits impaired LamB assembly, this defect is not as severe as the observed LptD assembly defect in this double mutant, especially compared to that in the *bamB* single mutant (Fig. 4). The Δ*fkpA bamB*::*kan* strain exhibits LamB levels that are essentially equivalent to those of the *bamB*::*kan* single-mutant strain (Fig. 4).

FhuA and LptD are similarly affected in double-mutant strains. The TonB-dependent siderophore transport protein FhuA (48) is, like LptD, one of the few OMPs whose assembly is strongly dependent on SurA (12). In fact, in a *surA* mutant, LptD and FhuA are the only known OMPs present at decreased levels that do not exhibit a concomitant decrease in transcript levels (12). FhuA is also structurally similar to LptD; it possesses a soluble N-terminal domain and a C-terminal barrel domain. Although we had already shown that the loss of *skp* and *fkpA* does not affect a number of model OMPs (LamB, OmpA, and TolC), the similarity of FhuA to LptD led us to investigate whether FhuA assembly is affected in the Δ*skp* Δ*fkpA* strain.

Indeed, like LptD, while FhuA levels are unaffected in the Δ*skp* and Δ*fkpA* single-mutant strains, there is an assembly defect in the Δ*skp* Δ*fkpA* double mutant (Fig. 4). FhuA also exhibits assembly defects that are similar to those of LptD in the absence of *bamB*. Although FhuA assembly is only moderately affected by the loss of BamB, its assembly is dramatically reduced in the Δ*skp bamB*::*kan* double-mutant strain (Fig. 4). This result suggests that FhuA and LptD are targeted to the Bam complex by similar chaperone assembly pathways.

A novel *baeS allele suppresses the defects of a Δ*skp* Δ*fkpA* double mutant.** In order to learn more about the role of Skp and FkpA in the assembly of LptD, we selected suppressors of the Δ*skp* Δ*fkpA* strain by plating on vancomycin. One vancomycin-resistant suppressor also restored growth of the temperature-sensitive Δ*skp* Δ*fkpA* Δ*degP*::*kan* strain at 37°C, in addition to restoring levels of LptD in the Δ*skp* Δ*fkpA* double mutant (Fig. 5A). This suppressor mapped to the *baeSR* locus, and sequencing revealed that amino acid 255 in BaeS had been changed from proline to leucine ($baeS^{P255L}$).

The BaeS protein is the sensor kinase of a two-component system; its cognate response regulator is BaeR (49). This two-component system is known to regulate several genes in response to a variety of stimuli, including spheroplast formation, PapG overexpression, and indole exposure (49). Among the genes regulated by this system are those specifying multidrug efflux pump

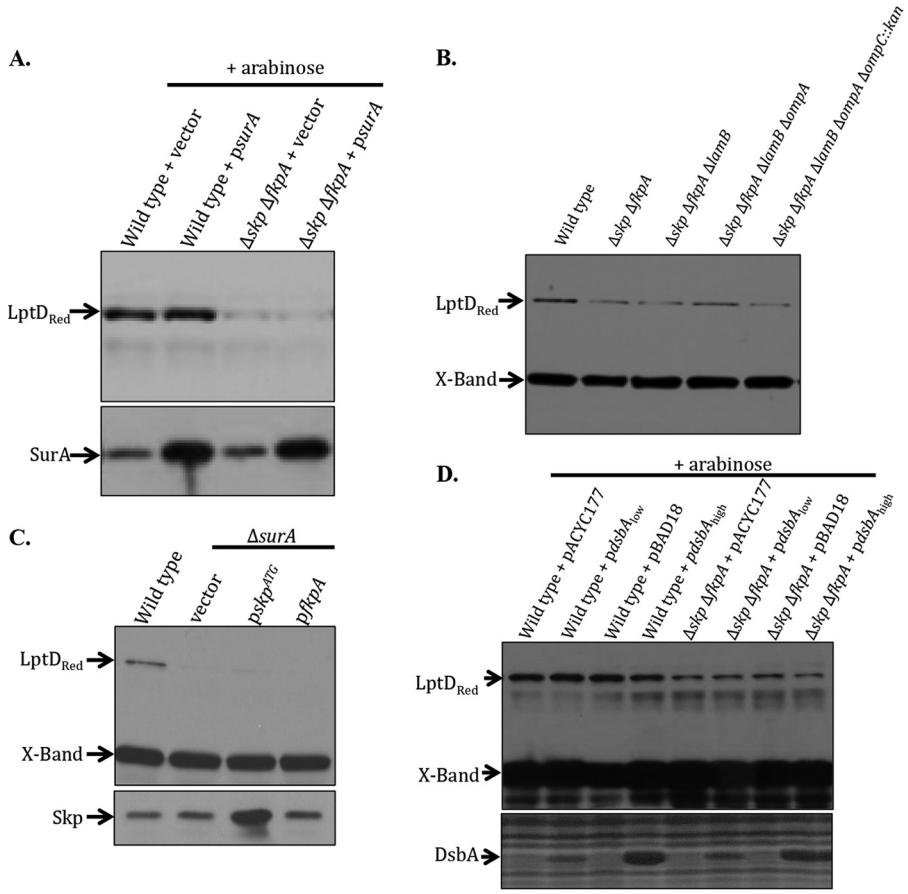

FIG 3 Skp/FkpA and SurA have distinct roles in LptD assembly. Cultures were grown to an OD_{600} of about 0.7 to 0.8 in LB medium. Whole-cell lysates were analyzed by SDS-PAGE and immunoblotted with the appropriate antibody. "X-Band" is a cross-reacting band of approximately 55 kDa, shown here as a loading control. (A) Medium was supplemented with 0.2% L-arabinose to induce the p*surA* plasmid. Overexpression of SurA in the Δ*skp* Δ*fkpA* strain has no effect on LptD levels. (B) The deletion of major OMPs has no effect on LptD levels in the absence of *skp* and *fkpA*. (C) Overexpression of Skp or FkpA does not increase LptD levels in the absence of *surA*. (D) Medium was supplemented with 0.2% L-arabinose to induce the plasmids. DsbA levels were visualized using Coomassie brilliant blue staining after SDS-PAGE analysis. Overexpression of DsbA in the Δ*skp* Δ*fkpA* strain does not affect LptD levels.

components (*mdtABCD* and *acrD*) and the periplasmic chaperone *spy* (50). Previously isolated *baeS* alleles, which dramatically upregulate the production of Spy, mapped to nearby amino acids (E264 and D268) (42). It has also been shown that Spy can protect substrate proteins from aggregation *in vitro* and that overexpression of Spy can stabilize an unstable variant of the colicin E7 immunity protein (Im7) *in vivo* (42). Thus, it seemed likely that *baeS*^{P255L} is a gain-of-function allele and that the LptD defect is suppressed due to the overexpression of Spy. Similar mutants in the Cpx two-component system have previously been called *cpx** mutants (51), and thus we will refer to this mutation as a *baeS** mutant.

The levels of Spy in this suppressor confirm that it is a *baeS** allele; in the Δ*skp* Δ*fkpA* strain containing the *baeS*^{P255L} suppressor, there is approximately 1,000-fold more Spy than in a wild-type strain (Fig. 5B). Spy levels are similarly induced when the suppressor allele is introduced into a wild-type background (Fig. 5C). When a Δ*spy*::*kan* allele is introduced into the Δ*skp* Δ*fkpA* *baeS*^{P255L} strain, levels of LptD return to the levels observed in the Δ*skp* Δ*fkpA* double mutant. When Spy alone is overexpressed in the Δ*skp* Δ*fkpA* strain, LptD levels are restored to nearly the levels observed in a wild-type strain (Fig. 5A). Thus, Spy is both necessary and sufficient to suppress the LptD assembly defect in the Δ*skp* Δ*fkpA* strain.

It seemed likely that the restoration of LptD levels in the Δ*skp* Δ*fkpA* strain when Spy is overexpressed is due to the general chaperone activity of the Spy protein. We investigated whether Spy overexpression is capable of suppressing a broader range of LptD assembly defects by overexpressing Spy in a Δ*surA* mutant. As shown in Fig. 5D, LptD levels remain unchanged even at high levels of Spy overexpression. This result reinforces the specificity of the roles played by Skp/FkpA and SurA in the LptD assembly process. Although Spy is capable of compensating for the role played by Skp and FkpA, it is not capable of compensating for the role played by SurA.

DISCUSSION

Several studies have shown that the periplasmic chaperone Skp can interact directly with OMPs (17, 23), and genetic analysis has revealed a role for this protein in the backup OMP assembly pathway in *E. coli* (11, 14). However, no substrates specifically dependent on Skp for their assembly have ever been identified. We have discovered a role for Skp in the assembly of LptD. This function was not previously identified, because it can be performed by an-

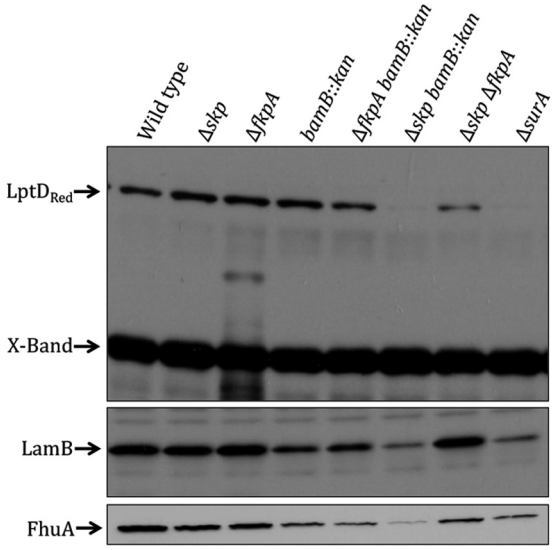

FIG 4 Loss of Skp and BamB results in synthetic LptD, LamB, and FhuA assembly defects. Cultures were grown to an OD_{600} of about 0.75 to 0.9 in LB medium. Samples were analyzed by SDS-PAGE and immunoblotted with anti-LptD, anti-LamB, and anti-FhuA antibodies. "X-Band" is a cross-reacting band of approximately 55 kDa, shown here as a loading control. The Δ*surA* strain is shown here as a control. The Δ*skp bamB*::*kan* and Δ*fkpA bamB*::*kan* strains exhibit decreased LptD and FhuA levels compared to those of the single-mutant strains.

other periplasmic chaperone, FkpA. The loss of both Skp and FkpA substantially reduces the levels of correctly assembled LptD.

Previous work has shown that LptD assembly is much more complicated than the assembly of a typical OMP. After translocation through the IM by the Sec machinery, LptD is escorted across the periplasm by SurA in a manner that no other chaperone can replicate (9, 12). Once LptD reaches the Bam complex, it must interact with its partner lipoprotein LptE in order for its β-barrel domain to be properly assembled (9, 10). Finally, oxidation, a process catalyzed by DsbA, must occur to form the disulfide bonds that connect the amino-terminal periplasmic domain to the carboxy-terminal β-barrel (9). Our results demonstrate that Skp or FkpA must act in concert with the main periplasmic chaperone SurA to efficiently assemble LptD but that SurA and Skp or FkpA perform distinct roles in this process. Overexpression of SurA or DsbA does not restore levels of LptD in a Δ*skp* Δ*fkpA* strain, nor can overexpressing Skp or FkpA restore LptD levels in a Δ*surA* strain. The loss of SurA, or of both Skp and FkpA, causes a profound defect in LptD assembly that cannot be compensated for by the other proteins.

The *baeS** suppressor isolated here provides further evidence of a novel role for Skp or FkpA in LptD assembly. This suppressor restores LptD assembly in mutants lacking Skp and FkpA, and the overproduction of Spy that occurs in this mutant is both necessary and sufficient for this restoration. However, Spy overexpression fails to restore LptD levels in the absence of SurA. We conclude that LptD assembly requires the participation of at least two different periplasmic chaperones, each of which must perform distinct functions. Spy is capable only of substituting for one of these two functions.

The loss of Skp, FkpA, or BamB has little effect on LptD assembly. However, unlike the loss of Skp or FkpA, the absence of BamB does cause defects in the assembly of many other OMPs, such as LamB and OmpA, and when LptD assembly is impaired, BamB

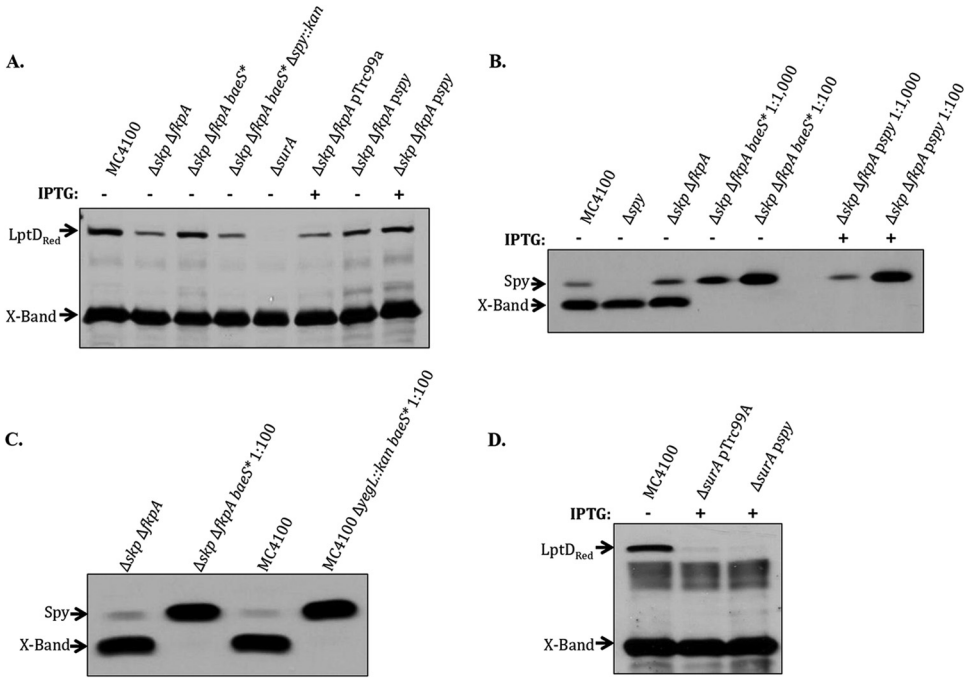

FIG 5 Spy overexpression suppresses the LptD assembly defect of a Δ*skp* Δ*fkpA* strain but not a Δ*surA* strain. Cultures were grown to stationary phase in LB medium. Where indicated, medium was supplemented with 50 μM IPTG. Samples were analyzed by SDS-PAGE and immunoblotted with anti-LptD and Spy antibodies. (A) The *baeS** suppressor (*baeS*^{P255L}) and Spy overexpression suppress the LptD assembly defect of a Δ*skp* Δ*fkpA* strain. (B) Spy is induced approximately 1,000-fold in the *baeS** and p*spy* strains. The X-band is too diluted to be visible in the 1:100 or 1:1,000 dilutions. (C) Spy is induced similarly when the *baeS** suppressor is transduced into a wild-type (MC4100) background. (D) Spy overexpression does not suppress the LptD defect of a Δ*surA* strain.

becomes essential (2). Although LptD assembly is not measurably compromised in strains lacking either Skp or BamB, assembly of this protein is decreased significantly in strains lacking both Skp and BamB. The synthetic phenotypes exhibited by these double mutants could indicate that Skp and BamB possess a redundant function in LptD assembly. Alternatively, there could be two different LptD assembly pathways: one that requires Skp (or FkpA) and another that uses BamB. At present, we cannot distinguish between these possibilities.

Strikingly, although other model OMPs, including LamB, are unaffected or less severely affected than LptD in mutant strains that lack Skp and FkpA, at least one other OMP, FhuA, exhibits assembly defects that are similar to those of LptD. Both LptD and FhuA are strongly dependent on SurA for their assembly, and both also require Skp or FkpA. It is tempting to speculate that Skp and FkpA aid in the assembly of these two proteins because of their structural similarities. One somewhat unique feature shared by LptD and FhuA is the presence of a large, periplasmic, N-terminal domain, which is the first to emerge into the periplasm during translocation. Skp has been shown to play a role in the folding and assembly of the N-terminal, soluble passenger domain of the autotransporter IcsA in *S. flexneri* (27), and both Skp and FkpA have been shown to interact with the N-terminal passenger domain of EspP in *E. coli* (24, 25, 35). It is possible that Skp and FkpA play a role in assembling proteins with these soluble N-terminal domains. Another possibility is that Skp and FkpA are involved at an early step in the assembly process and that only some OMPs require interaction with these chaperones at this step. Skp has been shown to associate with OMPs at the inner membrane (52) and to interact with EspP at an earlier step in its assembly than SurA (24) does.

There may be additional OMPs in *E. coli* that share the chaperone requirements of FhuA and LptD. Further study of these proteins and their structural characteristics could provide insight into the functional roles of Skp and FkpA. Moreover, because this is the first time a physiological substrate of the Spy protein has been identified, further study of this interaction may provide insight into the function of Spy in the cell.

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REFERENCES

1. **Silhavy TJ, Kahne D, Walker S.** 2010. The bacterial cell envelope. Cold Spring Harb. Perspect. Biol. **2:**a000414.
2. **Ruiz N, Falcone B, Kahne D, Silhavy TJ.** 2005. Chemical conditionality: a genetic strategy to probe organelle assembly. Cell **121:**307–317.
3. **Sampson BA, Misra R, Benson SA.** 1989. Identification and characterization of a new gene of *Escherichia coli* K-12 involved in outer membrane permeability. Genetics **122:**491–501.
4. **Braun M, Silhavy TJ.** 2002. Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. Mol. Microbiol. **45:**1289–1302.
5. **Wu T, Malinverni J, Ruiz N, Kim S, Silhavy TJ, Kahne D.** 2005. Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. Cell **121:**235–245.
6. **Sklar JG, Wu T, Gronenberg LS, Malinverni JC, Kahne D, Silhavy TJ.** 2007. Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **104:**6400–6405.
7. **Wu T, McCandlish AC, Gronenberg LS, Chng S-S, Silhavy TJ, Kahne D.** 2006. Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **103:**11754–11759.
8. **Chng S-S, Ruiz N, Chimalakonda G, Silhavy TJ, Kahne D.** 2010. Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. Proc. Natl. Acad. Sci. U. S. A. **107:**5363–5368.
9. **Ruiz N, Chng S-S, Hiniker A, Kahne D, Silhavy TJ.** 2010. Nonconsecutive disulfide bond formation in an essential integral outer membrane protein. Proc. Natl. Acad. Sci. U. S. A. **107:**12245–12250.
10. **Chimalakonda G, Ruiz N, Chng S-S, Garner RA, Kahne D, Silhavy TJ.** 2011. Lipoprotein LptE is required for the assembly of LptD by the β-barrel assembly machine in the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **108:**2492–2497.
11. **Sklar JG, Wu T, Kahne D, Silhavy TJ.** 2007. Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. Genes Dev. **21:**2473–2484.
12. **Vertommen D, Ruiz N, Leverrier P, Silhavy TJ, Collet J.** 2009. Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. Proteomics **9:**2432–2443.
13. **Dartigalongue C, Missiakas D, Raina S.** 2001. Characterization of the *Escherichia coli* sigma E regulon. J. Biol. Chem. **276:**20866–20875.
14. **Rizzitello AE, Harper JR, Silhavy TJ.** 2001. Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli*. J. Bacteriol. **183:**6794–6800.
15. **Denoncin K, Schwalm J, Vertommen D, Silhavy TJ, Collet J-F.** 2012. Dissecting the *Escherichia coli* periplasmic chaperone network using differential proteomics. Proteomics **12:**1391–1401.
16. **Holck A, Lossius I, Aasland R, Kleppe K.** 1987. Purification and characterization of the 17 K protein, a DNA-binding protein from *Escherichia coli*. Biochim. Biophys. Acta **914:**49–54.
17. **Chen R, Henning U.** 1996. A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. Mol. Microbiol. **19:**1287–1294.
18. **Jarchow S, Lück C, Görg A, Skerra A.** 2008. Identification of potential substrate proteins for the periplasmic *Escherichia coli* chaperone Skp. Proteomics **8:**4987–4994.
19. **Walton TA, Sousa MC.** 2004. Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. Mol. Cell **15:**367–374.
20. **De Cock H, Schäfer U, Potgeter M, Demel R, Müller M, Tommassen J.** 1999. Affinity of the periplasmic chaperone Skp of *Escherichia coli* for phospholipids, lipopolysaccharides and non-native outer membrane proteins. Role of Skp in the biogenesis of outer membrane protein. Eur. J. Biochem. **259:**96–103.
21. **Schäfer U, Beck K, Müller M.** 1999. Skp, a molecular chaperone of Gram-negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins. J. Biol. Chem. **274:**24567–24574.
22. **Bulieris PV, Behrens S, Holst O, Kleinschmidt JH.** 2003. Folding and insertion of the outer membrane protein OmpA is assisted by the chaperone Skp and by lipopolysaccharide. J. Biol. Chem. **278:**9092–9099.
23. **Walton TA, Sandoval CM, Fowler CA, Pardi A, Sousa MC.** 2009. The cavity-chaperone Skp protects its substrate from aggregation but allows independent folding of substrate domains. Proc. Natl. Acad. Sci. U. S. A. **106:**1772–1777.
24. **Ieva R, Tian P, Peterson JH, Bernstein HD.** 2011. Sequential and spatially restricted interactions of assembly factors with an autotransporter beta domain. Proc. Natl. Acad. Sci. U. S. A. **108:**E383–E391.
25. **Ieva R, Bernstein HD.** 2009. Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. Proc. Natl. Acad. Sci. U. S. A. **106:**19120–19125.
26. **Volokhina EB, Grijpstra J, Stork M, Schilders I, Tommassen J, Bos MP.** 2011. Role of the periplasmic chaperones Skp, SurA, and DegQ in outer membrane protein biogenesis in *Neisseria meningitidis*. J. Bacteriol. **193:**1612–1621.
27. **Wagner JK, Heindl JE, Gray AN, Jain S, Goldberg MB.** 2009. Contribution of the periplasmic chaperone Skp to efficient presentation of the autotransporter IcsA on the surface of *Shigella flexneri*. J. Bacteriol. **191:**815–821.

28. **Justice SS, Hunstad DA, Harper JR, Duguay AR, Pinkner JS, Bann J, Frieden C, Silhavy TJ, Hultgren SJ.** 2005. Periplasmic peptidyl prolyl *cis-trans* isomerases are not essential for viability, but SurA is required for pilus biogenesis in *Escherichia coli*. J. Bacteriol. **187:**7680–7686.
29. **Ow DS-W, Lim DY-X, Nissom PM, Camattari A, Wong VV-T.** 2010. Co-expression of Skp and FkpA chaperones improves cell viability and alters the global expression of stress response genes during scFvD1.3 production. Microb. Cell Fact. **9:**22.
30. **Saul FA, Arié J-P, Vulliez-le Normand B, Kahn R, Betton J-M, Bentley GA.** 2004. Structural and functional studies of FkpA from *Escherichia coli*, a *cis*/*trans* peptidyl-prolyl isomerase with chaperone activity. J. Mol. Biol. **335:**595–608.
31. **Zhang Z, Song L, Fang M, Wang F, He D, Zhao R, Liu J, Zhou Z, Yin C, Lin Q, Huang H.** 2003. Production of soluble and functional engineered antibodies in *Escherichia coli* improved by FkpA. Biotechniques **35:**1032–1038, 1041–1042.
32. **Hullmann J, Patzer SI, Römer C, Hantke K, Braun V.** 2008. Periplasmic chaperone FkpA is essential for imported colicin M toxicity. Mol. Microbiol. **69:**926–937.
33. **Barnéoud-Arnoulet A, Barreteau H, Touzé T, Mengin-Lecreulx D, Lloubès R, Duché D.** 2010. Toxicity of the colicin M catalytic domain exported to the periplasm is FkpA independent. J. Bacteriol. **192:**5212–5219.
34. **Helbig S, Patzer SI, Schiene-Fischer C, Zeth K, Braun V.** 2011. Activation of colicin M by the FkpA prolyl *cis-trans* isomerase/chaperone. J. Biol. Chem. **286:**6280–6290.
35. **Ruiz-Perez F, Henderson IR, Nataro JP.** 2010. Interaction of FkpA, a peptidyl-prolyl *cis*/*trans* isomerase with EspP autotransporter protein. Gut Microbes **1:**339–344.
36. **Veiga E, de Lorenzo V, Fernández LA.** 2004. Structural tolerance of bacterial autotransporters for folded passenger protein domains. Mol. Microbiol. **52:**1069–1080.
37. **Silhavy TJ, Berman ML, Enquist LW.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**2006.0008.
39. **Cherepanov PP, Wackernagel W.** 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.
40. **Singer M, Baker TA, Schnitzler G, Deischel SM, Goel M, Dove W, Jaacks KJ, Grossman AD, Erickson JW, Gross CA.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:**1–24.
41. **Amann E, Ochs B, Abel KJ.** 1988. Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301–315.
42. **Quan S, Koldewey P, Tapley T, Kirsch N, Ruane KM, Pfizenmaier J, Shi R, Hofmann S, Foit L, Ren G, Jakob U, Xu Z, Cygler M, Bardwell JCA.** 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat. Struct. Mol. Biol. **18:**262–269.
43. **Simpson RJ.** 2007. Staining proteins in gels with Coomassie blue. Cold Spring Harb. Protoc. **2007:**pdb.prot4719.
44. **Lipinska B, Fayet O, Baird L, Georgopoulos C.** 1989. Identification, characterization, and mapping of the *Escherichia coli htrA* gene, whose product is essential for bacterial growth only at elevated temperatures. J. Bacteriol. **171:**1574–1584.
45. **Spiess C, Beil A, Ehrmann M.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97:**339–347.
46. **Charlson ES, Werner JN, Misra R.** 2006. Differential effects of *yfgL* mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. J. Bacteriol. **188:**7186–7194.
47. **Ureta AR, Endres RG, Wingreen NS, Silhavy TJ.** 2007. Kinetic analysis of the assembly of the outer membrane protein LamB in *Escherichia coli* mutants each lacking a secretion or targeting factor in a different cellular compartment. J. Bacteriol. **189:**446–454.
48. **Sansom MS.** 1999. Membrane proteins: a tale of barrels and corks. Curr. Biol. **9:**R254–R257.
49. **Raffa RG, Raivio TL.** 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol. Microbiol. **45:**1599–1611.
50. **Leblanc SKD, Oates CW, Raivio TL.** 2011. Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. J. Bacteriol. **193:**3367–3375.
51. **Raivio TL, Silhavy TJ.** 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J. Bacteriol. **179:**7724–7733.
52. **Harms N, Koningstein G, Dontje W, Muller M, Oudega B, Luirink J, de Cock H.** 2001. The early interaction of the outer membrane protein Phoe with the periplasmic chaperone Skp occurs at the cytoplasmic membrane. J. Biol. Chem. **276:**18804–18811.
53. **Casadaban MJ.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. J. Mol. Biol. **104:**541–555.
54. **Rigel NW, Schwalm J, Ricci DP, Silhavy TJ.** 2012. BamE modulates the *Escherichia coli* beta-barrel assembly machine component BamA. J. Bacteriol. **194:**1002–1008.