Dissecting the *Escherichia coli* periplasmic chaperone network using differential proteomics

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Abstract

β-barrel proteins, or outer membrane proteins (OMPs), perform many essential functions in Gram-negative bacteria, but questions remain about the mechanism by which they are assembled into the outer membrane (OM). In *Escherichia coli*, β-barrels are escorted across the periplasm by chaperones, most notably SurA and Skp. However, the contributions of these two chaperones to the assembly of the OM proteome remained unclear. We used differential proteomics to determine how the elimination of Skp and SurA affects the assembly of many OMPs. We have shown that removal of Skp has no impact on the levels of the 63 identified OM proteins. However, depletion of SurA in the *skp* strain has a marked impact on the OM proteome, diminishing the levels of almost all β-barrel proteins. Our results are consistent with a model in which SurA plays a primary chaperone role in *E. coli*. Furthermore, they suggest that while no OMPs prefer the Skp chaperone pathway in wild-type cells, most can use Skp efficiently when SurA is absent. Our data, which provide a unique glimpse into the protein content of the non-viable *surA skp* mutant, clarify the roles of the periplasmic chaperones in *E. coli*.

Keywords

βeta-barrel; periplasm; proteomics; chaperone; outer membrane

1 Introduction

The outer membrane (OM) of Gram-negative bacteria is an essential organelle that functions as a permeability barrier protecting them from a wide variety of toxic molecules [1-3]. The OM is unique among biological membranes; it is an asymmetric lipid bilayer with lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet [4]. There are two major classes of proteins associated with the OM: lipoproteins, most of which are present in the periplasm but are anchored by a lipid moiety to the inner leaflet of the OM, and integral membrane proteins which generally adopt a β-barrel conformation, and are often referred to simply as β-barrels or OMPs [2, 5-7].

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Conflict of interest statement

The authors have declared no conflict of interest.

Many proteins that play essential roles in OM assembly have been recently identified, such as the LptD/E complex [4, 8, 9] and the 5-member Bam complex [10], which are involved in LPS and β-barrel insertion in the OM, respectively. Despite these recent breakthroughs, we still have a relatively poor understanding of how OM components, which are synthesized in the cytoplasm or at the inner membrane, are transported across the periplasm and inserted into the OM.

OMPs, which enter the periplasm as unfolded polypeptides, need to cross this hydrophilic compartment before being inserted into the OM. It is generally considered that unfolded β-barrels interact with periplasmic chaperones that prevent their aggregation in this compartment and facilitate their delivery to the Bam assembly complex [3, 11, 12].

In agreement with this model, several proteins that assist in the transport of OMPs across the periplasm have been identified. These proteins include protein-folding catalysts such as peptidyl-prolyl *cis/trans* isomerases (PPIases) [13] and proteins that catalyze the formation and isomerization of protein disulfide bonds [14, 15]. Two proteins, SurA and Skp, exhibit general chaperone activity and have been shown to play a role in β-barrel transport [13]. These two chaperones operate in two different, partially redundant, pathways, as indicated by the synthetically lethal phenotype of the *surA skp* double mutant [16].

SurA is a PPIase that has general chaperone activity, and is known to be involved in the assembly of OMPs [17-19]. Strains lacking *surA* have a decreased OM density [20] and are hypersensitive to detergents and hydrophobic antibiotics [18, 19], which is indicative of OM perturbations. Recently, we used a differential proteomic approach based on label-free two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) analysis to study the impact of *surA* deletion on the OM proteome [21]. We found that several OMPs, including FhuA and LptD, and the major β-barrels OmpA, OmpF and OmpX, were less abundant in strains lacking SurA. However, these studies were complicated by the fact that the lack of SurA causes massive induction of the σ^E stress response [18], resulting in the induction of sRNAs that decrease the synthesis of many OMPs [22-24]. FhuA and LptD were the only β-barrel proteins for which the decrease in abundance could not be attributed, at least in part, to decreased mRNA levels, which led us to identify these two proteins as substrates highly dependent on SurA for their assembly [21].

Skp is a small periplasmic protein that is homologous to the eukaryotic chaperone prefoldin and functions as a trimer [25]. Skp copurifies with numerous β-barrel proteins [26], binds denatured β-barrels such as OmpA, OmpC, OmpF and LamB with high selectivity [27] and forms stable complexes with refolded OmpA *in vitro* [28]. However, *skp* mutants have a much less severe phenotype than *surA* mutants. They are slightly more sensitive to hydrophobic antibiotics and detergents than wild-type strains, and although these mutants have been reported to have moderately decreased levels of the major β-barrels [27], other more recent papers demonstrate that the OMP defect in a *skp* mutant is minimal [20, 29]. Moreover, the loss of Skp has no effect on membrane density [20]. This could mean either that Skp plays a minor role in the transport of many OMPs, or that some minor OMPs are particularly dependent on Skp for their transport. Recent experiments in *Neisseria meningitidis* suggest that PorA and PorB, two major OMPs in that organism, use Skp preferentially [30].

The periplasm also contains a third protein, DegP, which plays a role in β-barrel transport and assembly. DegP forms large 12-mer and 24-mer oligomeric cages with an inner cavity that can accommodate and stabilize small folded proteins [31, 32]. DegP acts as a chaperone at low temperature, but as a protease at elevated temperature [33]. While the role of DegP as a protease is well-documented, the physiological role of its chaperone activity is less clear.

Previous studies have shown that both *surA degP* and *surA skp* double mutants have a synthetically lethal phenotype, but that *skp degP* mutants do not [16]. Moreover, it was shown that a protease-deficient DegP protein can complement the *degP surA* synthetic lethality at temperatures up to 37° C [16]. This demonstrates that it is the loss of the chaperone activity of the DegP protein that is the cause of the synthetically lethal *degP surA* phenotype, not the loss of the protease activity. Thus, in the absence of SurA, the chaperone activity of both Skp and DegP is required to support growth [16].

Two models have been put forward to explain the functional relationship between SurA and Skp in *E. coli*. In the first model, SurA and Skp/DegP function in two parallel, partially redundant pathways [16]; the SurA pathway transports most β-barrel proteins across the periplasm, while the Skp/DegP pathway rescues substrates that fall off the SurA pathway [20]. The major argument in favor of this model is that depletion of SurA in a *skp* strain significantly decreases OM density due to the decreased delivery of OMPs to the OM, and that this depletion leads to cell death. In an alternative model, it has been suggested that Skp and SurA cooperate sequentially in β-barrel assembly: Skp interacts first with unfolded OMPs as they enter the periplasm, and SurA is involved in a later stage, assisting the folding of the β-barrel proteins when they arrive at the Bam insertion machinery [34]. We do not favor this model, as we would expect that two proteins functioning sequentially would not show the synthetically lethal phenotype that has been observed.

Clearly, fundamental questions about the role of these chaperone pathways in β-barrel assembly remain unanswered and addressing them will require the use of novel experimental approaches to overcome the technical difficulties inherent in working with membrane proteins. We sought to clarify the relationship between SurA, Skp, and their substrates using a differential proteomic approach based on 2D-LC-MS/MS. By monitoring protein abundance on a global level, this technique provides invaluable insight into how the loss of a chaperone affects the protein content of the cell envelope. We had previously determined the impact of *surA* deletion on the OM proteome [21]. Here, we determined how the elimination of Skp or of both Skp and SurA affects the OM protein content.

2 Materials and methods

Bacterial strains

Bacterial strains used in this study are described in Table S1 (Supporting Information). All strains are derivatives of the *E. coli* K-12 strain MC4100 [35]. All alleles were moved by P1 transduction using standard procedures [36]. The SurA depletion strain was constructed by transducing the λInCh pBAD-*surA-bla* construct from MB292 [20] into an MC4100 strain containing Δ*(araBAD)567* from the BW25113 strain. The resulting strain was then transduced with the Δ*surA*::kan Keio allele from the Keio collection [37], and the kan cassette was flipped out using the unstable plasmid pCP20 [38], generating strain JAS260. JAS261 was constructed by transducing the Δ*skp*::kan Keio allele [37] into JAS260, and flipping out the kan cassette [38]. To achieve more rapid depletion, a lysate made from the *ara*+ strain NR754 [39] was transduced into both strains. JAS417 was constructed in a similar way: the *ara*+ mutation was transduced into an MC4100 strain containing the cured Δ*skp* allele from the Keio collection [37, 38] and the Δ*(araBAD)567* from the BW25113 strain.

Strains NR744 and NR745 were constructed by transducing the *zae*-502::Tn*10* insertion [40] either alone or linked to a Δ*skp* allele [27] into MC4100. To confirm the presence of the *skp* deletion in NR745, the resulting tet^R transductants were screened by PCR using primers external to the deletion. Unless indicated, all bacterial cultures were grown under aerobic

conditions at 37°C in LB media. When appropriate, media was supplemented with 0.2% L-arabinose (w/v).

Growth conditions

NR744 and NR745 cells were grown aerobically in LB medium at 37°C to an OD_{600} of 0.8. Overnight cultures of the *surA* depletion strain (JAS260) and the *surA* depletion *skp* strain (JAS261) were grown in LB medium in the presence of 0.2% L-arabinose (w/v). The overnight cultures were then washed three times with fresh LB medium and subcultured at a 1:100 dilution in LB media with or without 0.2% L-arabinose. After 3 hours of growth, the cultures were diluted 100 fold in LB with and without 0.2% L-arabinose to an OD_{600} of about 0.03-0.04. Cells were grown for two more hours at 37°C.

Preparation of outer membrane fraction and proteolytic digestion

Envelope proteins were extracted by using the osmotic shock procedure as described by Vertommen *et al.* (2009) [21] and OM proteins isolated by ultracentrifugation at $180,000 \times g$ for 1 h at 4°C. Protein concentration was determined using the Bradford assay.

150 μg of OM proteins were then precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% v/v, followed by incubation on ice for 30 min. Samples were centrifuged at $15,000 \times g$ for 20 min and the resulting pellets washed with 5% ice-cold TCA (v/v). The pellets were then resuspended in 100 μl of denaturing buffer (6M urea, 200 mM Tris-HCl pH 8.5, 10 mM EDTA) supplemented with 10 mM dithiothreitol (DTT) to reduce disulfide bonds. After a one hour incubation at 25°C, we added 100 μl of denaturing buffer supplemented with 100 mM iodoacetamide to titrate out the remaining DTT and alkylate all reduced cysteines. The reaction was stopped by addition of 10% TCA (v/v) and the proteins were collected by centrifugation at $15,000 \times g$ for 20 min. The resulting pellet was successively washed with 5% TCA (v/v) and 100% ice-cold acetone, dried in a Speedvac and resuspended by sonication in 50 μl 0.1 M NH_4HCO_3 pH 8.0, containing 0.2% (w/v) RapiGest (Waters). The samples were then diluted two fold with 50 mM NH_4HCO_3 pH 8.0 containing 3 μg sequencing grade trypsin, and digested overnight at 30°C. The acido-labile detergent was then removed by acidification with HCl according to the manufacturer's instructions and the samples were stored at −20°C.

Differential analysis of OM proteins by label-free 2D-LC-MS/MS

Peptides were loaded onto a strong cation exchange column GROM-SIL 100 SCX (100×2 mm, GROM, Rottenburg, Germany) equilibrated with solvent A (5% acetonitrile v/v, 0.05% v/v formic acid pH 3.5 in water) and connected to an Agilent 1100 HPLC system. Peptides were separated using a 50 min elution gradient that consisted of 0%-50% solvent B (5% acetonitrile v/v, 1 M ammonium formate adjusted to pH 3.5 with formic acid in water) at a flow rate of 200 μl/min. Absorbance was monitored at 280 nm to ensure that all samples contained similar amount of material. Fractions were collected at 2 min intervals (20 in total) and dried using a Speedvac. Peptides were resuspended in 10 μl of solvent C (5% acetonitrile v/v, 0.1% v/v TFA in water) and analyzed by LC-MS/MS as described below.

The LC-MS/MS system consisted of an LTQ XL ion trap mass spectrometer (ThermoFisher, San José, CA, USA) equipped with a microflow electrospray ionization source and interfaced to an LCPackings Ultimate Plus Dual gradient pump, Switchos column switching device, and Famos Autosampler (Dionex, Amsterdam, Netherlands). Two reverse phase peptide traps C18 Pepmap 100 Dionex (0.30 mm × 5 mm) were used in parallel with two analytical BioBasic-C18 columns from ThermoFisher (0.18 mm × 150 mm). Samples (6.5 μl) were injected and desalted on the peptide trap equilibrated with solvent C at a flow rate of 30 μl/min. After valve switching, peptides were eluted in backflush mode from the trap

onto the analytical column equilibrated in solvent D (5% acetonitrile v/v, 0.1% v/v formic acid in water) and separated using a 100 min gradient from 0% to 70% solvent E (80% acetonitrile v/v, 0.1% formic acid in water) at a flow rate of 1.5 µl/min.

The mass spectrometer in positive mode was set up to acquire one full MS scan in the mass range of 400-2000 m/z, followed by five MS/MS spectra of the five most intense peaks in the mass range 400-1500 m/z. The dynamic exclusion feature was enabled to obtain MS/MS spectra on co-eluting peptides, and the exclusion time was set at 1 min.

Protein identification and quantification

Raw data collection of approximately 130,000 MS/MS spectra per 2D-LC-MS/MS experiment was followed by protein identification using Sequest. In detail, peak lists were generated using spectrum selector in Proteome Discoverer 1.2. From raw files, MS/MS spectra were exported as individuals files in .dta format with the following settings: peptide mass range: 400-3500 Da, minimal total ion intensity: 500, minimal number of fragment ions: 12, precursor mass tolerance: 1.1 Da. The resulting peak lists were searched against a target-decoy *E. coli* protein database (release 18.01.2007, 8690 entries comprising forward and reversed sequences) obtained from Swissprot (ftp://ftp.expasy.org/databases/complete_proteomes/fasta/) using Sequest by comparison with the theoretical spectra of all possible peptides fragments from the target-decoy database. The following parameters were used: trypsin was selected with proteolytic cleavage only after arginine and lysine but not at Arg/Pro or Lys/Pro, number of internal cleavage sites was set to 1, mass tolerance for precursor and fragment ions was 1.1 Da and 1.0 Da, respectively, considered variable modifications were +15.99 Da for oxydized methionine and +57.02 Da for carboxyamidomethylcysteine. Peptide matches were filtered using charge-state *versus* cross-correlation scores (Xcorr) ensuring an estimated false-positive rate below 5% calculated by target-decoy database searching. The filtered Sequest output files for each peptide were grouped according to the protein from which they were derived using the multiconsensus results tool within Proteome Discoverer. The analysis was repeated on three biological replicates for each strain. Sampling statistics such as unique peptides, spectral counts and sequence coverage were exported in Microsoft Excel spreadsheets. The spectral counts data were normalized by dividing the protein spectral count in a particular experiment by the average spectral count across all the proteins in that experiment. Relative quantification of protein abundance was estimated by calculating, after normalization, the ratio of spectral counts. Statistical significance was tested with the unpaired Student's *t*-test and significance was defined as a $P < 0.05$ (two-tail two-sample equal variance test). The extracted peak list for all datasets were exported in Mascot Generic Format and made freely available at the Tranche Network (http//proteomecommons.org) under the following Hash code: c/huk7kU9mKvkgIJ7A7RtYb6YfvAAWe1vnnwk5jPhOlj2uwlzvxrmnuZxvi4DbpvpKFu5eoc xb mC8LoK3FVyuFSldeAAAAAAAAAGuQ==

Immunoblotting

Complete depletion of SurA in either the *surA* depletion strain (JAS260) or the *surA* depletion *skp* strain (JAS261) was achieved as described in *Growth conditions*. The *skp* strain (JAS417) was subjected to the same treatment as the depletion strain. At 5 hours after the initial subculture, 1 mL samples were pelleted (16,000 × *g*, 1 min), and re-suspended at a volume equal to the $OD_{600}/7$ in SDS sample buffer containing β-mercaptoethanol and boiled. 10 µL of each re-suspended sample was analyzed by SDS-PAGE. Immunoblotting was done using rabbit anti-LamB antibody (which cross-reacts with OmpA) [41] at a 1:30,000 dilution and rabbit anti-BamA antibody [42] at a 1:20,000 dilution. Donkey anti-rabbit antibody conjugated to horseradish peroxidase (GE Life Sciences) was used at a

dilution of 1:8,000, with the ECL antibody detection kit (GE Life Sciences) to visualize the Western blots.

RNA extraction and RT-qPCR

Depletion of SurA was achieved as described under *Growth conditions*. RNA was prepared from 1 mL of a 5 mL culture 5 hours after the initial subculture using the Ambion Ribopure™-Bacteria Kit, including the optional DNase I treatment described in the manual. cDNA was made from the RNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit using the protocol described in the manual. The Reverse Transcriptase was inactivated by the addition of 15 µL 1 N NaOH. After a 30 minute incubation at 65°C, 15 µL of 1 N HCl were added. The cDNA was purified using the Qiagen MinElute PCR Purification Kit in an elution volume of 15 µL. The purified cDNA was then diluted 1:40. The qRT-PCR was performed using Applied Biosystems 7900 HT Fast Real-Time PCR System, in 25 µL reactions using PerfeCTa™ SYBR® Green FastMix™ ROX by Quanta Biosciences, Inc. Analysis was done using Applied Biosystems SDS software, version 2.3. Cycle threshold (Ct) values were determined after automatic adjustment to the baseline. For comparative, quantitative analysis, transcript levels were normalized to the level of *rpoD* and the ΔCt was determined. The comparative quantitation method (ΔΔCt) was used to compare the different strains and transformed to absolute values for obtaining relative fold changes. All the assays were performed in triplicate on each of three independent RNA preparations.

3 Results

Proteomic analysis of the skp strain

We first examined the impact of *skp* deletion using a more comprehensive and precise approach than had been previously undertaken by comparing the OM protein content of the NR745 *skp* strain to that of the NR744 wild-type strain by label-free 2D-LC-MS/MS. This approach allows a global and semi-quantitative analysis of protein expression ratios. This is a reliable and powerful technique that recently allowed us to identify the substrates of the protein folding catalysts DsbA and DsbC [43] and to characterize the role of the chaperone SurA in β-barrel assembly [21]. For quantification of protein abundance, we used the number of spectral counts (SC) reported for every protein (See Supporting Information Tables S2 and S3). The number of SC for a protein is the total number of MS/MS spectra taken on peptides from this protein in a given 2D-LC-MS/MS analysis. This value is linearly correlated with the protein abundance [21, 43, 44]. It is important to note that the number of SC can be used to compare the abundance of a given protein between two different strains but not to compare the abundance of different proteins in the same biological sample [21].

In order to identify the β-barrel proteins that are affected by the loss of Skp, we prepared OM extracts from wild-type and *skp* strains using a protocol similar to that described in [21]. Briefly, an extract containing periplasmic and OM proteins was prepared, the OM proteins were isolated by ultracentrifugation and acid-precipitated. After solubilization with an acid-labile surfactant, the OM proteins were digested with trypsin and the pool of tryptic peptides was analyzed by 2D-LC-MS/MS. The experiments were repeated using three independent cultures for both strains. In total, 120 raw files were submitted to a database search using Sequest against a target-decoy *E. coli* database obtained from SwissProt. Results were filtered using charge state versus cross-correlation scores (Xcorr) and peptide probabilities to obtain a false positive rate below 5% calculated according to the formula (FPR= 2x false positives (FP)/(false positives (FP) + true positives (TP)).

An average of 786 ± 98 and 766 ± 102 proteins were identified for the wild-type and *skp* strain, respectively. These proteins included up to 90 OM proteins (lipoproteins and β-barrel proteins) as well as soluble proteins that contaminated the OM fraction. Importantly, 19 of the 20 proteins with the highest SC values corresponded to envelope proteins. However, only 63 OM proteins (23 β-barrel proteins and 40 lipoproteins) that could be reproducibly identified at least twice in the experiments under either condition were kept for further analysis. The SC values of those proteins are presented in the Supporting Information Table 2. We noted that the SC values of most proteins are similar in all three independent analyses of the same strain, which highlights the reproducibility of our approach. The average spectral count across all the proteins was 24.9 ± 1.0 and 22.2 ± 3.8 for the wild-type and *skp* strain, respectively, which denotes a similar sampling rate between samples from the two strains. We used these values for normalization (see Supporting Information Table 2). Moreover, the total number of SC reported for the OM proteins was similar for the wild-type and *skp* strains (see Supporting Information Table S2), which confirms that the amount of OM material used for the proteomic analysis was similar.

To identify β-barrel proteins that are substrates of Skp, we searched for proteins that were significantly less abundant in the OM of the *skp* strain. We consider that the abundance of a protein is changed if it is decreased or increased by at least 2-fold. To test the significance of the data, we used the unpaired Student's *t* test and defined significance as a $P < 0.05$ (2-tail 2-sample equal variance test). We did not identify a single OMP that was significantly affected by the deletion of *skp*. As we might have expected, given the dependence of lipoproteins on the LolA pathway rather than the SurA or Skp/DegP pathways [7], only one lipoprotein (LptE) out of the 40 identified was present at decreased levels and none were present at increased levels. However, the decrease in LptE levels is barely within our defined criteria for significance described above, and we were unable to confirm this decrease by Western blot analysis. Thus, the *skp* mutant has an envelope proteome that is indistinguishable from that of the wild type, as reflected by the linear distribution observed when the SC values from the *skp* strain are plotted against those from the wild type (Figure 1). We confirmed the results of the 2D-LC-MS/MS analysis for a few selected proteins such as LamB, OmpA and BamA using Western blot analysis. Because OMPs that do not reach the OM are degraded in the periplasm, the levels of these proteins in whole-cell extracts are indicative of levels in the OM. We confirm that the levels of those proteins remain largely unchanged in a *skp* strain (JAS417) (Figure 4).

Proteomic analysis of the *surA* depletion *skp* strain

We further investigated Skp's role, as well as its functional relationship to SurA, by determining how the elimination of both SurA and Skp impacts the OM proteome. As explained above, *surA skp* mutants have a synthetically lethal phenotype [16]. In order to circumvent the lethality of the double mutant, we used a depletion strain. This strain is a double *surA skp* mutant in which a copy of the *surA* gene under the control of the *araBAD* promoter is inserted into the chromosome at the λ attachment site (strain JAS261). When cultured in the presence of L-arabinose, this strain is viable because SurA is produced. However, subculture of the strain in the absence of L-arabinose leads to the progressive depletion of SurA, growth of the strain slows and then ceases (Figure 2). Thus, this strain offers the unique opportunity to look at the physiological consequences of the loss of both SurA and Skp.

The *surA* depletion *skp* strain was first grown overnight with L-arabinose, and diluted 1:100 in the presence or absence of L-arabinose. After 3 hours, the cells were diluted 100-fold in fresh medium (with or without L-arabinose). After two additional hours of growth, corresponding to the time at which the cells cultured in the non-permissive condition cease

growing (Figure 2), envelope extracts were prepared and the OM proteins were analyzed by 2D-LC-MS/MS as explained above. This experiment was repeated on three independent cultures.

An average of 710 ± 183 and 1158 ± 461 proteins were identified when the *surA* depletion *skp* strain was grown under permissive and non-permissive conditions, respectively. Most of the additional proteins that were identified in the sample prepared under non-permissive conditions correspond to cytoplasmic proteins that were released during the osmotic shock procedure, probably because of the decreased envelope integrity of the *surA skp* double mutant. Here also, we analyzed only the 51 OM proteins (20 β-barrel proteins and 31 lipoproteins) that could be reproducibly identified at least twice in the experiments under either condition. The SC values of those proteins are presented in the Supporting Information Table S3. The average spectral count across all the proteins (9.6 ± 2.8 and 5.7 ± 1.5 when the strain was grown with or without L-arabinose, respectively) was not statistically different ($p=0.09$), indicating that the sampling rate is similar between samples prepared under the two conditions. We used these values for normalization (see Supporting Information Table S3).

Depleting SurA in a *skp* strain has a marked impact on the OM proteome and affects the abundance of both β-barrel proteins and lipoproteins. This is reflected by the dispersed distribution of the SC values when those reported for proteins from the *surA* depletion *skp* mutant grown with L-arabinose are plotted against those from the same strain grown in the absence of L-arabinose (Figure 3A).

We found that SurA depletion in the *skp* strain leads to a significant decrease in the abundance of 14 β-barrel proteins (representing about 70% of the identified β-barrels) (Figure 3B and Table 1). These proteins include the abundant proteins OmpA, OmpF and LamB, the unusual β-barrel protein TolC, as well as FhuA and LptD. The latter two proteins, which were identified previously as SurA-dependent substrates [21], were the most affected by the depletion of SurA. An additional protein, FadL, is also decreased but fails the *t*-test. The proteomic data were confirmed for a few selected proteins, such as LamB and OmpA, using Western blot analysis (Figure 4).

Interestingly, whereas most β-barrel proteins were affected by the loss of SurA and Skp, the levels of a subset of OM proteins did not appear to be significantly decreased. The most striking example is the essential β-barrel protein BamA, which appears to be present at approximately wild-type levels. This was also confirmed by Western blot analysis (Figure 4). The other β-barrel proteins that appear less affected by the simultaneous absence of SurA and Skp include the colicin IA receptor protein CirA, the phospholipase PldA, the MltA-interacting protein MipA and OmpW.

To further examine the contributions of transcription and assembly to the levels of select members of this group, we compared the levels of BamA and PldA mRNA by RT-qPCR when the *surA* depletion *skp* strain is grown with or without L-arabinose. As shown in Figure 5, we observed that growth under non-permissive conditions leads to the induction of *bamA* and *pldA* transcription by 1.9 and 2.4-fold, respectively. As we do not observe this same increase in the levels of BamA and PldA in the OM, it suggests that SurA depletion in the *skp* strain does affect the assembly of these proteins, neutralizing their increased synthesis.

Upon analysis of the OM lipoproteins, we found that the abundance of 14 lipoproteins is significantly increased (Table 1) when the cells are grown under non-permissive conditions. These proteins include the Bam complex lipoproteins BamD (formerly known as YfiO) and

BamC (formerly known as NlpB) and the LptD partner LptE, which all belong to the σ^E regulon [24]. This increase in levels, together with the increased synthesis of *bamA*, suggested that SurA depletion in the *skp* strain activates the σ^E response, which was verified by RT-qPCR (Figure 5). However, no lipoproteins are significantly decreased upon SurA depletion in the *skp* strain, which is consistent with their assembly depending on the dedicated chaperone LolA [7].

4 Discussion

The roles of SurA and Skp in β-barrel assembly have been studied using bacterial genetics and biochemistry. However, it has never been possible to fully characterize the OMP assembly defect in strains lacking these chaperones. Instead, previous studies have examined only a few model proteins. Clearly, there is a need for more comprehensive approaches in order to determine how the absence of SurA and Skp affects the global OM protein content and not only the few β-barrel proteins that constitute the bulk mass of OMPs.

In our analysis of the OM proteome of the *skp* mutant, we found no β-barrel proteins that were present at significantly decreased levels in the OM when compared to the wild-type strain. This indicates that SurA alone is sufficient to transport most β-barrel proteins across the periplasm. Perhaps surprisingly, given the strong dependence of some OM proteins (such as LptD) on the SurA pathway [21], no β-barrel proteins seem to strongly depend on the Skp/DegP pathway for their assembly, suggesting that there are no OMPs that use the *skp* pathway preferentially.

This new data is striking when compared to recent analyses of Skp's involvement in OMP biogenesis in other species of bacteria. In *Neisseria meningitidis*, levels of the OMPs PorA and PorB are decreased in a *skp* mutant, but not in a *surA* mutant [30]. It was also reported that there is no synthetic lethality associated with the *surA skp* strain in that bacterial species [30]. In *Shigella flexneri*, Skp is crucial for the assembly of the autotransporter IcsA. Remarkably, however, it is the N-terminal passenger domain of this protein that requires Skp, not the C-terminal β-barrel domain [45, 46]. Conversely, in *E. coli* at 25°, it has recently been shown that Skp interacts with the β-barrel domain of the autotransporter EspP [47]. It is clear that even homologous chaperones in different species function quite differently.

The new data also reveal that many OMPs in *E. coli* show no pathway preference. CirA, PldA and others were previously shown to be unaffected in a *surA* mutant [21] and are now shown to be unaffected in the *skp* mutant. However, in the absence of both chaperone pathways, the levels of almost every OMP we were able to analyze were decreased in the OM. Clearly, some proteins can use either pathway available to them, but require at least one of the two for efficient transport.

The ability of the Skp/DegP backup pathway to transport OMPs in the absence of the main SurA pathway may be additionally aided by the decrease in levels of the mRNAs for some of the most abundant β-barrels, such as OmpA and LamB, mediated by the σ^E stress response [24]. The reduced synthesis of these β-barrel proteins decreases the number of OMPs that the Skp/DegP backup pathway must chaperone, and so this pathway is sufficient, even in the absence of SurA. In fact, the levels of some OMPs are even higher in the OM of the *surA* strain [21], which is likely due to the combination of an increase in the synthesis of these proteins and the decreased load placed on the remaining chaperones and the Bam assembly complex, as has been suggested previously [21, 48, 49].

Although most OMPs are affected by the loss of both Skp and SurA, for a small number of proteins including BamA and PldA, there is no apparent decrease in OM abundance in the *surA* depletion *skp* strain. These proteins could depend on a third, unknown, chaperone pathway for their assembly, or they could be slower to deplete from the OM once SurA and Skp are gone. Alternatively, the unchanged levels of these proteins could result from the combination of two opposing processes: an increased synthesis of the protein and a decreased assembly in the cell envelope. Upon examining the synthesis of BamA and PldA, we found that their mRNA levels were indeed increased in the *surA* depletion *skp* mutant. Taking this increase in mRNA levels into account, we find that the seemingly unaffected PldA levels represent an assembly defect of about 75%. It is possible that all of the proteins with levels that seem relatively unaffected in the *surA* depletion *skp* strain follow this same pattern: an increase in synthesis masks their assembly defect.

It should be noted, however, that BamA appears to be an exceptional case. Taking the increase in mRNA levels into account, we find that BamA levels could represent an assembly defect of 50%. Clearly, even in the absence of both chaperones, a significant portion of it is able to reach the OM. As has been suggested by Bennion *et al.* [50], BamA's five Polypeptide-Transport-Associated (POTRA) [51] domains may play a role in preventing its aggregation in the periplasm. Thus, more work is required to clarify the chaperone requirement in the assembly pathway for BamA.

Consistent with the idea that the Skp/DegP pathway does not play a central role in β-barrel assembly when SurA is present, we did not observe the same increase in the levels of σ^E-regulated OM lipoproteins in the *skp* strain that was observed in a *surA* strain [21] or in the *surA* depletion *skp* strain. This suggests that the σ^E response is not strongly induced, and consistent with earlier reports, that the OM is not greatly perturbed in the absence of Skp [29]. However, levels of many σ^E-regulated OM lipoproteins are increased in the *surA* depletion *skp* strain [24]. Because OM lipoproteins are transported by the Lol pathway, independently of the β-barrel chaperones, an increase in transcription due to increased σ^E activity can be directly observed as an increase in levels at the OM (Figure 5). It is clear that even when SurA is depleted quickly, the OM assembly defect is severe enough to induce the σ^E response when Skp is also absent.

In the absence of SurA, the Skp/DegP pathway becomes essential for the transport of OMPs. In the absence of both pathways, levels of all the β-barrels that we could detect are decreased. Apparently, when the SurA and Skp/DegP pathways are compromised simultaneously, there is no chaperone pathway available to transport β-barrels to the OM. The global impact of the loss of both of these pathways means that we could obtain no evidence supporting the presence of an additional chaperone pathway in *E. coli*. There are a number of other periplasmic protein folding factors, including FkpA, PpiA, and Spy [1, 52]. These proteins may play a role in OMP assembly. However, these proteins by themselves cannot support the assembly of any OMP we could identify.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

OM	Outer Membrane
OMP	Outer Membrane Protein (β-barrel)
LPS	Lipopolysaccharides
PPIase	Peptidyl-Prolyl *cis-trans* Isomerase
LB	Luria Bertani
2D-LC-MS/MS	Two-Dimensional Liquid Chromatography Tandem Mass Spectrometry
EDTA	Ethylenediaminetetraacetic Acid
DTT	Dithiothreitol
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic Acid
SC	Spectral Count
IM	Inner Membrane
POTRA	Polypeptide-Transport-Associated

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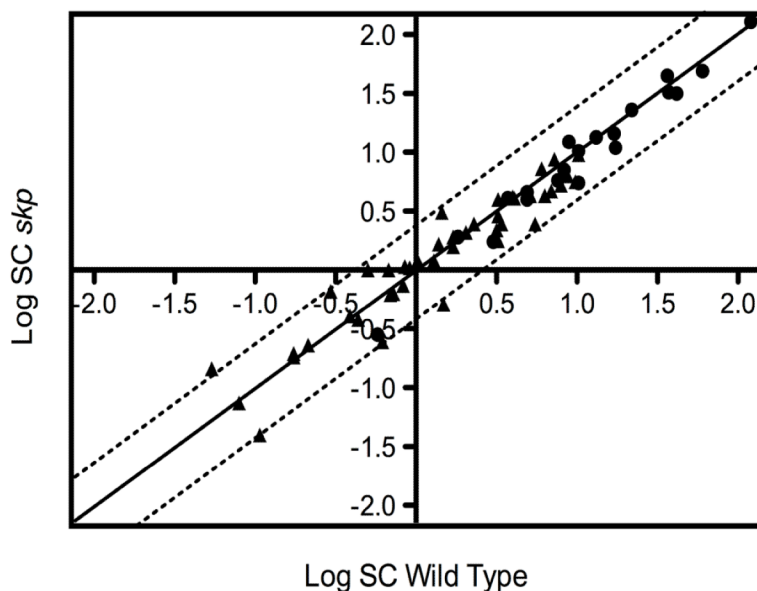
Figure 1. *skp* deletion has no effect on the OM proteome
Two-dimensional logarithmic plot for the mean SC values of the identified lipoproteins and β-barrel proteins. Spots on the diagonal and between the two dotted lines correspond to proteins that are not significantly affected by the loss of Skp (they differ in their SC values by a factor of less than 2). Closed circles (●) correspond to β-barrel proteins and closed triangles (▴) to lipoproteins. Only one spot corresponding to the LptE lipoprotein (above the upper dotted line) is decreased by more than twofold in the *skp* strain (NR745).
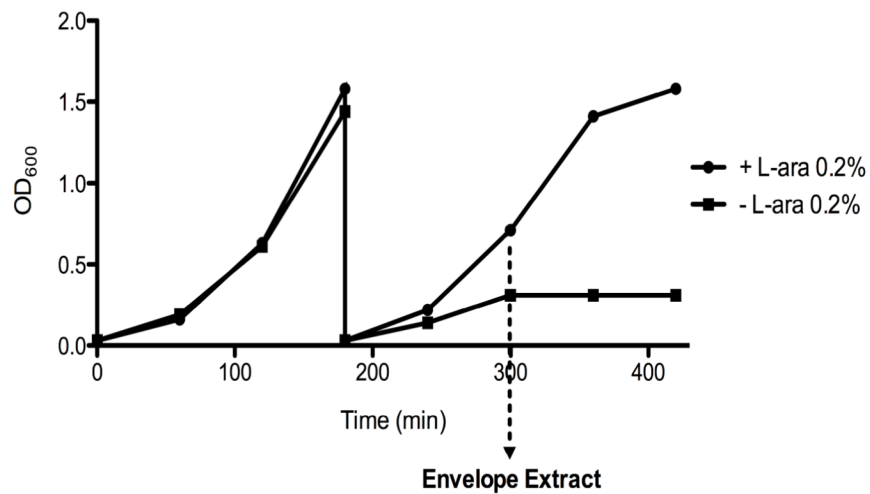
Figure 2. Bacteria cease growing upon SurA depletion in the *skp* strain
After 3 hours of growth at 37°C, cells were diluted 100-fold in a medium with or without 0.2% of L-arabinose. After two more hours, envelope proteins were extracted by an osmotic shock procedure. Growth of the *surA* depletion *skp* strain requires L-arabinose for viability. In the presence of L-arabinose (●), the depletion strain exhibits normal growth. In the absence of L-arabinose (■), cells cease growing. The data of a representative experiment are shown.
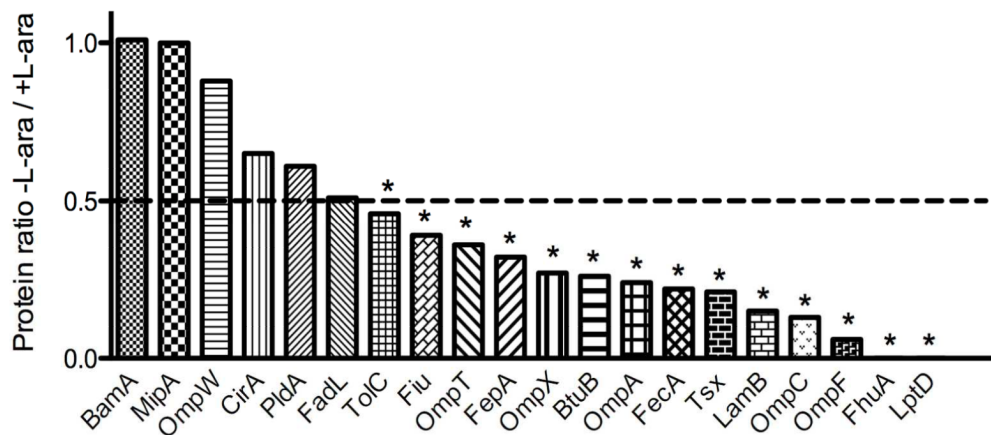
A.
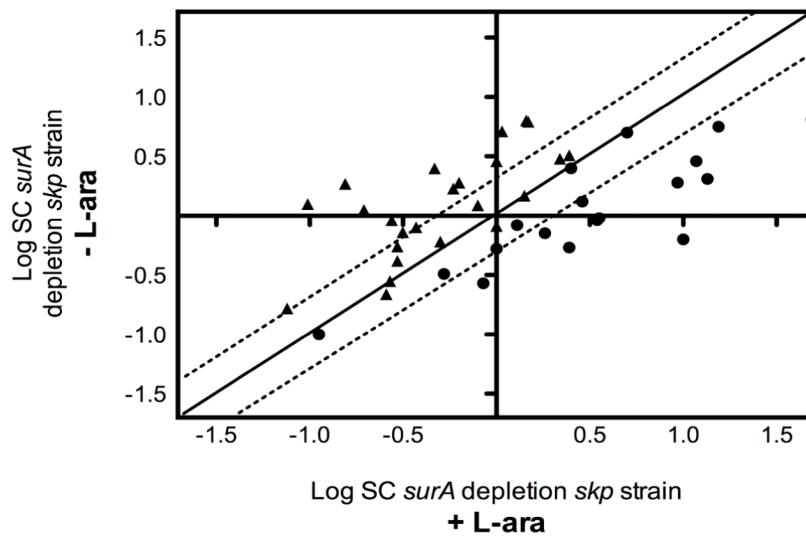
B.

Figure 3. The loss of SurA and Skp has a severe impact on the OM proteome
A. Two-dimensional logarithmic plot for the mean SC values of the identified lipoproteins and β-barrel proteins from the *surA* depletion *skp* strain grown in the presence or absence of L-arabinose. Spots on the diagonal and between the two dotted lines correspond to proteins that are not significantly affected by the depletion of SurA (they differ in their SC values by a factor of less than 2). Spots that are above the upper dotted line or below the lower dotted line correspond to proteins whose abundance is increased or decreased by more than two fold in the *surA* depletion *skp* strain, respectively. Closed circles (●) correspond to β-barrel proteins and closed triangles (▴) to lipoproteins. **B.** Ratio of OM proteins calculated by considering that SC values obtained for proteins from the *surA* depletion *skp* strain grown with L-arabinose are equal to 1. SurA depletion in the *surA* depletion *skp* strain leads to a significant decrease (more than two fold) in the abundance of 14 β-barrel proteins. The OM

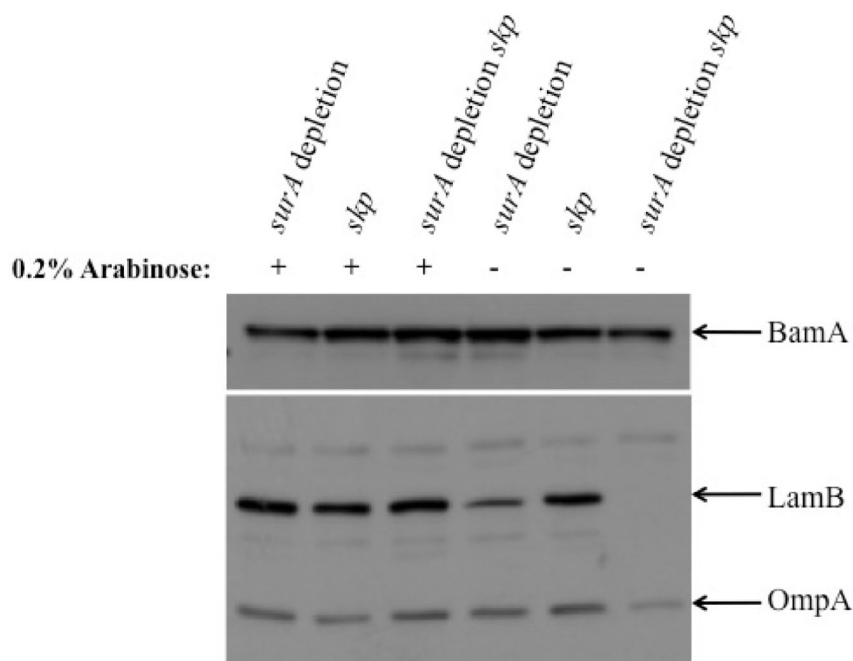proteins for which the decrease is statistically significant ($p < 0.05$) are indicated by an asterisk.

Figure 4. Western blot analysis confirms the proteomic results
Depletion of SurA in JAS260 (*surA* depletion strain) leads to increased levels of BamA (compare lane 4 to lane 1), as reported in our previous proteomic analysis of that strain [21]. When SurA is depleted in the *surA* depletion *skp* strain (JAS261), BamA levels appear only slightly decreased (compare lane 6 to lane 3), in good agreement with our 2D-LC-MS/MS analysis where they are not changed. The levels of LamB and OmpA are greatly decreased when the *surA* depletion *skp* strain (JAS261) is grown under non-permissive conditions (compare lane 6 to lane 3), as observed in the 2D-LC-MS/MS experiments. The levels of all proteins examined remained largely unchanged in the *skp* strain (JAS417) (lanes 2 and 5).
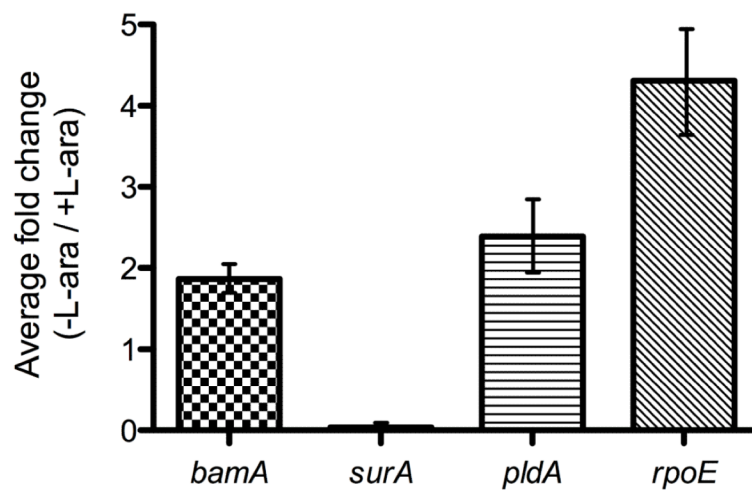
Figure 5. Synthesis of *bamA* and *pldA* increases upon SurA depletion in the *skp* strain and masks the assembly defect

The mRNA levels of BamA and PldA are increased (about 1.9 and 2.4-fold, respectively). Depletion of SurA also induces the *rpoE* gene, responsible for the induction of the σ^E response (about 4.3-fold induction).

Table 1

Outer membrane proteins identified by 2-D LC-MS/MS in the *surA* depletion *skp* strain

β-barrel proteins	Ratio[a]	Lipoproteins	Ratio[a]
BamA	1.01	YbjP	13.07[c]
MipA	1.00	YajI	11.94[c]
OmpW	0.88	LptE	5.78[c]
CirA	0.65	YedD	5.33[c]
PldA	0.61	BamD	4.78[c]
FadL	0.51	BamC	4.31[c]
TolC	0.46	YajG	4.17[c]
Fiu	0.39	OsmE	3.35
OmpT	0.36	LolB	3.04[c]
FepA	0.32	BamB	2.90[c]
OmpX	0.27	YdcL	2.89[c]
BtuB	0.26	MltC	2.26
OmpA	0.24	NlpI	2.17
FecA	0.22	CutF	2.13
Tsx	0.21	NlpD	1.89
LamB	0.15	MltA	1.56
OmpC	0.13	MltB	1.43
OmpF	0.06	Pal	1.38
FhuA	0.00	SlyB	1.31
LptD	0.00	VacJ	1.20
		YggN	1.04
		Lpp	1.04
		YgeR	0.83
		RlpA	0.82
		YeaY	0.00
		YncD	0.00
		YddW	0.00
		YgdI	*b)*
		YbaY	*b)*
		YgdR	*b)*
		YifL	*b)*

a) The ratios were calculated by considering that the SC values obtained for proteins from the depletion strain grown with L-arabinose are equal to 1.

b) Lipoproteins that were only detected when the strain was grown in the absence of L-arabinose.

[c)]Lipoproteins for which the ratio is statistically significant.