Analyzing the role of periplasmic folding factors in the biogenesis of OMPs and members of the type V secretion system

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Summary

The outer membrane (OM) of Gram-negative bacteria is highly packed with OM proteins (OMPs) and the trafficking and assembly of OMPs in Gram-negative bacteria is a subject of intense research. Structurally, OMPs vary in the number of β-strands and in the size and complexity of extra-membrane domains, being extreme examples the members of the type V protein secretion system (T5SS), such as the autotransporter (AT) and intimin/invasin families of secreted proteins, in which a large extracellular "passenger" domain is linked to a β-barrel that inserts in the OM. Despite their structural and functional diversity, OMPs interact in the periplasm with a relatively small set of protein chaperons that facilitate their transport from the inner membrane (IM) to the β-barrel assembly machinery (BAM complex), preventing aggregation and assisting their folding in various aspects including disulfide bond formation. This chapter is focused on the periplasmic folding factors involved in the biogenesis of integral OMPs and members of T5SS in *E. coli,* which is used as a model system in this field. Background information of these periplasmic folding factors is provided along with genetic methods to generate conditional mutants that deplete these factors from *E. coli* and biochemical methods to analyze the folding, surface display, disulfide formation and oligomerization state of OMPs/T5SS in these mutants.

Introduction

The biogenesis of OMPs initiates in the bacterial cytoplasm and encompasses their translocation across the IM to the periplasm via the Sec pathway before final folding and assembly in the OM by the β-barrel assembly machinery (BAM complex) (1-4). OMPs contain an N-terminal cleavable signal sequence that is recognized by the SecYEG translocon. In the cytoplasm, OMPs bind to SecB, which maintains them in a fully unfolded state until they reach the translocase that drives translocation of the polypeptide across the IM in an unfolded conformation (5). Subsequently, in the periplasm the polypeptides are assisted by different types of folding factors and escorted to the BAM complex for OM assembly (6, 7).

The identification of extracytoplasmic folding factors started in the early nineties with the discovery of disulfide bond formation by DsbA and the peptidylprolyl-cis-trans-isomerase PpiA (8, 9). In 1996, as a result of the search for proteins that decreased the σE-dependent response constitutively induced by the accumulation of misfolded proteins in the periplasm, SurA, FkpA and Skp proteins were identified as periplasmic folding factors (10). In that same year, SurA and Skp were shown to bind and assist the folding of OMPs, confirming their function as molecular chaperones (11, 12). Since these initial discoveries, the work of the past decades has led to the identification of numerous periplasmic folding factors, the obtention of their crystalized structures and elucidation of the molecular mechanisms and events that participate in the biogenesis of OMPs (1-4, 13-17).

The periplasm contains numerous folding catalysts, that in some cases present overlapping functions: chaperones for preventing undesirable off-pathway interactions or aggregation of improperly folded polypeptides (e.g. Skp, SurA, DegP, HdeA and Spy), peptidyl–prolyl cis/trans isomerases (PPIases) catalyzing cis/trans isomerization of proline peptide bonds in proteins (e.g. SurA, FkpA, PpiA, PpiD), oxidorreductases that mediate the formation and exchange of disulfide bonds (e.g. DsbA, DsbC), and proteases (e.g. DegP). These proteins assist the folding of OMPs but also other types of periplasmic and membrane-associated proteins. The structure and function of the above mentioned folding factors have been reviewed recently (3, 13, 17, 18). In addition to the above-mentioned proteins, the periplasm of Gram-negatives contains other types of folding factors that are specifically dedicated to assist the subunits of surface organelles (e.g. fimbria, pili, etc.). The function of dedicated periplasmic chaperones of fimbrial subunits (e.g. PapD, FimC) has been reviewed recently (19).

The periplasmic chaperones are also involved in the protection of OMPs under stress conditions. Envelope stress induces the accumulation of misfolded or aggregated OMPs in the periplasm, which is lethal to the cell. To avoid cellular damage, Gram-negative bacteria have evolved signal transduction pathways (e.g. σE and Cpx systems) that sense and respond to stress, in part by upregulating periplasmic folding factors (e.g. SurA, Skp, FkpA) and proteases (DegP) (13, 20).

The SurA polypeptide is composed of four domains: an N-terminal domain, two central PPIase domains (PPIase 1 and 2) of the parvulin family and a short C-terminal domain (21). The N- and the C-terminal regions have chaperone activity, whereas the PPIase activity exclusively resides in the PPIase domain 2 (22), the PPIase domain 1 could be responsible for substrate selection because it was shown

to selectively bind peptides that are rich in aromatic residues and characteristic for OMPs (23). SurA has a strong preference for binding Ar-X-Ar tripeptide motifs, commonly found in the C-terminus of OMPs, being Ar any aromatic and X can be any residue (24). SurA binds OMPs immediately after they leave the Sec translocon before signal sequence cleavage (25), and it has been shown to interact with the AT serine protease EspP from enterohemorrhagic *E. coli* (EHEC) O157:H7 (26). It is also the may chaperone assisting folding of EHEC Intimin (27). Interestingly, SurA was also detected in association with BamA, the central component of the BAM complex (26, 28, 29). These and other experimental observations (30), have led propose that SurA binds unfolded OMPs in the periplasm and transports them to BamA, where they are taken by the periplasmic polypeptide transport-associated (POTRA) domains through a β-augmentation mechanism. SurA and BamB have been also suggested to function in the same folding pathway of numerous OMPs (25, 31-33). Recently, Ricci and collaborators have shown that defects in OMP assembly caused by mutations of BamA or BamB can be corrected by gain-of-function mutations in the PPIase 1 domain of SurA, suggesting that the activity of SurA could be regulated by interactions between its PPIase 1 domain and BamA/BamB (34).

Skp is a small protein of about 17 kDa that forms a homotrimer, which binds its substrate in a 1:1 stoichiometry. The crystallized trimer shows a structure with three α-helical tentacles protruding from a basal trimerization interface forming a central cavity that holds substrates protecting them from aggregation (35, 36). Recent structural and biophysical analysis showed that Skp forms transient interactions with OMP polypeptides that are sequence unspecific and weak, thus allowing Skp to interact with a broad range of different substrates. The authors speculate that the flexibility of the interaction between Skp and the OMP substrates could also facilitate its release to a downstream receptor in the Bam complex bearing higher affinity for a particular sequence in the OMP (37). *In vivo*, Skp binds unfolded OMPs while they are still engaged in translocation through Sec translocon (38, 39). Site-specific photocrosslinking experiments have demonstrated that Skp interacts with the AT EspP, which is at the same time engaged with components of the BAM complex (26, 40). Intriguingly, although Skp was shown to interact with more than 30 envelope proteins of *E. coli* (40-42), to date no OMP appears to depend preferentially on Skp for folding in *E. coli* (43). However, recent experiments in *Neisseria meningitidis* demonstrated that two major OMPs, PorA and PorB, use Skp preferentially (44), and Skp was demonstrated to be required for the efficient assembly of the AT IcsA in *Shigella flexneri* (45).

DegP belongs to the serine proteases high temperature requirement (HtrA) family and is considered both a chaperone and a protease (46-48). The DegP monomer is composed of a N-terminal trypsin-like protease domain and two C-terminal PDZ domains allowing DegP to recognize its substrates (47). DegP exists in different oligomeric forms, ranging from a 6-mer to a 24-mer with a 3-mer as the fundamental building block. In the presence of the substrate, the homotrimers further oligomerize via PDZ1–PDZ2 interactions into oligomeric cage-like structures that exhibit both protease and chaperone activity (48-53). The likely reason for the different oligomeric states of DegP is to provide functional control of its efficient but rather indiscriminate proteolytic activity (52). DegP is upregulated

by the σE and the Cpx systems in response to heat shock or to other envelope stresses performing both the antagonistic functions of protein repair and degradation (13). DegP was proposed to act as a molecular chaperone mainly based on the observations that captures folded OmpA and OmpC (48). Interestingly, complementation experiments with a DegP protein mutated in its protease active site (DegP-S236A) rescued full expression of EspP in a *degP* mutant strain with severe reduction of EspP secretion (42). In the case of EHEC Intimin, DegP was shown to act mainly as a protease to degrade unfolded polypeptide in the periplasm (27). Recently, Ge and collaborators used genetically incorporated non-natural amino acids for the identification of DegP substrates by photocrosslinking and co-purification in *E. coli* cells, and identified several OMPs including OmpC, F, A, X, W and NmpC (54). In this work, the authors stated that DegP mainly functions as a protease with hardly any appreciable chaperone function (54).

Two pathways have been shown to assist the biogenesis of OMPs: SurA and Skp/DegP pathways. Genetic analysis performed in *E. coli* indicated that bacterial cells that lack either SurA or Skp are viable, but cells that lack SurA and Skp or SurA and DegP are not viable (synthetic lethality). This discovery suggested that DegP/Skp and SurA operate in parallel pathways that are functionally redundant (55). In *E. coli*, OMP assembly preferentially depends on SurA, suggesting that the DegP/Skp pathway functions as a backup to rescue OMPs that fall off of the SurA pathway, particularly under stressful conditions (27, 28, 43). The major arguments in favor of this hypothesis is that whereas strains lacking *surA* have a decreased OM density (28) and are hypersensitive to detergents and hydrophobic antibiotics, an indicative of OM perturbations (43), deletion of the *skp* gene only leads to a moderate reduction in OMPs (56) and shows a much less severe phenotype than *surA* mutants (28). In an alternative model, it has been proposed that Skp and SurA cooperate sequentially in the same pathway (57). Interestingly, it has been reported that both SurA and Skp perform distinct roles in the biogenesis of the essential OMP LptD, indicating that both pathways might act in concert to efficiently assemble certain OMPs (58). Despite of the numerous findings, the exact role of the various players involved is far to be clear (59). It is still difficult to provide a detailed description of the role of the periplasmic folding factors that is universally applicable to all OMPs. The preference for periplasmic folding factors seems to be protein and potentially species-specific (14).

Besides SurA, the periplasm of *E. coli* contains several other PPIases: PpiD and PpiA, whose function in OMP biogenesis remains to be clarified (17), and FkpA. FkpA belongs to the FKBP family of PPIases, presents general chaperone activity (60) and is upregulated by the σE system in response to envelope stress (61). Structuraly, FkpA is a V-shaped homodimer comprised of two monomers, each containing a C-terminal domain with PPIase activity and an N-terminal dimerization domain constituted by three α-helices that accommodate the chaperone activity (62). The activity of FkpA as a periplasmic chaperone was initially demonstrated using heterologous proteins expressed in *E. coli*. Although there is limited data regarding the natural substrates of this folding factor, FkpA was shown to bind unfolded EspP passenger domain with high affinity (63), and recently it has been proposed that this chaperone plays a role in the folding of LptD and FhuA (59, 63).

The periplasmic disulfide bond catalysts DsbA and DsbC that carry out disulfide bond formation and isomerization have been shown to improve both the speed of otherwise slow steps in protein folding and the stability of proteins. DsbA is a monomeric protein with a thioredoxin fold and has a CXXC catalytic motif with a redox-active cysteine pair (Cys30– Cys33) (64). It has been suggested that DsbA is the main oxidant in the periplasm having more than 300 *in vivo* substrates in *E. coli* (65), including Imp (66), OmpA (67), or the passenger domains of intimin (27) and IcsA (68). DsbA also forms disulfide bonds in LptD, the OMP translocon of LPS (69). It has been shown that DsbA introduces disulfide bonds nonspecifically between consecutive cysteines into proteins entering the periplasm (70), without regard for the correct disulfide pairing that is found in the native conformation. The isomerization of incorrect disulfide bonds is catalyzed by the disulphide isomerase DsbC (71). DsbC is a V-shaped homodimeric protein, and each monomer consists of two domains: an N-terminal dimerization domain and a C-terminal thioredoxin-like domain with a CXXC motif bearing with a redox-active cysteine pair (Cys98-Cys101) (72), which should be maintained in its reduced state for proper isomerase activity in a process mediated by the disulfide reductase DsbD (73). Currently, it is becoming apparent the importance of Dsb systems for bacterial pathogenesis, and numerous Gram-negative pathogens either encode several copies of their Dsb genes or posses functional Dsb paralogs (74). The study of *dsb* mutants of various bacterial pathogens has shown an impaired assembly of different adhesive organelles (18) and attenuation (75, 76), revealing the implication of Dsbs in bacterial virulence and, importantly, that disulfide formation systems are interesting targets for the design of new antimicrobial drugs.

Overall, a possible picture for OMP biogenesis is that major OMPs and certain ATs and LptD may recruit Skp and/or FkpA at an early stage for preventing aggregation. SurA plays a role for targeting the OMP β-domain and the AT passenger domain of EspP to the BAM complex (3, 40, 57, 77). Although the function of FkpA is still unclear, it has been reported that this protein assists Skp in the folding of certain OMPs (59). FkpA may be a novel quality control factor under heat-shock conditions (78). DsbA and DsbC catalyze the formation of disulfide bonds in cysteine-containing polypeptides. The role of DegP as a chaperone still remains to be elucidated but it clearly plays an important role as a protease in the quality control of OMP biogenesis. In general, translocation incompetent OMPs are degraded by DegP, relieving the cell from the toxic effects associated with the presence of misfolded polypeptides.

Despite the recent advances and the intense research in OMP biogenesis, we still have a relatively poor understanding of how OMPs and ATs, as structural challenging substrates, are maintained in a translocation competent state and transported across the periplasm by the different folding factors. Also, defining the exact role of the different players and how they interact and regulated are important questions that remain unanswered. It is becoming apparent that even homologous chaperones in different species function quite differently. Hence, further studies are thus required to allow an in-depth understanding of the molecular mechanisms that govern the periplasmic transit and assembly of OMPs.

Section I. Genetic strategy for analyzing the role of periplasmic folding factors

1. Obtention of conditional depletion mutants of periplasmic folding factors in *E. coli*

The evidences for the functional role of periplasmic chaperones have been defined *in vivo* using genetically modified strains bearing either null mutations, or in conditional depletion strains, in which the expression of the chaperone gene can be regulated allowing control of protein levels (28, 55).

The procedure presented here describes the generation of a conditional mutant strain in which an inducible araC-P_{BAD} promoter cassette replaces the native promoter of *surA* on the chromosome (Figure 1). The transcriptional activity of the P_{BAD}::*surA* allele can be controlled by the presence in the growth medium of the inducer L-arabinose or the repressor D-glucose. This strategy was used to obtain conditional mutants in *surA* (27, 33) and *bamA* (27). This approach can be easily adapted to the periplasmic folding factor of interest in each case.

In this approach we adapted a method developed by the laboratory of Dr Jean-Marc Ghigo that combines the lambda-red linear DNA recombination method with site-directed insertion of a repression and expression (RExBAD) cassette that replaces a native promoter with a functional pBAD promoter (79). The mutant strains described here were obtained by one-step inactivation of chromosomal genes with PCR products in *E. coli* cells bearing the bacteriophage lambda-red recombination system (80, 81).

To avoid recombination in the *araC* gene we generated an *E. coli* UT5600 knockout strain devoid of *araC* termed UT5601. Subsequently, this strain was used for generating the RExBAD conditional mutant with regulated *surA* expression termed UTP_{BAD}::s*urA*.

2. Materials

2.1. Bacterial culture

1. Incubators and shakers set at 30°C, 37°C or 42°C.

2. Spectrophotometer and cuvettes for measuring optical densities of bacterial cultures.

3. LB medium: 10 g tryptone, 5 g yeast extract and 10 g NaCl. Low salt LB medium: 10 g tryptone, 5 g yeast extract and 5 g NaCl. Combine the dry reagents above and add distilled water to 950 ml. Adjust pH to 7.5 with 1 N NaOH and bring the volume up to 1 liter. SOC medium: Add the following to 900 ml of distilled H_2O, 20g Tryptone, 5 g Yeast Extract, 2 ml of 5M NaCl, 2.5 ml of 1M KCl, 10 ml of 1M $MgCl_2$, 10 ml of 1M $MgSO_4$. Adjust to 1L with distilled H_2O. BHI broth medium: 37 g of powder with 1 liter of distilled water. Autoclave on liquid cycle at 15 psi and 121°C for 20 min and store at room temperature. For LB agar plates, add 15 g agar to 1 liter of LB, autoclave as above and allow it to cool at room temperature for at least 55°C. Add antibiotics as needed, and pour into 100 mm×15 mm petri plates using 25–30 ml per plate.

4. Ampicillin (sodium salt). Dissolve 1 g of sodium ampicillin in distilled deionized water to a final volume of 10 ml. Filter-sterilize the ampicillin solution through a 0,22 μm filter. Store the ampicillin in aliquots at -20°C (or at 4°C for 3 months).

5. Kanamycin (monosulfate). Make kanamycin to a final concentration of 50 mg/ml in distilled deionized water. Filter-sterilize as above and store in aliquots at −20°C.

6. Zeocin. The stock solution is dissolved at 100 mg/ml in distilled deionized water. Filter-sterilize as above and store in aliquots at −20°C. Do not store at 4°C for more than a week. (See Note 1).

7. 20% L-Arabinose stock. Dissolve 10 g in 50 ml of distilled deionized water and filter sterilize.

8. 20% D-Glucose stock. Dissolve 10 g in 50 ml of distilled deionized water and filter sterilize.

9. TG1zeoRExBAD *E. coli* bacterial strain is a gift of Dr. Jean-Marc Ghigo. The zeoRExBAD (zeoR araC PBAD) promoter cassette is a modification of the catRExBAD cassette (79).

10. UT5600 *E. coli* bacterial strain (82).

11. pKD4 plasmid: Template for gene disruption. OriR6Kgamma, (AmpR, KmR), rgnB(Ter). The Km resistance gene is flanked by FRT sites. Gene Bank: AY048743 (81).

12. pKD46 plasmid: Lambda Red recombinase expression plasmid. repA101(ts), araBp-gam-bet-exo, oriR101, (AmpR). Gene Bank: AY048746 (81).

13. pCP20 plasmid: Has the yeast Flp recombinase gene, ts-rep, [cI857](lambda)(ts), (AmpR, CmR) (83).

2.2. Recombineering reagents

1. Thermocycler (e.g., T100 Thermal Cycler, BioRad).

2. Biorad MicroPulser Electroporator (#165-2100).

3. Electroporation cuvettes – sterile, 0.2 cm gap, package of 50 (Bio-Rad, 165-2086).

4. Agarose. Use at 0.75–1.5% for analysis of PCR products.

5. Vent DNA polymerase (NEB, M0254S). Enzyme used for generating PCR recombineering substrates.

6. Taq DNA polymerase. Enzyme used for colony PCR.

7. DpnI enzyme (NEB, R0176S). Restriction enzyme used to cleave methylated DNA.

8. QIAprep Spin Miniprep kit (Qiagen). Used for the isolation of plasmids.

9. QIAquick PCR purification kit (Qiagen) and Qiaex II gel extraction kit (Qiagen). Used for the purification of PCR products to be used as substrates for recombineering.

10. Elution buffer: 10 mM Tris–HCl, pH 8.5.

11. dNTPs: 2.5 mM each of dATP, dCTP, dGTP, dTTP.

12. Sterile distilled water and sterile distilled deionized water.

13. Glycerol.

14. Electroporation washing buffer: Dilute 50 ml of glycerol in 450 ml distilled deionized water and sterilize using 0,22 μm filters. Store at 4°C.

2.3. Denaturing SDS-PAGE

1. Electrophoresis system: Bio-Rad Mini-PROTEAN 3 or equivalent. Electrophoresis power supply (PowerPac Basic Power Supply. Bio-Rad, 164-5050EDU) or equivalent.

2. Running gel buffer (1 M Tris-HCl, pH 8.8): Dissolve 90.9 g Tris-base in 1 L of dH_2O, adjust to pH 8.8 with HCl.

3. Stacking gel buffer (1 M Tris-HCl, pH 6.8): Dissolve 30.3 g Tris-base in 1 L of dH_2O, adjust to pH 6.8 with HCl.

4. 30% Acrylamide solution: 29.2% acrylamide/0.8% bis-acrylamide (See Note 2).

5. Ammonium persulfate (APS) 25%. Dissolve 2.5 g APS in a total volume of 10 ml of distilled water. Filter (0.45 μm) and make 1-ml aliquots. Store a working aliquot at 4°C and the rest of the aliquots at –80°C.

6. 10% SDS. Dissolve 10 g of SDS in 80 ml of distilled water, and then add distilled water to 100 ml. This stock solution is stable for 6 months at room temperature. Caution: Wear a dust mask for protection against breathing SDS powder.

7. Tetramethylethylenediamine (TEMED).

8. 5X Running buffer: 5X Electrophoresis buffer: 15.1 g Tris base, 72 g glycine, and 5 g SDS in 1 liter of distilled water.

9. 2× sample buffer: Dissolve 2 mg bromophenol blue in 1.25 ml of 1 M Tris–HCl (pH 6.8), 2 ml of glycerol, 4 ml of 10% SDS, 0.5 ml of β-mercaptoethanol (2-ME), and 2.25 ml of dH_2O. Store in aliquots at −20°C.

10. Molecular weight marker (e.g., a prestained protein markers, Bio-Rad, or equivalent).

2.4. Western blotting

1. Semidry transfer apparatus (e.g., Trans-Blot SD, Bio-Rad, or equivalent).

2. 5X transfer buffer: 15.1 g Tris and 72 g glycine in 1 liter of distilled water.

3. 1X transfer buffer: Mix 300 ml of H_2O, 100 ml of 5X transfer buffer, 100 ml of methanol, and 1.9 ml of 10% SDS.

4. PVDF membrane (e.g., Immobilon-P, Millipore).

5. Methanol

6. Extra-thick blot paper (e.g., Bio-Rad); 2–3 mm thickness.

7. Primary antibody: anti-GroEL monoclonal antibody-peroxidase (POD) conjugate (1:5,000; Sigma) and anti-OmpA (1:20,000; a gift of Hiroshi Nikaido), anti-Skp (1:1,000; a gift of Matthias Mueller), anti-MBP-DegP (1:5,000; a gift of Michael Ehrmann), anti-SurA (1:10,000; a gift of Roberto Kolter), anti-Fim D (33).

8. Secondary antibody: bound rabbit antibodies were detected with protein A-POD conjugate (1:8,000; Zymed) (See Note 3).

9. PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml of distilled water. Adjust pH to 7.4 with HCl; add distilled water to 1 liter and autoclave.

10. PBS-0.1% Tween 20 (PBST): Mix on magnetic stirrer 1 ml Tween 20 (Sigma, P-1379) in 1 liter of PBS.

11. Blocking and antibody dilution solution: dissolve 5 g of non-fat dry milk in 100 ml of PBST.

12. X-ray film.

3. Methods

3.1. Obtention of UT5600 ΔaraC (UT5601) *E. coli* strain

1. The targeting substrate for recombineering is a PCR product consisting in a Kanamycin (Km) drug marker flanked by upstream and downstream regions of *araC* target site amplified from pKD4 plasmid template. The primers for the PCR are shown below. The 20 bases on the 3´end of each oligonucleotide (lower case) anneal to and amplify the Km cassette, the rest of the bases on the 5 end of each primer (upper case) contain the upstream sequence and the reverse complement of the downstream sequence.

AraC KO1 (5'- CCC TAT GCT ACT CCG TCA AGC CGT CAA TTG TCT GAT TCG TTA Cgt gta ggc tgg agc tgc ttc) and AraC KO2 (5'- CCG CCA AAG CTC GCA CAG AAT CAC TGC CAA AAT CGA GGC Cat atg aat atc ctc ctt agt) were used as primers for PCR on pKD4 template.

2. Prepare a PCR reaction (75 μl) as follows: 60,75 μl sterile deionized water, 7,5 μl 10× Vent PCR buffer (NEB), 1,5 μl dNTPs (10 mM), 1,5 μl primer AraC KO1 (20 μM), 1,5 μl primer AraC KO2 (20 μM), 1,5 μl pKD4 (10 ng) and 0,75 μl Vent polymerase (NEB) used as a high fidelity polymerase.

3. Perform standard PCR as follows: (step 1) 95°C, 4 min; (step 2) 94°C, 30 s; (step 3) 59°C, 30 s; (step 4) 74°C, 2 min. Repeat last three steps 29 times; (step 5) 74°C,

5 min; (step 6) hold at 4°C. The extension times should be increased for products expected to be longer than 1 kb. When completed, load 10 μl of the PCR on a 1% agarose gel to check for correct size and purity of the PCR product. Clean the PCR product with QIAquick PCR purification kit (Qiagen) and elute DNA in 50 μl of elution buffer.

4. Degrade chromosomal DNA that may be present in the sample with DpnI (NEB), a methylation-dependent restriction enzyme that cleaves Gm6A^TC sites. Mix 43 μl of the PCR product with 1 μl of DpnI (NEB) and 5 μl of 10X CutSmart Buffer (NEB) and incubate at 37°C for two hours.

5. Gel-purify the PCR product using a gel extraction kit (Qiaex II, Qiagen) following manufacturer´s instructions and elute in 50 μl of sterile deionized water.

6. Transform UT5600 *E. coli* bacterial strain (82) with Red-recombineering plasmid pKD46 (AmpR) following a protocol suited for this purpose. Plate transformation at 30°C on LB plates containing 100 μg/ml Amp overnight. Inoculate a fresh colony into 25 ml LB containing 100 μg/ml Amp and grow overnight at 30°C.

7. In a 125 ml flask, inoculate 20 ml of LB containing 100 μg/ml ampicillin and 0.2% (v/v) L-Arabinose with 200 μl of the 5 ml overnight (ON) culture containing pKD46. Grow cells shaking (210 rpm) at 30°C to an OD of 0.5 (~10^8 cells/ml).

8. Chill the culture on ice for 5 minutes and pour it into pre-chilled sterile 50 ml Falcon tubes. Collect cells by centrifugation in swinging bucket bench top centrifuge at 3,800 × g for 20 minutes and 4°C. Handle tubes gently so as not to disturb the cell pellet. Pour off supernatant slowly, resuspend the cells in 25 ml of ice-cold 10% glycerol and collect the cells by centrifugation as above. Resuspend the cell pellet in 5 ml of ice-cold 10% glycerol and centrifuge. Resuspend the cells in 100 μl of ice-cold 10% glycerol by gently pipeting back and forth. Make sure no clumps are present. Place cells on ice and use within 30 min. This amount of cells is good for two to three trials using 50 μl of electrocompetent cells per electroporation.

9. Prechill the electroporation cuvettes (0.2 cm, Bio-Rad) in ice for 10 min. In a prechilled sterile Eppendorf tube, mix 50 μl of electrocompetent cells with 3 μl of DNA containing 0.1 to 0.5 ng of PCR product. Do not exceed this volume of DNA-containing sample since it will cause arcing during electroporation.

10. In a Micropulser electroporation apparatus (Bio-Rad) select EC2, a preset protocol for transformation of *E. coli* cells using 0.2 cm cuvette.

11. Transfer the DNA-bacteria mixture to a prechilled cuvette and incubate on ice for 1 min. Quickly dry the cuvette with kimwipes, place the cuvette into the electroporation chamber, and release charge. Immediately add 1 ml of SOC medium to the cuvette. Pipet back and forth a few times and transfer cells to a 15 ml sterile Falcon tube or similar. Add another ml of SOC medium to the cuvette and repeat the process.

12. Grow the 2 ml bacterial culture for 120 min shaking at 30°C. This step allows phenotypic expression of the resistance marker gene prior to exposure to Km selection. Spread 0.2 ml aliquots of the culture on LB Amp (100 μg/ml) Km (25 μg/ml) plates and incubate at 30°C overnight. Alternatively, the pKD46 plasmid could be cured by growing the cells on Km (25 μg/ml) plates at 37°C overnight.

13. Screening of recombinants: Restreak candidate *AraC* gene knockout colonies on LB Amp (100 μg/ml) Km (25 μg/ml) plates and incubate at 30°C overnight.

14. Colony PCR can be used to verify the structure of the recombinant. Use a standard Taq polymerase and a primer located upstream or downstream of the sequences used for targeting the gene replacement: AraC2 (5'- CTG GTG GCG ATC TCT TCA CCG GTA GC), and a primer annealing to the drug marker cassette: k2 (5'-CGG TGC CCT GAA TGA ACT GC). Perform standard PCR as follows: (step 1) 95°C, 4 min; (step 2) 94°C, 30 s; (step 3) 55°C, 1 min; (step 4) 72°C, 90 s. Repeat last three steps 29 times; (step 5) 72°C, 10 min; (step 6) hold at 4°C. When completed, load 10 μl of the PCR on a 1% agarose gel.

3.2. Obtention of UTP_{BAD}::surA *E. coli* strain

1. The zeoRExBAD (zeoR araC-P_{BAD}) cassette was amplified from the chromosome of *E. coli* strain TG1zeoRExBAD using specific oligonucleotide primers hybridizing with the upstream promoter region of the *surA* gene (5′ primer) and the beginning of their coding sequences (3′ primer), named ZEOBAD surA1 (5′-CGC AAG AGA TGC TGC GTT CGA ACA TTC TGC CGT ATC AAA ACA CTT TGT GAa gca atg ctt gca taa tgt gcc tgt c-3′) and ZEOBAD surA2 (5′-CTG GTA TTC GCG ATC ATG GCG ATA CCG AGA AGC AGC GTT TTC CAG TTC TTC **CAT** cgt ttc act cca tcc aaa aaa acg ggt-3′). The uppercase letters correspond to the sequence hybridizing to surA upstream or coding sequences. The lowercase letters correspond to the sequence hybridizing to the zeoRExBAD cassette. In bold is the first codon of *surA* coding sequence. Use standard PCR conditions, as described above. The PCR fragment with zeoR araC-P_{BAD} cassette is approximately 1.9 kb.

2. Clean the PCR product with QIAquick PCR purification kit (Qiagen) and elute DNA in 50 μl of elution buffer. Digest chromosomal DNA with DpnI (NEB). Mix 43 μl of the PCR product with 1 μl of DpnI (NEB) and 5 μl of NEB buffer 4 (10X) and incubate at 37°C for two hours. Gel-purify the PCR product using the Qiaex II gel extraction kit and elute in 50 μl of sterile deionized water.

3. Preparation of electrocompetent UT5601 *E. coli* cells carrying pKD46: Inoculate a fresh colony of UT5601 *E. coli* cells carrying pKD46 into 25 ml LB containing Amp (100 μg/ml) and Km (25 μg/ml). Grow overnight shaking at 30°C.

4. In a 125 ml flask, inoculate 20 ml of LB containing Amp (100 μg/ml), Km (25 μg/ml) and 0.2% (v/v) L-Arabinose with 200 μl of a 5 ml overnight culture. Grow cells shaking (210 rpm) at 30°C to an OD of 0.5 (~10^8 cells/ml).

5. Proceed as described in sections 8 to 11 from subheading 3.1.

6. After electroporation, grow 2 ml of the bacterial culture for 120 min shaking at 30°C. Spread 0.2 ml aliquots of the culture on low salt LB medium containing 0.2%

(v/v) arabinose and Zeo (40 μg/ml). The presence of L-arabinose is required for *surA* expression in putative UTP_{BAD}::*surA* cells. Incubate at 37°C overnight to remove pKD46 plasmid.

7. Screening of recombinants: Restreak candidate UTP_{BAD}::*surA* colonies on low salt LB Zeo (40 μg/ml) plates containing 0.2% (v/v) arabinose and incubate at 30°C overnight.

8. The insertion of the zeoRExBAD cassette in the promoter region of surA can be tested by colony PCR with oligonucleotides Zeo1 (5′-CAC TGG TCA ACT TGG CCA TGG TTT AG-3′) and SurA2 (5′-CAT TAA TCC ATC AAC GTC GCT TTC CAG CAC-3′). Use standard PCR conditions, as described above. When completed, load 10 μl of the PCR on a 1% agarose gel. (See Note 4)

Elimination of Km selection marker: if desired, the Km selective marker can be removed after transformation with temperature-sensitive helper plasmid pCP20. This FLP expression plasmid is resistant to ampicillin at 30°C (81).

9. Grow transformed UTP_{BAD}::*surA* bacteria with pCP20 plasmid on LB Amp (100 μg/ml) plates containing 0.2% (v/v) arabinose and incubate at 30°C overnight. Induce the flipase and plasmid loss by shifting the temperature to 42°C and overnight incubation. The resulting integrants are then spread on nonselective LB medium agar at 40°C for overnight. Cell colonies appearing on plates are again picked and patched onto LB agar plates containing Amp and Km, respectively. Consequently, the integrants are picked for exhibiting sensitivity to both Amp (indicating loss of the helper plasmid) and Km (indicating removal of the marker).

3.3. *Functional analysis of SurA depletion*

As mentioned before the expression of SurA in this conditional mutant can be controlled by the presence in the growth medium of the inducer L-arabinose or the repressor D-glucose. The conditional expression of SurA and its impact in the OM folding of the usher protein FimD of type 1 fimbriae are monitored as follows.

1. Inoculate a single colony of UTP_{BAD}::*surA* in 20 ml of liquid brain heart infusion (BHI) medium containing 0.4% (w/v) L-arabinose and Kanamycin (25 μg/ml) (See Note 5). Grow the culture at 37°C under static conditions for 16 hours. Dilute the preinoculum cultures to an OD_{600} of 0.05 in 10 ml of fresh medium containing either 0.4% (w/v) L-arabinose (inducing conditions) or 0.4% (w/v) D-glucose (repressing conditions) and Kanamycin (25 μg/ml). Grow the cultures at 37°C without shaking for type 1 fimbriae expression (FimD) and after 3 hours dilute them in 10 ml of inducing or depletion medium to an OD_{600} of 0.05 and grew for an additional 3 hours. Repeat this process one more time. Follow the growth of the bacterial cultures by taking OD_{600} readings each hour. Before culture dilution (indicated by roman numbers I, II, and III in Figure 2A) remove samples (0.25 OD_{600} units) for western botting.

2. Harvest bacteria by centrifugation (3,300 × g, 3 min) and prepare whole cell extracts as follows. Resuspend the cell pellet in 25 μl of PBS and mix it with the same volume of 2× SDS sample buffer.

3. Sonicate the samples on ice (5-10 s) with a thin needle at maximum power and spin (14,000 x g, 5 min) to remove insoluble material before loading onto an SDS-PAGE gel.

4. Load samples in 12% SDS-PAGE and perform Western blotting to detect the levels of folded and unfolded FimD, OmpA; the periplasmic chaperones SurA, Skp, and DegP; and cytoplasmic GroEL as an internal loading control. The procedure for SDS-PAGE and Western blotting has been precisely described elsewhere (84, 85).

3.4. *Example of results.*

As shown in Figure 2B, the steady-state level of SurA during continuous exponential growth with L-arabinose is clearly sufficient for proper expression and folding of FimD and OmpA. However, depletion of SurA from bacteria grown with D-glucose diminished the levels of FimD. The OmpA expression also decreased in depleting medium, although not as dramatically as FimD, since folded OmpA was clearly detectable when SurA was depleted from bacteria. Since DegP is not significantly upregulated under these conditions, these results indicate that SurA is required for FimD folding and insertion in the OM of *E. coli*.

Section II. Biochemical assessment of OMP folding

1. *Mobility shift and protease accessibility assays for OMPs*

Herein, we describe two simple methods traditionally used to evaluate the folding of OMPs, which are based in the compact folding adopted by correctly folded OMPs in the OM that confers distinct biochemical properties.

Usually natively folded OMPs do not denature in SDS sample buffer if not heated before SDS-PAGE, whereas OMPs displaying folding defects are sensitive to SDS. Thus, independently of their oligomeric state, the folding conformation of OMPs can be easily distinguished by analyzing their heat-modifiable property in SDS-PAGE (86, 87). Correctly folded OMPs acquire a compact SDS-resistant structure that migrates faster in SDS-PAGE gels than the unfolded polypeptides.

Also, OMPs are usually highly resistant to proteases when they are correctly assembled in the OM, being either totally protected against the proteolytic treatment or yielding discrete degradation products, such as the membrane embedded β-barrel portion of the OMP. This can be assessed *in vivo* by incubating bacterial cells with proteases such as trypsin or proteinase K. An increased sensitivity to protease treatment is often seen as an indicative of an alteration in the folding of the protein.

As an example, we will show the heat-modifiable mobility of the OMP intimin and its resistance to the treatment with proteinase K.

Intimins are large polypeptides (ca. 95 kDa) located at the surface of enteropathogenic and enterohemorrhagic *E. coli* strains (EPEC and EHEC, respectively) (Figure 3A). The N region of intimins (residues 1 to 550) anchors the protein in the OM and is highly conserved among intimins from different strains

(>95% identity). This region contains a β-barrel domain embedded in the OM (88). The extracellular C region of intimins is less conserved (ca. 50 to 70% identity) and forms a rigid rod that binds to the translocated intimin receptor (Tir) (89, 90). This region contains three immunoglobulin-like domains (named D0, D1, and D2) and a C-type lectin like domain (D3) (91). $Intimin_{EPEC}$ contains two Cys residues (Cys-860 and Cys-937) in domain D3 that form an intra-domain disulfide bond (92, 93) catalyzed by DsbA the periplasm (27).

2. Materials

2.1. *Bacterial culture*

1. Incubators and shakers

2. Spectrophotometer and cuvettes for measuring optical densities of bacterial cultures.

3. LB medium and LB agar plates as describe above on paragraph 2.1 Bacterial culture of Section I.

4. *E. coli* UT5600 wild type (82), UT5600*dsbA* (94) and EPEC E2348/69 strain (95).

5. pCVD438 plasmid expressing $intimin_{EPEC}$ (*eae*) (96). This plasmid carries the *eaeA* gene from EPEC under the control of its natural promoter and was previously used for complementation of Δeae mutants of EPEC and *C. rodentium* (97).

6. Chloramphenicol (34 mg/ml). Dissolve 340 mg chloramphenicol stock in 10 ml (100%) ethanol. If necessary, vortex solution to ensure the antibiotic is fully dissolved. Filter sterilze this solution using a 0.2 μm syringe filter. Aliquot (500 μl - 1 ml aliquots are useful) and store at -20°C until use.

8. β-mercaptoethanol (2-ME).

2.2. *Protease digestion*

1. Sonicator.

2. Proteinase K (PK) at 10 mg/ml (Sigma). See Note 6.

3. Phenyl-methylsulfonyl fluoride (PMSF) 100 mM. Dissolve 17.4 mg PMSF in 1 ml of ethanol. Caution: Wear gloves as PMSF is highly toxic and carcinogenic.

4. PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 ml of distilled water. Adjust pH to 7.4 with HCl; add distilled water to 1 liter and autoclave.

2.3. *Denaturing SDS-PAGE*

Prepare materials as in Subheading 2.3, Section I, with the following modifications

1. Prepare urea-SDS sample buffer (2×) for achieving complete denaturation of intimin polypeptides. The urea-SDS sample buffer (1×) contains 60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 4 M urea, 5 mM EDTA, 5% (v/v) glycerol, 0.005% (w/v) bromophenol blue, and 1% (v/v) 2-ME. See Note 7.

2.4. *Western blot*

Prepare materials as in Subheading 2.4, Section I. The antibodies are as follows:

1. Primary antibody: rabbit anti-Int280EPEC polyclonal serum (1:200; a gift from Gad Frankel).

2. Secondary antibody: bound rabbit antibodies were detected with protein A-POD conjugate (1:8,000; Zymed).

3. Methods

3.1. *Mobility shift assay and Proteinase K digestion of intimin expressed in EPEC and E. coli K12 bacteria*

1. For expression of intimin in EPEC bacteria, inoculate a fresh colony of EPEC E2348/69 strain (95) in 25 ml LB medium and grow statically at 37°C for 16 h.

2. Dilute the bacterial culture in fresh LB medium to a final optical density at 600 nm (OD600) of 0.1 and grow under identical conditions for 2 hours in the presence or absence of 10 mM 2-ME. The reducing agent 2-ME is included for reducing the disulphide bond (Cys-860 and Cys-937) present in intimin that renders this polypeptide sensitive to PK.

3. Measure the optical density at 600 nm of both cultures (+/- 2-ME) and centrifuge 0.5 ODs (3,300 × g, 3 min). Resuspend the cell pellet in 1 ml of PBS and harvest the cells by centrifugation (3,300 × g, 3 min).

4. Redisperse the cell pellet in 1 ml of PBS and divide into three aliquots of 330 μl. Resuspend two aliquots in 25 μl of PBS and add the same volume of urea-SDS sample buffer (2×). Incubate one of the aliquots at 100°C for 30 minutes (boiled sample), and kept the other on ice (unboiled sample).

5. Add 40 μg/ml of PK to the third aliquot on ice (PK treated). Incubate at 37°C for 20 minutes. Stop the reaction with 1 μl of PMSF (100 mM). Resuspend it in 25 μl of PBS and add the same volume of urea-SDS sample buffer (2×). Boil it for 30 minutes.

6. Sonicate the samples on ice (5-10 s) with a thin needle at maximum power and spin (14,000 x g, 5 min) to remove insoluble material before loading onto an SDS-PAGE gel.

7. Load samples in 8% SDS-PAGE and perform Western blotting to detect EPEC intimin with anti-Int280EPEC polyclonal serum (raised against the secreted domains D1, D2, and D3). The procedure for SDS-PAGE and western blotting has been precisely described elsewhere (98).

8. For heterologous expression of EPEC intimin in wild type *E. coli* UT5600 or in the isogenic dsbA mutant, transform UT5600 and UT5600*dsbA* bacteria with pCVD438 plasmid (CmR) following a protocol suited for this purpose. Plate transformation at 30°C on LB plates containing 34 μg/ml Cm and grow overnight.

9. Inoculate a fresh colony of UT5600/pCVD438 or UT5600*dsbA*/pCVD438 bacteria into 25 ml LB medium containing Cm.

10. Grow overnight shaking (160 rpm) at 37°C for constitutive expression.

11. Dilute the bacterial culture in fresh LB medium to a final optical density at 600 nm (OD600) of 0.1 and grow under identical conditions for 2 hours in the presence or absence of 10 mM 2-ME.

12. Repeat from step 3.

3.2. *Example of results.*

The intimin band shows a mobility of ~95 kDa in the boiled samples, corresponding to the expected mass of full-length intimin and indicating that the polypeptide was completely denatured after boiling in the SDS-urea buffer (Figure 3B and C, lane 2). In those samples kept at 4°C intimin showed a faster mobility in SDS-PAGE as expected for a folded β-barrel protein (Figure 3B and C, lane 1). Intimin requires boiling in the presence of 4 M urea and 2% SDS for complete unfolding indicating the formation of a very stable β-barrel in both EPEC and *E. coli* K-12.

Intimin also shows high resistance to extracellularly added proteases when expressed in EPEC and *E. coli* UT5600/pCVD438. Full-length intimin bands did not exhibit any sign of proteolytic digestion after incubation of intact bacteria with PK (Figure 3B and C, lane 3). Nonetheless, intimin is sensitive to PK when the reducing agent 2-ME was added to the growth medium of EPEC and *E. coli* UT5600/pCVD438. Bacteria grown with 2-ME and incubated with PK showed almost the complete digestion of the full-length intimin band and the simultaneous appearance of a ~66-kDa proteolytic product (Figure 3B and C, lane 6). The growth of EPEC and *E. coli* UT5600/pCVD438 bacteria with 2-ME did not affect folding of the β-barrel of intimin, as demonstrated by its resistance to urea-SDS denaturation and its heat-modifiable mobility (Figure 3B and C, lanes 4 and 5). These data indicate that such reducing conditions render intimin accessible in the extracellular medium to the action of the protease, and suggest that the increased sensitivity to PK was caused by alterations elsewhere in the protein.

Intimin shows identical heat-modifiable mobility and resistance to urea-SDS denaturation in wild-type *E. coli* UT5600/pCVD438 bacteria and *dsbA* mutant UT5600*dsbA*/pCVD438 bacteria (Figure 4). However, as observed under reducing growth conditions (Figure 3B and C), intimin produced in *dsbA* mutant bacteria was sensitive to PK digestion (Figure 4). Therefore, the absence of DsbA makes intimin less stable and susceptible to protease digestion, likely due to the misfolding of the secreted D3 domain that contains the single disulfide bond between Cys-860 and Cys-937.

Section III. Disulfide bond formation in OMPs

1. *Analysis of the redox state of cysteine residues in OMPs with alkylating agents*

In this method we determine whether two cysteine residues present in a protein are oxidized by DsbA to form a disulfide bond. This is an important question to address when investigating the molecular mechanisms that take place during the periplasmic transit of OMPs. For instance, DsbA catalyzes the formation of the disulfide bond (Cys-860-Cys-937) present in the D3 lectin-like domain of EPEC intimin, indicating that this secreted C-terminal domain is at least partially folded prior to its translocation across the OM (27).

For this purpose, an alkylation assay is performed with the polyethylene glycol (PEG)-conjugated maleimide (mPEG-MAL, ca. 5000 Da) or with 4-acetamido-40-maleimidylstilbene-2,20- disulfonic acid (AMS, ca. 500 Da) that covalently binds to free sulfydryl groups in proteins.

Maleimide-activated crosslinkers (mPEG-MAL or AMS) react with the free thiolate groups of reduced cysteine residues (-SH) in proteins at near neutral conditions (pH 6.5-7.5) to form covalent thioether linkages increasing their molecular mass and preventing any further oxidation. Hence, disulfide bonds in protein structures (e.g., between cysteines) must be reduced to free thiols to react with such maleimide reagents. In contrast, cysteine residues involved in a disulfide bond are not modified. Thus, oxidized proteins migrate with the expected size in non-reducing SDS-PAGE, whereas reduced proteins are shifted towards higher molecular weights. See Note 8.

2. Materials

2.1. Bacterial culture

1. Incubators and shakers

2. Spectrophotometer and cuvettes for measuring optical densities of bacterial cultures.

3. LB medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl. Combine the dry reagents above and add distilled water to 950 mL. Adjust pH to 7.5 with 1 N NaOH and bring the volume up to 1 liter. Autoclave and store at room temperature.

4. LB agar plates: add 15 g agar, autoclave as above and allow to cool at room temperature for at least 55 ^{o}C. Add antibiotics as needed, and pour into 100 mm×15 mm petri plates using 25–30 ml per plate.

5. Three *E. coli* strains are used in our study: *E. coli* UT5600 wild type (82), UT5600*dsbA* (94) and EPEC E2348/69 strain (95).

6. The pCVD438 plasmid expressing intimin$_{EPEC}$ (*eae*) (96).

7. Chloramphenicol. Dissolve 340 mg chloramphenicol stock in 10 mL (100%) ethanol. If necessary, vortex solution to ensure the antibiotic is fully dissolved.

Filter sterilze solution using a 0.2 μm syringe filter. Aliquot (500 μL - 1 mL aliquots are useful) and store at -20 °C until use.

2.2. *Alkylation assay*

1. 1 M dithiothreitol (DTT): Dissolve 0,15 g in 1 ml H_20

2. Alkylation buffer (AK): 80 mM Tris-HCl [pH 6.8], 150 mM NaCl

3. 10 mM mPEG-MAL: Dissolve 0,05 g in 0,25 ml AK buffer. It is prepare on the moment, ready to use because lost fastly the alkylation activity. See Note 9.

4. 100% (w/v) Trichloroacetic acid (TCA): dissolve 500g TCA (as shipped) into 350 ml dH2O, store at RT.

5. Acetone

6. Glycerol

7. PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 ml of distilled water. Adjust pH to 7.4 with HCl; add distilled water to 1 liter and autoclave.

2.3. *Non-reducing SDS-PAGE*

Prepare materials as described in Subheading 2.3 of Section I.

1. Prepare non-reducing urea-SDS-sample buffer (2×) (without 2-ME) The non-reducing urea-SDS sample buffer (1×) contains 60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 4 M urea, 5 mM EDTA, 5% (v/v) glycerol and 0.005% (w/v) bromophenol blue.

2.4. *Western blotting*

Prepare materials as in Subheading 2.4, Section I. The antibodies are as follows:

1. Primary antibody: rabbit anti-Int280EPEC polyclonal serum (1:200; a gift from Gad Frankel).

2. Secondary antibody: bound rabbit antibodies were detected with protein A-POD conjugate (1:8,000; Zymed).

3. Methods

3.1. *Alkylation assay of intimin expressed in EPEC and E. coli K12 bacteria with mPEG-MAL*

1. Transform UT5600 and UT5600*dsbA E. coli* bacterial strains with pCVD438 plasmid (CmR) following a protocol suited for this purpose. Plate transformation at 30°C on LB plates containing 34 μg/ml Cm overnight.

2. For expression of intimin in *E. coli* K-12 strains, inoculate a fresh colony of UT5600 or UT5600*dsbA* bacteria carrying pCVD438 plasmid into 25 ml LB medium containing Cm (34 μg/ml).

3. Grow overnight shaking (160 rpm) at 37°C for constitutive expression.

4. For expression of intimin in EPEC bacteria, inoculate a fresh colony of EPEC E2348/69 strain (95) in 25 ml LB medium and grow statically at 37°C for 16 h.

5. Dilute the bacterial culture in fresh LB medium to a final optical density at 600 nm (OD600) of 0.05, and grown under identical conditions for additional 4 hours.

6. Harvest UT5600*dsbA*/pCVD438, UT5600/pCVD438 and EPEC bacteria by centrifugation (3,300 × g, 3 min). Resuspend the cells to an OD600 of 1.0 in 2 ml of PBS and pellet again by centrifugation (3,300 × g, 3 min). Wash twice with 1 ml of PBS by centrifugation (3,300 × g, 3 min).

7. Resuspend the cell pellet in 0.5 ml of buffer AK and divide the suspension into four aliquots of 100 µl. Incubate two of them with 100 mM dithiothreitol (DTT) on ice for 10 min and then incubate at 60°C for an additional 10 min (DTT-treated samples). See Note 10.

8. Wash DTT-treated and untreated samples with 1 ml of PBS two times by centrifugation (3,300 × g, 3 min). Resuspend one sample from each group in 50 µl of AK buffer containing 10 mM mPEG-MAL (mPEG-Mal, Mr 5,000; Nektar Therapeutics, San Carlos, CA), and resuspend the remaining samples in 50 µl AK buffer. Perform the alkylation reaction for 30 minutes at room temperature.

9. Precipitate the proteins with trichloroacetic acid (10% [wt/vol]) for 1 h at 4°C, and recover the precipitates by centrifugation (14,000 × g, 15 min).

10. Wash the protein pellets with 1 ml of ice-cold acetone, followed by centrifugation (14,000 × g, 15 min).

11. Dry pellet by placing tube in 95°C heat block for 5-10 min to drive off acetone and resuspend in 30 µl of AK buffer containing 1% (w/v) SDS and 5% (v/v) glycerol. Samples were mixed with the same volume of nonreducing urea-SDS-sample buffer (2×) (without 2-ME) before being loaded onto SDS-PAGE gels.

12. The redox state of intimin is analyzed by standard SDS-PAGE and Western blot protocols as describedd previously. Immunodetection of intimin is performed with rabbit polyclonal anti-intimin-280EPEC antibody (1:200; a gift from Gad Frankel). Bound rabbit antibodies were detected with protein A-POD conjugate (1:8,000; Zymed).

3.2. *Example of results.*

To gain a direct evidence of the formation disulfide bond (Cys-860-Cys-937) by DsbA, the *in vivo* redox state of intimin was compared in wild-type and dsbA mutant *E. coli* K-12 strains carrying pCVD438. The experiment shows that intimin reacts to mPEG-MAL when expressed in the dsbA mutant, in which a high-molecular-weight band corresponding to alkylated intimin appears (Figure 5, lane 7). In contrast, intimin expressed in wild-type EPEC or UT5600/pCVD438 bacteria was not reactive to the alkylating agent (Figure 5C, lanes 2 and 5) unless the disulfide bond in D3 was reduced by the incubation of bacteria with the reducing agent DTT (Figure 5C, lane 3).

Section IV. Analysis of cuaternary structure of OMPs.

1. *Blue-native PAGE (BN-PAGE) and cross-linking with DSP to follow the cuaternary structure of OMPs*

BN-PAGE relies on the solubilization of protein complexes from the membrane with mild non-ionic detergents (99). These detergents also help to prevent disruption of the protein–protein interactions. The protein complexes are negatively charged with Coomassie brilliant blue G-250 facilitating thus their migration towards the anode and separation according to its size. During electrophoresis, protein complexes separation is obtained with high resolution by the decreasing pore size in the polyacrylamide gradient gel. Always the sample is kept on native condition, the polyacrylamide gels are native (without SDS) and the electrophoresis is under native conditions (without SDS and with Coomassie blue G-250). To this aim, the sample is kept on ice during the preparation steps and running gel. This procedure avoids protein degradation as well as loss of the quaternary structure because separation of protein subunits of the complex.

Intimin is reported to form a homodimer when purified (100). N-terminal fragments of intimin (Int550, and construct Neae with the D0 Ig like domain) and full length intimin were tested by BN-PAGE to evaluate their dimeric structure (27) (Figure 6).

To stabilize protein complexes, intact *E. coli* cells can treated by means of chemical cross-linking *in vivo* with specific agent as dithiobis-succinimidyl propionate (DSP). DSP is a homobifunctional amine-reactive N-hydroxysuccinimide (NHS) ester which has a spacer arm of 12 Å and a disulfide bond that can be cleaved with reducing agents (e.g. 2-ME). Reducing agents allow identification of the individual components within an oligomeric complex. The advantage of this technique is that it can do it with intact cells without any treatment or purification. The concentration of crosslinker, as well as the length and temperature of the crosslinking reaction, are critical parameters and should not be varied. Few crosslinking results in a ladder of partially crosslinked products whereas over crosslinking can produce a wrong conclusion because on non-specific cross-reaction that do not reflect true interactions.

C-terminal β-domain of ATs were used as a model to test quaternary structure into the OM. Each construct for of the C-terminal domain are tagged with an epitope (E-tag) to allow the detection with specific mAb antibody in a Western blot. After induction of these constructs with IPTG, cell cultures were incubated with DSP and bacterial suspensions were mixed with either reducing or nonreducing SDS-PAGE sample buffer, boiled and subjected to Western blotting with anti-E tab mAb-POD (101) (Figure 7).

2. Materials

2.1. Bacterial culture

1. Incubators and shakers

2. Spectrophotometer and cuvettes for measuring optical densities of bacterial cultures.

3. LB medium and LB agar plates as describe above on paragraph 2.1 Bacterial culture of Section I.

4. *E. coli* UT5600 wild type (82) and EPEC E2348/69 (95).

5. pInt550 and pNeae plasmids expressing intimin$_{EPEC}$ (eae) polypeptides (27). ATs plasmids with C-terminal domain fused to 6xHistidine-tag and E-tag (101). All the plasmid are CmR and derivated from pAK-Not (82).

6. Chloramphenicol (Cm) as previously describe.

7. 1 M IPTG: dissolve 1 g in 5 ml dH_2O. Filter-sterilize the IPTG solution through a 0,22 μm filter. Store the IPTG in aliquots at -20°C and one aliquot ready to use at 4°C.

2.2. BN-PAGE

1. Electrophoresis system: Bio-Rad Mini-PROTEAN 3 or equivalent.

2. Electrophoresis power supply (PowerPac Basic Power Supply. Bio-Rad, 164-5050EDU) or power supply capable of providing constant voltage of 150 V or higher.

3. Electrophoresis buffer: 50 mM Bis-Tris-HCl, 500 mM 6-aminocaproic acid, 10% (v/v) glycerol pH 7.0.

4. The cathode buffer consisted of 50 mM Tricine, 15 mM Bis-Tris-HCl pH 7.0 and 0.002% (w/v) Coomassie blue G250.

5. The anode buffer contained 50 mM Bis-Tris-HCl pH 7.0.

6. Protein standards of high molecular mass (66 to 669 kDa) for native electrophoresis (commercial) were resuspended at a 2.5 mg/ml final concentration in 50 mM Bis-Tris-HCl pH 7.0 containing 750 mM 6-aminocaproic acid.

7. Sample buffer: 20 mM Tris-HCl pH 8.0, 10 mM NaCl, 1% (w/v) Zwittergent 3-14, 8.7% (v/v) glycerol and 0.5% (w/v) Coomassie blue G-250.

Stocks solution: 10% (w/v) Zwittergent 3-14, 87% (v/v) glycerol and 5% (w/v) Coomassie blue G-250.

8. To prepare the separating (polyacrylamide gradient gel) and stacking gel is necessary the following solutions:

- AB solution: 49.5% T and 3% C

%T, total concentration of both monomers acrylamide and bis-acrylamide.

%C, percentage of cross-linker relative to the total concentration.

- Buffer 3x: 150mM Bistris/HCl pH 7.0, 1.5 M 6-aminocaproic acid

- Glycerol 87% (v/v)

- 10% (w/v) ammonium persulfate (APS) solution. Store at -20°C for long time and at 4°C for up to 2 weeks.

- N,N,N,N´-tetramethyl-ethylenediamine (TEMED)

2.3. Cross-linking with DSP

1. PBS: Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml of distilled water. Adjust pH to 7.4 with HCl; add distilled water to 1 liter and autoclave.

2. Buffer 1 M Tris-HCl pH 7.5 to quenched crosslinking reaction.

3. DSP (dithio-bis[succinimidyl propionate]) crosslinker must be dissolved in an organic solvent, such as DMSO, and then added to an aqueous crosslinking reaction. Stock solution was prepared at 250 mM on DMSO by rigorous vortex-mixing plus 1 min incubation at 37°C. A few particles may remain still insoluble.

2.4. Denaturing SDS-PAGE and Western blotting

As previously describe on Materials Section I.

1. Primary antibody: E-tag mAb-POD conjugate (1:2,000; GE Bioscience) and rabbit anti-$Int280_{EPEC}$ polyclonal serum (1:200; a gift from Gad Frankel).

2. Secondary antibody: bound rabbit antibodies were detected with protein A-POD conjugate (1:8,000; Zymed).

3. Methods

3.1. *BN-PAGE of outer membrane extract*

1. Prepare polyacrylamide gradiente gels between 4-20% with a gradient forming and based on (99). This set up allows the separation of protein complexes in the molecular mass range from ~50 kDa to 700 kDa.

Prepare separately the higher (20%) and lower (4%) percentage of acrylamide, the same volume. Lower percentage of separating gel (4% acrylamide) and stacking gel (4% acrylamide) are prepared equal: 242 μl AB solution, 1 ml buffer 3x, 25 μl APS, 2.5 μl TEMED and 1730 μl water, without glycerol. The higher percentage of separating gel mix (20% acrylamide): 1212 μl AB solution, 1 ml buffer 3x, 690 μl glycerol, 15 μl APS, 2 μl TEMED and 81 μl distilled water. Addition of TEMED and APS at the end of the process, just before open valves. Always the higher percentage of acrylamide solution to do the gradient gel must have 20% of glycerol and put in the position close to the exit in the gradient forming. Place the gradient forming with a magnet rod inside the higher concentration on a magnetic stirrer and connect with the casting stand. Ensure that all ports are closed, between the collectors of acrylamide solutions and with the casting stand. Use soft agitation to avoid air bubbles inside acrylamide solution. Open the two valves at the same time and let the solutions rinse between the glass plates.

Add distilled water to the top of separating gel during the polymerization process of the gradient gel that should be done around 90 min. Remove distilled water and dry the area above the separating gel completely with thick blot paper (e.g. Whatman) and add the stacking gel with 12-well comb.

2. To obtain the sample, pellet the membrane fraction of your cell culture by centrifugation after lysis step (with French Press or sonication). Remove the supernatant containing all soluble or faint associated proteins to the membranes. Solubilize the pellet on 20 mM Tris-HCl pH 8.0, 10 mM NaCl and 1% (w/v) Zwittergent 3-14 by gentle agitation and kept on ice. Concentrate sample 100x when resuspended membrane pellet with respect the initial volume culture. Sonicate the sample in a cold sonication bath improve the solubilization of the samples and reduce the time to spend on it. It is important to avoid foam formation during solubilization process. In case of foam it can hinder full sample solubilization. Centrifuge during 30 min at 4°C and 100,000 x g to pellet the unsolubilized material that can affect in a negative way to the subsequent electrophoretic separation of the protein complexes. Before gel loading, 20 μl of this OMP extract was mixed with 2.5 μl glycerol 87% (v/v) and 2.5 μl Coomassie blue G-250 5% (w/v) and kept on ice.

Protein complex solubilization is the most critical step, for this is important select the appropriate detergent in which is going to solubilize and at what concentration. Protein interaction must be stable with the detergent selected.

3. Load 5 to 10 μl of OMPs and resuspended protein standards per well, and electrophoresis was run for 45 min at 100 V and for ca. 1 h at 500 V. As soon as the blue Coomassie dye front has reached half of the separating gel, pause the electrophoretic run and remove the blue cathode buffer from the upper buffer chamber. Refilled with colorless blue native cathode buffer and continue the electrophoretic run. The electrophoresis finish when the blue Coomassie dye front line has reached the bottom of the separating gel (See Note 11).

4. Disassemble the buffer chamber unit and the glass plate sandwich too and remove the stacking gel. Remove blue staining of the gel with distilled water. The blue-gel can transferred directly onto a PVDF membrane using a semidry electrophoresis transfer apparatus to check protein complexes with antibodies.

3.2. *Treatment with DSP of intact E. coli cells*

1. 1 ml of a culture of *E. coli* strain selected on late exponential growth phase, OD600nm around 1-1.5, is centrifuge at 3300 x g 3 min. The pellet is resuspended in 100 μl PBS with or without DSP 2.5 mM, concentrated the sample 10 times. DSP must be prepared freshly, ready to use.

2. Cross-linking was carried out for 30 min at room temperature (RT) with occasional vortex-mixing.

3. Crosslinking reaction must be stooped to ovoid undesirable interactions of the oligomeric complex. For this reason, it was added 5 μl of 1 M Tris-HCl pH 7.5, to a final concentration of 50 mM. The incubation was done for 15 min at RT to quench the reaction.

5. The cells were washed twice with 10 mM Tris-HCl pH 7.5 and resuspended in the same buffer at the same volume.

6. One volume of SDS-PAGE with or without 2-ME 5% (vol/vol) was added to the samples, and then they were boiled 10 min before gel loading (See Note 12).

7. After chemical crosslinking monomeric and oligomeric species be immunoprecipitated and/or analyzed by Western blotting using specific antibodies.

3.2. *Example of results.*

The intimin polypeptides run in the BN-PAGE with a mobility corresponding to the expected size of dimers (Figure 6).

With DSP we evaluated the quaternary structure of C-terminal domains of different ATs from Gram-negative bacteria expressed in *E. coli* (101). DNA fragments encoding the C-terminal domain of the selected ATs, were cloned into the expression vector pAK-Not (82). The plasmids obtained, named pHEA (C-terminal domain of EhaA), pHES (C-terminal domain of ShdA), pHEI (C-terminal domain of IgAP), pHEN (C-terminal domain of NalP) and pHEBA (C-terminal domain of BruA), encode polypeptides containing the N-terminal signal peptide (sp) of PelB, followed by the His tag (H) and E tag (E) epitopes and their respective C-terminal domains. The expression of these HE-tagged C-terminal domains was induced with IPTG in *E. coli* K-12 strain UT5600. After incubation with DSP and with either non-reducing (Figure 7A) or reducing SDS-PAGE buffer (Figure 7B) the samples were analyzed by Western blotting and developed with anti-E-tag antibody. Only HES showed oligomeric state with a strong crosslinking band around 98 kDa corresponding to the predicted size for a dimer. On the contrary, the rest of C-terminal domain ATs were weakly cross-linked (HEA and HEBA) or not cross-linked at all (HEI and HEN), suggesting that they mainly form monomers in the OM *in vivo*.

V. Notes.

1. Any *E. coli* strain that contains the complete Tn5 transposable element (i.e. DH5αF´IQ, SURE, SURE2, MC1066) encodes the ble (bleomycin) resistance gene. These strains will confer resistance to Zeocin. Plates containing Zeocin are stable for 1 month when stored at 4°C.

2. SDS-PAGE on polyacrylamide gels of 8% for OMPs around 90-100 kDa or 10% for OMPs around 45-65 kDa.

3. For POD-developing, a chemiluminescence reaction was prepared using a mixture of 1.25 mM luminol (Sigma) and 42 μM luciferin (Roche) in 100 mM Tris–HCl (pH 8.0). Following a rapid rinse in PBS, the membranes were soaked in this mixture and H_2O_2 added at 0.0075% (v/v). Alternatively enhanced peroxidase chemiluminescence developer (Roche) was employed. In all cases, after a one minute incubation in the dark, the PVDF-membranes were exposed to an X-ray film (X-OMAT, Kodak).

4. In the chromosome of wild-type *E. coli*, the surA gene is transcribed with downstream genes pdxA, ksgA, apaG and apaH. These genes are involved in functions unrelated to surA (e.g. pyridoxal 5'-phosphate biosynthesis and methylation of 16S rRNA). In addition, these genes are transcribed in pdxA- ksgA-apaG-apaH and apaG-apaH transcripts due to the presence of promoters upstream pdxA and apaG (http://biocyc.org/ECOLI).

5. The BHI medium is used for optimal production of type 1 fimbriae (102) and for expression of endogenous FimD in *E. coli* (33). LB medium was also used for depletion studies of BamA and SurA (27).

6. In this protocol we used Proteinase K as the protease of choice. Other proteases can be used (e.g., trypsin). In brief, induced *E. coli* cells were washed and resuspended in PBS, trypsin was added externally (10 µg/ml). Samples were incubated for 20 min at 37°C and stopped by adding trypsin inhibitor (5 µg/ml; Sigma). Total protein extracts from these cells can be analyzed by Western blotting.

7. We found that intimin requires boiling in the presence of 4 M urea and 2% SDS for complete unfolding, indicating the formation of a very stable β-barrel in both EPEC and *E. coli* K-12 (27).

8. Extraneous thiols (most reducing agents) must be excluded from maleimide reaction buffers, because they will compete for coupling sites.

9. mPEG-Mal can be substituted with AMS. 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS, Mw ~500 Da), as described in (103).

10. The DTT treatment is performed as a control in order to determine the mobility of the reduced form of the polypeptide.

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Figure Legends

Figure 1. Construction of SurA depletion *E. coli* mutant strain. (A) Scheme of the RExBAD casette amplified by PCR from the TG1zeoRExBAD strain using primers ZEOBAD SurA1 and ZEOBAD SurA2 and insertion of the RExBAD cassette by λred-driven homologous recombination after transformation with the PCR product containing the zeoRExBAD cassette and flanking DNA of the *surA* promoter region. (B) Schematic of P_{BAD} controlled *surA* allele in the UTP_{BAD}::surA strain.

Figure 2. Expression of FimD in the conditional *E. coli* surA mutant. (A) Growth curves of static cultures of UTP_{BAD}::surA strain grown in rich BHI medium containing D-glucose (Glu) or L-arabinose (Ara) and maintained in exponential phase by repeated dilutions with the same medium. Samples from these cultures were taken at the indicated times (I, II, and III) for Western blot analysis. (B) Whole-cell protein extracts from bacteria harvested at the indicated times (I, II, and III) from cultures shown in panel A were analyzed by Western blotting with anti-FimD, anti-OmpA, anti-SurA, anti-Skp, anti-DegP, and anti-GroEL antibodies. Control samples were obtained from cultures of UTP_{BAD}::surA grown in medium with L-arabinose. Reproduced from (85) with permission from ASM.

Figure 3. Intimin domain organization and expression in EPEC (E2348/69) and *E. coli* K-12. (A) Schematic drawing of intimin illustrating its domain organization. The first 550 amino acid residues of intimin (Int550) contain a signal peptide (sp) located at the N terminus (N), a putative peptidoglycan-binding domain (LysM), and an OM-embedded domain predicted to fold as a β-barrel. The immunoglobulin-like (D0, D1, and D2) and the lectin-like (D3) domains are displayed on the bacterial surface. Domain D3 contains a disulfide bond indicated with an "S-S". (B) Western blotting performed with a rabbit anti-$Int280_{EPEC}$ polyclonal serum (detecting domains D1, D2, and D3) of whole-cell protein extracts from EPEC bacteria grown in LB in the presence (+) or absence (−) of 10 mM 2-ME. Intact bacteria, harvested from this culture, were incubated with (+) or without (−) PK. The whole-cell extracts were prepared in urea-SDS sample buffer (2% SDS, 4 M urea) and boiled (+) or not (−) as indicated. The mobility of unfolded (U) and folded (F) intimin is labeled on the right, and the masses of protein standards are shown on the left (in kilodaltons). (C) Western blot probed with anti-$Int280_{EPEC}$ of whole-cell protein extracts from *E. coli* K-12 strain UT5600 carrying pCVD438. Reproduced from (27) with permission from ASM.

Figure 4. Intimin expression in a *dsbA* mutant of *E. coli* K-12 and PK sensitivity. Western blot with anti-$Int280_{EPEC}$ of whole-cell protein extracts in urea-SDS sample buffer of *E. coli* UT5600 and its isogenic *dsbA* mutant carrying pCVD438. Samples were treated as in Figure 3. Reproduced from (27) with permission from ASM.

Figure 5. Alkylation with mPEG-MAL of intimin expressed in EPEC, UT5600/pCVD438 and UTdsbA/pCVD438. Treated samples were subjected to non-reducing SDS-PAGE and Western blotting developed with anti-Int280$_{EPEC}$ serum. Samples incubated with the reducing agent DTT and/or with the alkylating agent mPEG-MAL are indicated (+). The bands corresponding to alkylated intimin polypeptide are labeled with arrows. The masses of protein standards are shown on the left (in kDa). Reproduced from (27) with permission from ASM.

Figure 6. Outer membrane localization and dimerization of intimin polypeptides. Blue-native PAGE of solubilized OMPs from the outer membrane fractions of *E. coli* UT5600/pInt550, UT5600/pNeae and EPEC bacteria. Intimin polypeptides were detected by Western blot incubating the membrane with anti-Int280$_{EPEC}$ serum followed by incubation with a mixture of protein A-POD and anti-E-tag mAb-POD. The mass of native protein standards (GE Amersham) is shown on the left (in kDa). Reproduced from (27) with permission from ASM.

Figure 7. Analysis of the quaternary structure of the AT C-terminal domains *in vivo*. (A) Cross-linking with DSP of ATs C-terminal domains expressed in *E. coli* to determine the formation of oligomeric complexes *in vivo.* DSP-treated (+) and untreated (-) samples were subjected to non-reducing SDS-PAGE, and the Western blot was probed with anti-E tag mAb-POD. Samples incubated with the reducing agent 2-ME are indicated (+). (B) Samples treated as in A but subjected to reducing SDS-PAGE. Reproduced from (101) with permission from ASM.

Figure 1

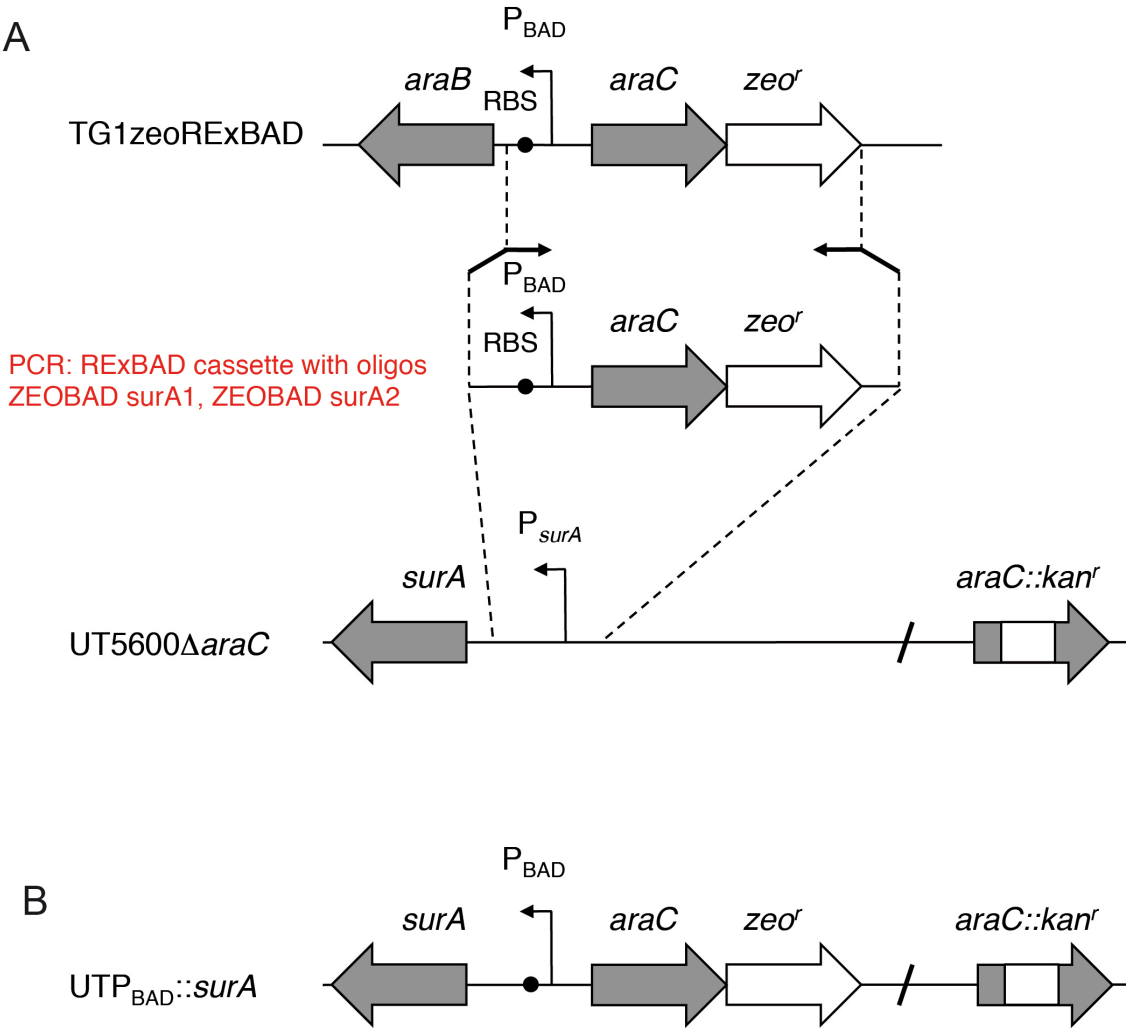
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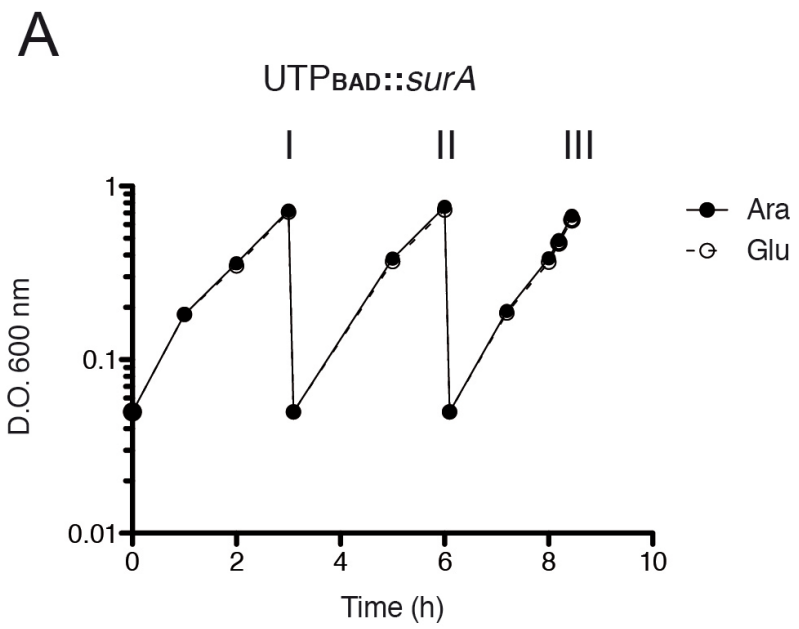
Figure 2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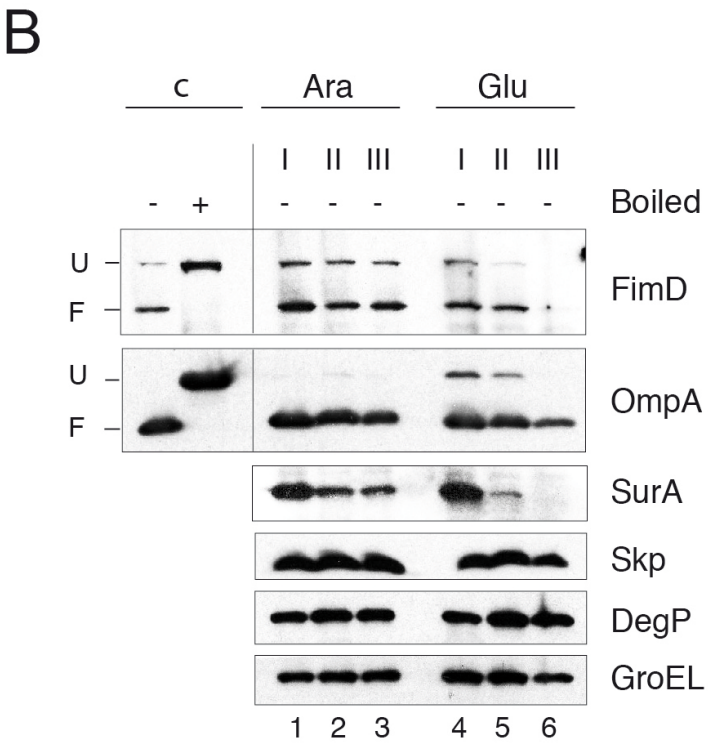
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Figure 3

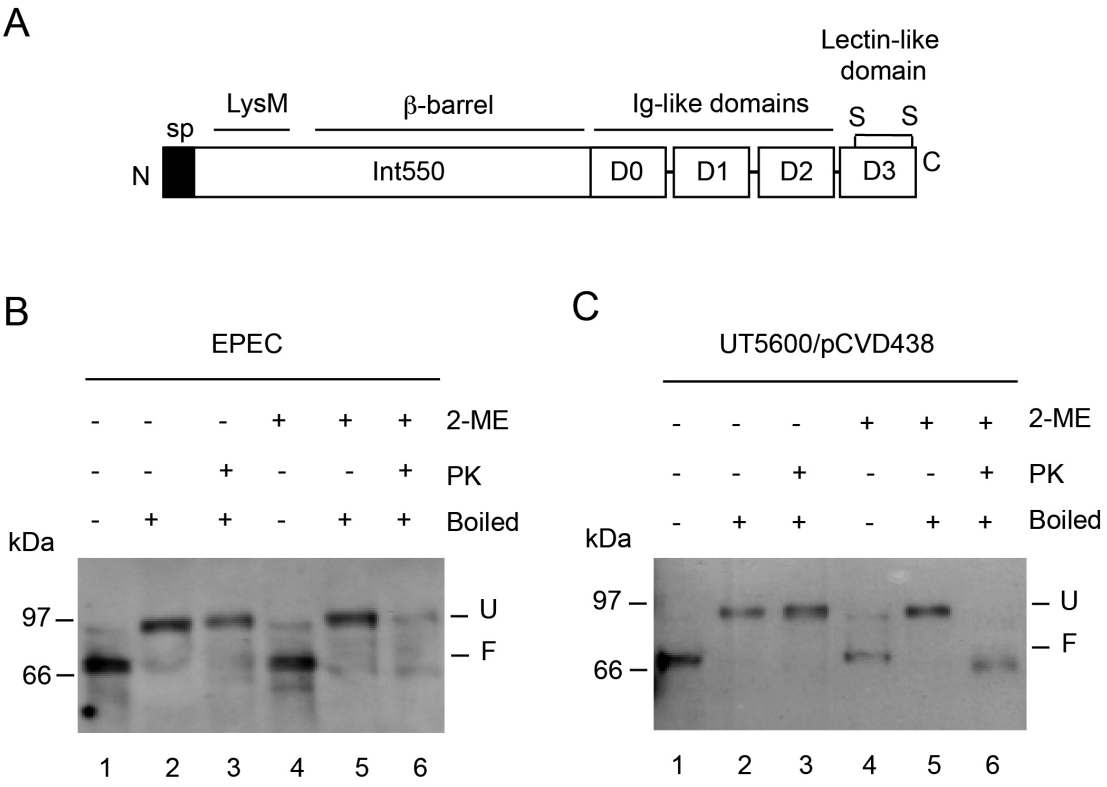
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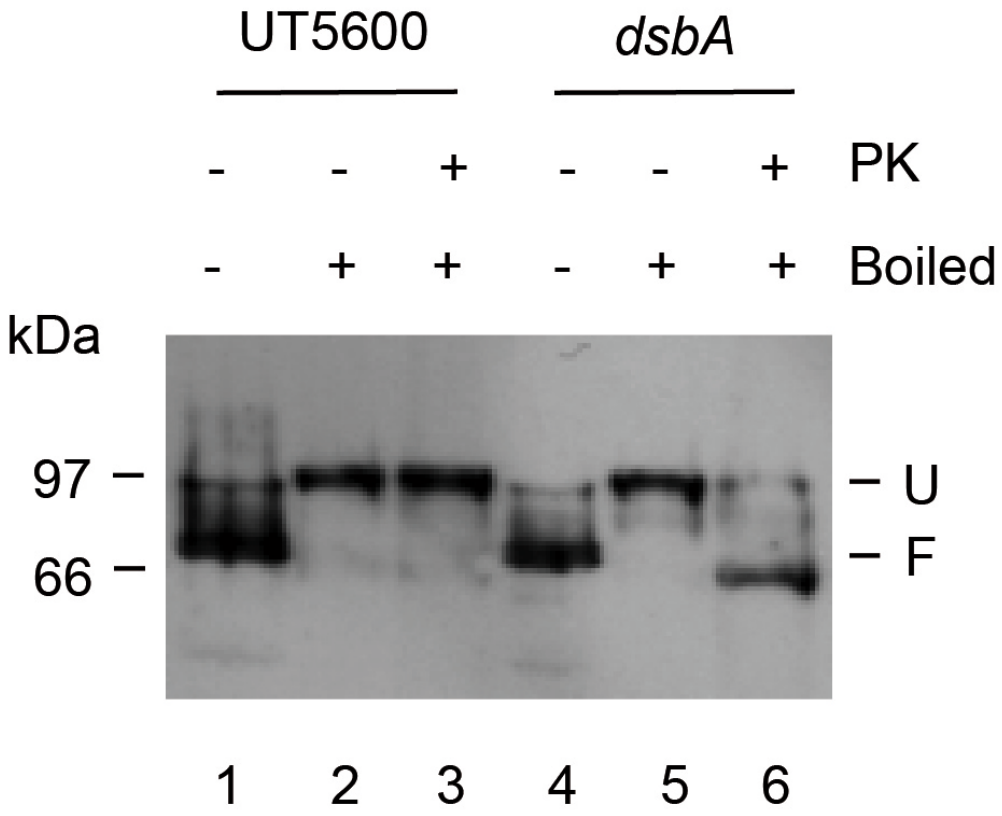
Figure 4



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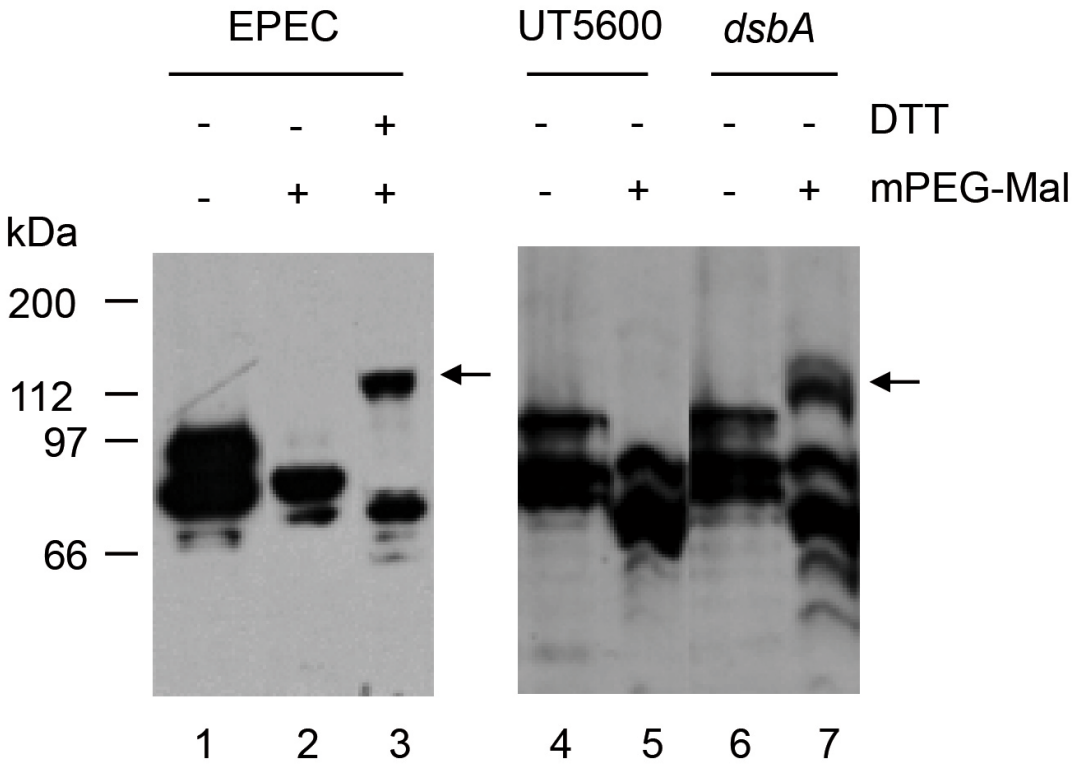

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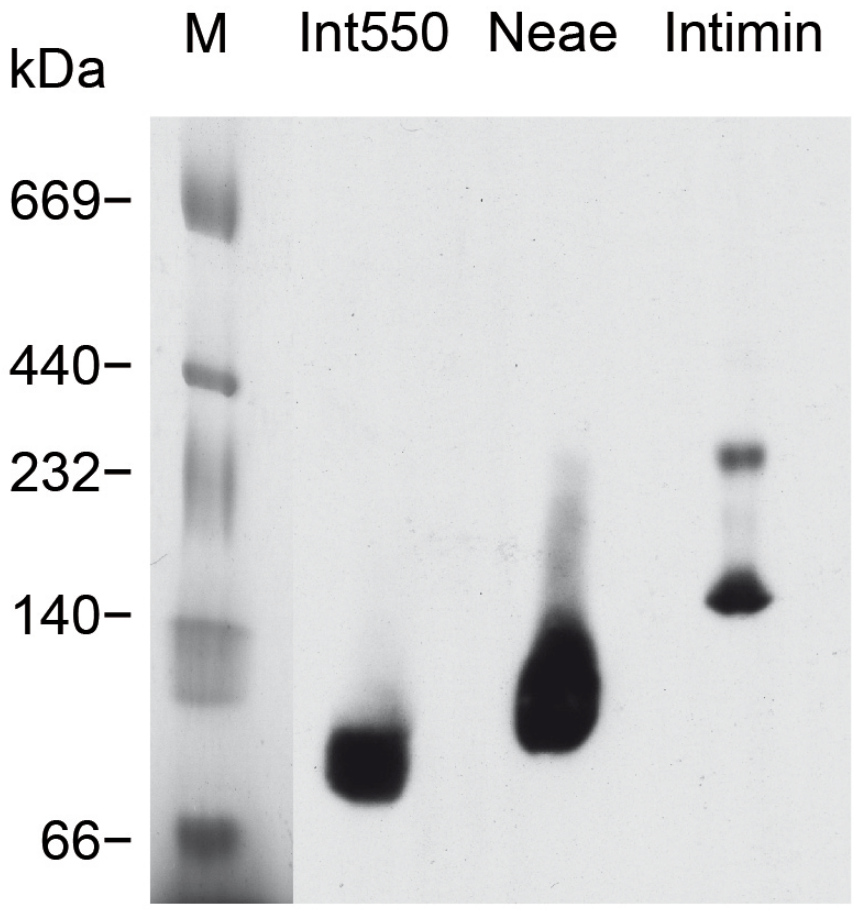
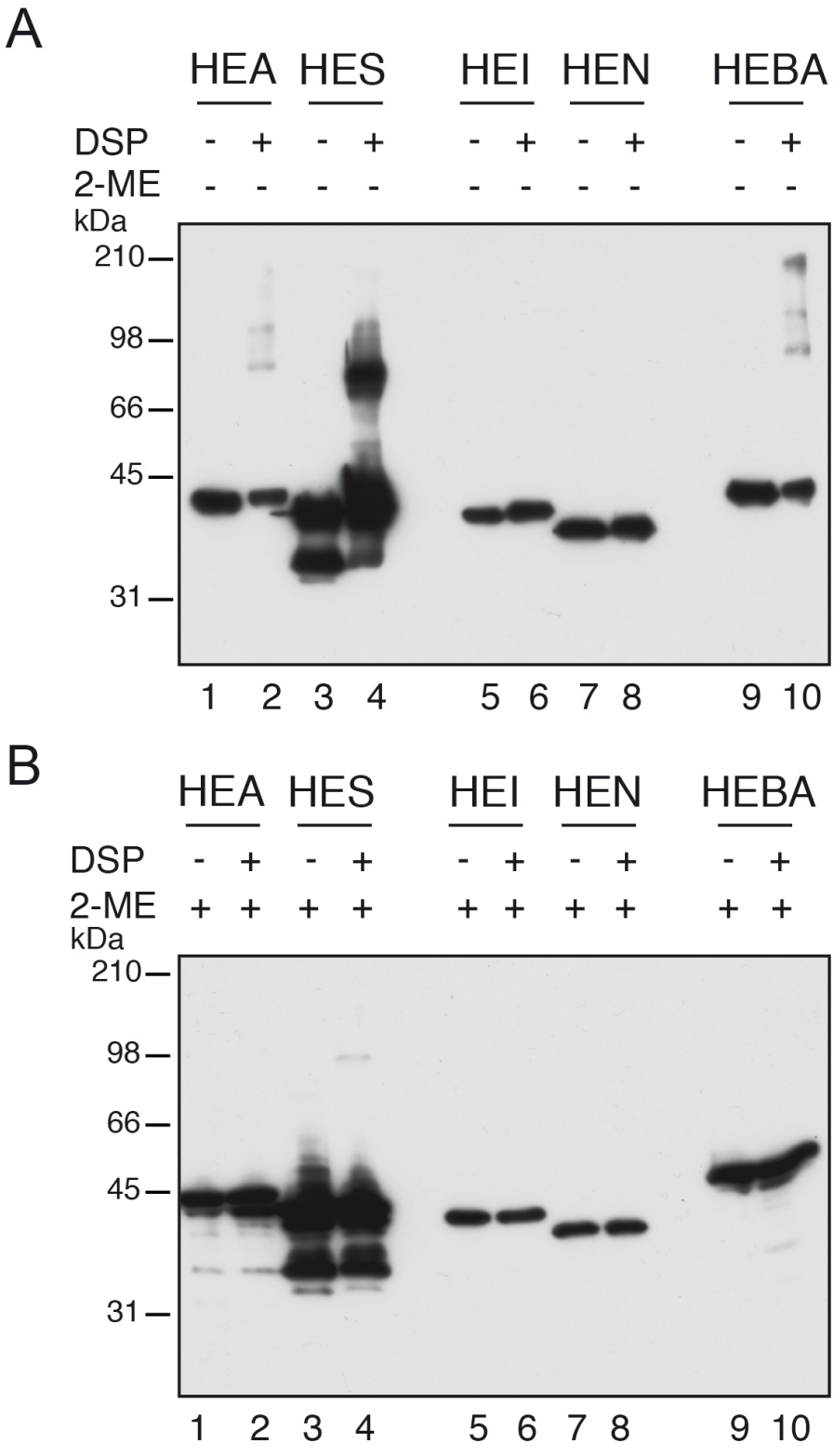


Figure 7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