Two-partner Secretion of Gram-negative Bacteria

A SINGLE β-BARREL PROTEIN ENABLES TRANSPORT ACROSS THE OUTER MEMBRANE*

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Background: The filamentous hemagglutinin (FHA) of the whooping cough agent Bordetella pertussis requires the outer membrane protein FhaC for secretion.

Results: Purified FhaC is sufficient for transport into reconstituted proteoliposomes.

Conclusion: FhaC, a member of the Omp85 β-barrel protein integrators, functions as independent protein translocator.

Significance: This is the first in vitro system for analyzing protein secretion across the outer bacterial membrane.

The mechanisms of protein secretion by pathogenic bacteria remain poorly understood. In Gram-negative bacteria, the two-partner secretion pathway exports large, mostly virulence-related “TpsA” proteins across the outer membrane via their dedicated “TpsB” transporters. TpsB transporters belong to the ubiquitous Omp85 superfamily, whose members are involved in protein translocation across, or integration into, cellular membranes. The filamentous hemagglutinin/FhaC pair of Bordetella pertussis is a model two-partner secretion system. We have reconstituted the TpsB transporter FhaC into proteoliposomes and demonstrate that FhaC is the sole outer membrane protein required for translocation of its cognate TpsA protein. This is the first in vitro system for analyzing protein secretion across the outer membrane of Gram-negative bacteria. Our data also provide clear evidence for the protein translocation function of Omp85 transporters.

Gram-negative bacteria harbor a multitude of molecular systems, by which cytosolically synthesized proteins are secreted onto the bacterial surface or into the surrounding medium (1, 2). Many of these secretion systems are dedicated to a small number of substrate proteins or even to only a single protein. The molecular mechanisms of the secretion machineries largely remain to be deciphered. One such pathway, the so-called type V secretion pathway, secretes proteins such as proteases, adhesins, or toxins that mostly function as virulence factors of pathogenic bacteria. Type V secretion is commonly divided into at least two branches, the autotransporter pathway (reviewed in Ref. 3) and the two-partner secretion pathway (reviewed in Ref. 4). Common characteristics of both pathways are that they secrete unusually large proteins (>100 kDa) that after secretion adopt characteristic β-solenoid structures consisting of multturn parallel β-helices or β-rolls (5–7). Autotransporters are two domain proteins consisting of an N-terminal passenger domain and a C-terminal β-domain, which inserts as a β-barrel into the outer membrane and was originally believed to be sufficient for the translocation of the upstream passenger domain (hence the name autotransporters). On the contrary, in two-partner secretion, the passenger domain (TpsA protein) and the β-domain (TpsB protein) are encoded by two separate genes.

TpsB proteins are members of the superfamily of Omp85 protein transporters (8, 9). Some of these transporters, including TpsB of Gram-negative bacteria and Toc75-III of the chloroplast outer envelope, mediate the translocation of their protein substrates across a membrane (10). Other members, exemplified by BamA of Escherichia coli and Sam50 of the mitochondrial outer membrane, are involved in membrane integration of their substrates (11–16). Recently, the integration function of BamA was reconstituted in vitro (17). In contrast, the translocation function of any of these transporters has not been directly demonstrated, and the only complete structure available, that of the TpsB protein FhaC, has revealed an occluded channel (18).

FHA2 and FhaC are the TpsA and TpsB proteins of a well studied two-partner secretion system from the whooping cough agent Bordetella pertussis. FHA, the filamentous hemagglutinin, represents the major extracellular protein of B. pertussis (19). This 230-kDa, ∼50 nm long, rigid β-helical protein mediates the attachment of the bacterium to epithelial cells of the respiratory tract. FHA is initially synthesized as a 367-kDa precursor (termed FhaB) that is transported into the periplasm via its Sec signal sequence (20). With the help of the cognate TpsB transporter FhaC, it is subsequently secreted onto the surface of B. pertussis, where it is C-terminally processed to yield the 230-kDa mature protein (21, 22).

To enable an in-depth biochemical analysis of the two-partner secretion pathway, we set out to establish a cell-free system for studying secretion of FhaB across its cognate outer membrane transporter FhaC embedded in a lipid bilayer. Our studies clearly indicate that FhaC is the sole translocator of the passenger protein FhaB.

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2 The abbreviations used are: FHA, filamentous hemagglutinin; OMP, outer membrane proteins; POTRA, polypeptide translocation associated.
**Experimental Procedures**

**Plasmids—**Plasmid pFJD12 (23) encodes an ~80-kDa N-terminal fragment of *B. pertussis* FhaB preceded by a hybrid OmpA-FhaB tandem signal peptide for efficient secretion from *E. coli* cells. To introduce five methionines for optimized radioactive labeling C-terminally to a suitably sized FhaB truncate, a DNA fragment encoding the OmpA-FhaB hybrid signal peptide and the first 370 amino acids of mature FhaB was amplified from template plasmid pFJD12 using the primers OmpBspHCSC (5’-CTGGAAGCTTTTCAATGGTTG-3’) and FhaBmetNCS (5’-CCGCTGAGCATGCTATCATCATTGATCGACCCGTCGCGGACGC-3’). The PCR product was ligated into the cloning vector pGEM-T (Promega) to generate pGem::FhaB45met. The insert was then excised by use of BspH1 and Xhol and ligated upstream of a His tag into a pTrc99a derivative to generate pTrc99a::FhaB45met-His. The pTrc99a derivative was obtained by Ncol/Xhol restriction of pTrc99a::espC*, which had been constructed using the primers pETCS (5’-CATGTCGACCCGTCGCGTCGCCGACAGC-3’) and pEThisNCS (5’-CGCCTCGAGTGGAGCCACCCG-3’). Plasmid pFJD149 is a pET22b derivative (Novagen) that harbors a 1-kb Ndel-Xhol 5’-fragment of espC obtained by PCR using plasmid pLADC1 as a template. To finally change the His tag to a streptag, a PCR product containing an XhoI site, the Strep tag, two stop codons, and the first 370 amino acids of mature FhaB was amplified from template pFJD12 using the primers OmpBspHCS (5’-CTGGAAGCTTTTCAATGGTTG-3’) and pETstrepC (5’-CATGTCGACCCGTCGCGTCGCCGACAGC-3’). The PCR product was ligated into the cloning vector pGEM-T (Promega) to generate pGem::FhaB45met-Strep. The insert was then excised by use of BspH1 and Xhol and ligated upstream of a His tag into a pTrc99a derivative to generate pTrc99a::FhaB45met-Strep. All plasmids were verified by DNA sequencing.

**Reconstitution of Two-partner Secretion**

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**FhaC was expressed in a T7 RNA polymerase-dependent manner from plasmid pFJD118 (24) using the porin-deficient *E. coli* host strain BL21(DE3)-omp5 (24). Plasmids coding for FhaC variants lacking either POTRA domain 1 (pAS01) or loop L6 (pAS03) have been described elsewhere (18). Plasmid pEC30 encodes FhaC with an OmpA signal peptide for efficient expression in *E. coli* cells (25).

**Purification of FhaC—** *E. coli* BL21(DE3)-omp5 cells containing pFJD118 were grown in LB medium to an absorbance at 600 nm of 0.8 units/ml. Expression of FhaC was then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside for 2.5–3 h. Cells were collected, and the broken and outer membranes were isolated by sucrose gradient centrifugation similarly as described (26). To allow for a better separation of inner and outer membranes, the volume of the 0.77 m sucrose cushion was reduced from 12 to 10 ml and that of the 1.44 m sucrose layer increased from 12 to 14 ml. Collected outer membranes were suspen-
ded in inside-out inner membrane vesicle buffer (50 mM triethanolamine acetate buffer, pH 7.5, 250 mM sucrose, 1 mM DTT) to a concentration of 40 A260 units/ml. Usually six 40-μl aliquots of outer membranes were diluted 10-fold each with solution E (20 mM Na2HPO4, 1% Elugent) and solubilized for 2 h at room temperature by end-over-end rotation. Subsequently, insoluble material was removed by centrifugation at 100,000 × gmax for 1 h at 4 °C (Sorvall rotor S100-AT4, 43,000 rpm). The six supernatants were combined and applied to a Mono S® HR 5/5 column equilibrated with solution E. Proteins were eluted at 4 °C by use of a 0–1 M NaCl gradient in solution E, at a flow rate of 0.5 ml/min. FhaC eluted between 220 and 400 mM NaCl. FhaC-containing fractions were combined, desalted on Sephadex G-25 (NAP 5 columns, GE Healthcare) against solution E, and applied at 4 °C to 2 ml of nickel-Sepharose (GE Healthcare) equilibrated with solution E. The column was washed three times with 3 ml of solution E containing 5 mM imidazole, and bound proteins were eluted in 3-ml steps using 200 mM imidazole in solution E. Most FhaC was recovered from the first elution fraction. It was dialyzed three times against 1 liter of solution E and stored at 4 °C.

**Reconstitution of Proteoliposomes—** *E. coli* phospholipids were solubilized with detergent by diluting a sonicated 100 mg/ml aqueous stock solution of Avanti polar lipids 25-fold into 16 mM Na2HPO4, 0.8% Elugent. For reconstitution, 400 μl of solubilized outer membrane vesicles from 40 μl of outer membrane vesicles as described above for the purification of FhaC were mixed with 200 μl of Elugent-treated phospholipids, 300 μl of 20 mM Na2HPO4, and 50–65 mg of Biobeads SM-2 Adsorbents (Bio-Rad) that had been pretreated as described (27). Protein-free liposomes were obtained in the same way by replacing outer membrane proteins by 400 μl of solution E. After 2 h of incubation at 4 °C by end-over-end rotation, Biobeads were removed by centrifugation for 2 min at 13,000 × rpm at room temperature. Removal of detergent by Biobeads was repeated twice with one of the two subsequent incubations being performed overnight. The final supernatant was spun at 186,000 × gmax for 30 min at 4 °C in a Beckman TLS-55 rotor. The pelletted proteoliposomes were resuspended in 100 μl of inside-out inner membrane vesicle buffer by repeated pipetting and brief sonication and then used for translocation studies. For the reconstitution of purified FhaC, the desired amount of FhaC was diluted with solution E to give a final volume of 400 μl and then processed as described for solubilized outer membrane proteins.

**Secretion of FhaB* from Spheroplasts and Translocation into Outer Membrane Vesicles—**Spheroplasts were obtained from *E. coli* BL21(DE3) cells transformed with pTrc99a::FhaB45met-Strep. Cells were grown overnight in 10 ml of minimal E-medium (28) supplemented with 100 μM each of 18 amino acids, omitting methionine and cysteine, 0.4% glucose, and 100 μg/ml ampicillin. After measuring the absorbance of the cell suspension at 580 nm, an aliquot that was equivalent to 2 ml of cell culture grown to an absorbance at 580 nm of 1, was spun at 8,000 rpm for 1 min in a table top centrifuge, and the
pelleted cells were resuspended in 500 μl of 100 mM Tris/HCl, pH 7.5, 0.5 M sucrose. They were converted to spheroplasts by adding 500 μl of 0.1 mg/ml lysozyme in 8 mM EDTA, pH 8.0, gentle end-over-end mixing, and incubating for 15 min on ice. Spheroplasts were collected at 10,000 rpm for 10 min in a table top centrifuge and resuspended in 500 μl of E-medium supplemented with 100 μM each of 18 amino acids, 2% glycerol, and 0.25 M sucrose. Following a 15-min preincubation period at 37 °C, spheroplasts were induced using 4 mM isopropyl-β-D-1-thiogalactopyranoside for 3 min for the synthesis of FhaB*, then gently mixed with 4 μl of [35S]methionine/cysteine (11 μCi/μl) and varying amounts of liposomes or proteoliposomes (cf. figure legends), and incubated at 37 °C typically for 15 min. Spheroplasts were removed from the reaction mixture by centrifugation at 13,000 rpm for 6 min. One-half of each supernatant was precipitated on ice with 5% trichloroacetic acid (TCA), and the other half was digested with 500 μg/ml proteinase K at 25 °C for 25 min and then precipitated with 5% TCA first at 56 °C for 10 min and then on ice for 30 min. TCA pellets were each dissolved in 15 μl of SDS loading buffer (26) and boiled at 95 °C for 5 min, and proteins were resolved by SDS-electrophoresis using 12.5% polyacrylamide slab gels. The translocation efficiency was calculated by quantifying the amount of PK-resistant FhaB* relative to the nontreated FhaB* species using ImageQuant 5.2 (GE Healthcare).

For post-secretional assays, spheroplasts were induced with isopropyl β-D-1-thiogalactopyranoside as described above and pulse-labeled with [35S]methionine/cysteine for 2 min. After pelleting the spheroplasts, supernatants were mixed with 1 μl each of OMP proteoliposomes and incubated for 15 min at 37 °C. Reactions were divided in halves and treated with TCA and PK as detailed above.

Flotation centrifugation was performed as described previously (29) except that 50-μl samples were diluted 2-fold with sucrose to give a final concentration of 1.3 M and then overlaid with each 200 μl of 1 M sucrose and 100 μl of sucrose-free buffer according to Ref. 30.

RESULTS

To set up a cell-free system for studying secretion of the FHA from B. pertussis, we made use of a 37-kDa N-terminal fragment of FhaB, denoted FhaB*. This was possible because even shorter N-terminal fragments were shown to contain all the information necessary for an FhaC-dependent secretion from E. coli cells and subsequent extracellular folding (6). A signal sequence-containing pre-form of FhaB* was expressed and radioactively labeled in E. coli spheroplasts that due to the lack of a continuous outer membrane released FhaB* into the surrounding medium. To follow transport of FhaB* across its TpsB transporter FhaC, we overexpressed FhaC from the T7 promoter in E. coli host cells, isolated FhaC-containing outer membranes, and reconstituted the pool of detergent-solubilized OMP with E. coli phospholipids into proteoliposomes.

These OMP proteoliposomes were added to the FhaB*-expressing spheroplasts during pulse-labeling with [35S]methionine/cysteine. After 15 min, spheroplasts were removed by low speed centrifugation, and the proteoliposome-containing supernatant was divided into halves. One aliquot was directly precipitated with trichloroacetic acid and prepared for SDS-PAGE, and the other one was first digested with 500 μg/ml proteinase K (PK) to remove all soluble proteins. Under the experimental conditions chosen, the 37-kDa FhaB* was the major newly synthesized protein released from the spheroplasts into the supernatant (Fig. 1A, lane 1). When no vesicles were added, the secreted FhaB* was completely digested by PK (Fig. 1A, lane 2). Virtually the same was true for samples that had received protein-free liposomes (Fig. 1A, lanes 3 and 4). If, however, the FhaB*-secreting spheroplasts were incubated with proteoliposomes that had been reconstituted from outer membrane proteins of an FhaC-expressing E. coli strain, a significant fraction of FhaB* became PK-resistant (Fig. 1A, lanes 5 and 6). The protease resistance of FhaB* was abolished when the PK treatment was performed in the presence of 1% Triton X-100 to disrupt the OMP proteoliposomes (Fig. 1A, lane 7), suggesting that FhaB* was translocated into a protease-protected environment provided by the membrane vesicles. Quantitation of five independent experiments yielded a mean translocation efficiency of 28% under these experimental conditions. Notably, the acquisition of PK resistance of FhaB* in the presence of the vesicles was strictly dependent on FhaC, because no significant PK resistance was obtained when proteoliposomes had been prepared from outer membrane proteins that were devoid of FhaC (Fig. 1A, lanes 8 and 9).

To solubilize outer membrane proteins, various detergents were tested (Fig. 1B). When compared with Triton X-100, n-octyl-β-D-glucopyranoside, and dodecyl maltoside, Elugent yielded OMP proteoliposomes with the highest translocation activity toward FhaB*.

To demonstrate that a functionally active FhaC was required for FhaB* to become protease-resistant, we expressed inactive variants of FhaC in E. coli and reconstituted proteoliposomes from outer membrane proteins of these strains. FhaCΔPOTRA1 lacks the first of its two POTRA domains, and FhaCΔloop6 lacks the extracellular loop L6 of its β-barrel that folds into a hairpin structure within the barrel interior. Both deletion mutations abolish secretion in vivo (18). As illustrated in Fig. 1C (lanes 1–6), OMP proteoliposomes containing these two fhaC mutants yielded drastically reduced PK protection when compared with wild-type FhaC.

Secreted FHAs, as well as N-terminal fragments such as FhaB*, fold extracellularly into stable β-helical filamentous structures. If the mere interactions with FhaC on the outside of our reconstituted proteoliposomes were sufficient to induce β-helix formation of FhaB*, it was conceivable that such stable structures displayed protease resistance even without being translocated into the lumen of the vesicles. This possibility is, however, not consistent with the finding that FhaB* remained almost completely sensitive to PK, when FhaC lacked its loop L6 (Fig. 1C, lanes 5 and 6). Because this mutation abolishes transport (18) but does not affect the two POTRA domains of FhaC required for binding of FhaB, a mere association of FhaB* with FhaC does not seem to be sufficient to induce protease resistance. Furthermore, single amino acid substitutions in FhaB have been described that affect secretion of FhaB without impairing its interaction with FhaC (31). We also tested one of these translocation-defective mutants of FhaB* carrying the
Reconstitution of Two-partner Secretion

FIGURE 1. FhaB* secreted from E. coli spheroplasts translocates into proteoliposomes when reconstituted from FhaC-containing E. coli outer membrane extracts. A, proteoliposomes were reconstituted from E. coli phospholipids and Elugent-solubilized outer membrane proteins of E. coli cells that either expressed or were devoid of FhaC (OMP proteoliposomes; +/− FhaC). Next, E. coli spheroplasts were induced for the synthesis of a 37-kDa N-terminal precursor truncate of the B. pertussis filamentous hemagglutinin FHA (FhaB*). Spheroplasts (100 μl prepared as described under “Experimental Procedures”) were mixed with 1 μl of plain liposomes (lanes 3 and 4), 1 μl of OMP proteoliposomes (lanes 5–9), or 1 μl of inside-out inner membrane vesicle buffer (lanes 1 and 2) and pulse-labeled with [35S]methionine/cysteine. After 15 min of incubation at 37 °C, spheroplasts were removed by low speed centrifugation, and supernatants containing membrane vesicles were divided into halves, one being precipitated with 5% TCA and the other one first digested with 500 μg/ml proteinase K (PK). In the sample shown in lane 7, PK treatment was performed in the presence of 1% Triton X-100. Radioactively labeled proteins were separated by SDS-PAGE and are displayed following phosphorimaging. Translocation efficiencies were determined as described under “Experimental Procedures” and represent the means of five (lanes 1–6) and three (lanes 8 and 9) independent experiments. Protease protection is obtained only with proteoliposomes containing FhaC and is abolished upon detergent-mediated disruption of the vesicles. B, as in A, lanes 5 and 6, except that outer membranes were solubilized with the indicated detergents Elugent, Triton X-100, n-octyl-β-D-glucopyranoside (OG), and dodecyl maltoside (DDM). C, as in A except that OMP proteoliposomes were prepared from outer membrane extracts of E. coli cells expressing wild-type FhaC (FhaC) as well as inactive FhaC variants lacking either the POTRA domain 1 (FhaCΔPOTRA1) or the loop L6 (FhaCΔLoop6). Lanes 7–10 depict results obtained with the secretion-defective FhaB* mutation N66I (31). D, E. coli UT5600 cells (E. coli Genetic Stock Center, Yale University) expressing FhaC and FhaB* from plasmids pEC30 and pTrc99a::FhaB45met-Strep, respectively, were pulse-labeled with [35S]methionine/cysteine at 37 °C. At the indicated times, cells were removed by centrifugation, and the secreted FhaB* was assayed for its susceptibility to PK. Secreted FhaB* remains fully sensitive toward 500 μg/ml PK at least for 2 h after translocation across FhaC. E, flotation analysis of FhaB* secreted from spheroplasts in the absence or presence of FhaC-containing OMP proteoliposomes. For details see “Experimental Procedures.” Vesicle-associated FhaB* is recovered from the top two fractions of the sucrose gradient. F as in A, lanes 5 and 7, except that the concentration of PK was varied as indicated. Resistance to 5 μg/ml PK after disruption of the proteoliposomes by Triton X-100 suggests folding of FhaB* after translocation into the vesicles.
mutation N66I and found it completely digested by PK in the presence of FhaC-containing proteoliposomes (Fig. 1C, lanes 9 and 10). Thus, also in the case of FhaB*(N66I), the expected defect in secretion was paralleled by its sensitivity toward PK. Finally, we examined if FhaB* when in fact folded into a β-helix structure would be resistant to the amount of PK (500 µg/ml) that we routinely used in our protease protection assays. Because FhaB* when secreted from FhaC-expressing E. coli cells was shown to fold into a β-helical structure (6), we recovered FhaB* from the growth medium of E. coli cells and determined its susceptibility toward 500 µg/ml PK. Fig. 1D demonstrates that even 2 h after secretion, FhaB* remained fully protease-sensitive. These results strongly indicate that the PK resistance of FhaB* obtained in the presence of active FhaC-containing OMP proteoliposomes (Fig. 1, A and C) does not stem from folding on the surface of the OMP proteoliposomes but rather from its translocation into the lumen of the vesicles.

Another theoretical possibility for FhaB* to become protease-resistant was the formation of aggregates. This was addressed by the flotation analysis shown in Fig. 1E. FhaB* secreted from spheroplasts in the absence or presence of OMP proteoliposomes was subjected to flotation centrifugation using sucrose gradients. Following centrifugation, gradients were fractionated from the top in four equal aliquots, and the pellet was directly dissolved in SDS-PAGE loading buffer. Under the conditions chosen, membrane vesicles float to the top of these gradients represented by fractions 1 and 2 (29). In fact, 60% of FhaB* was recovered from the membrane fractions, whereas the equivalent fractions of the proteoliposome-free gradient remained empty. This result indicates that in the presence of OMP proteoliposomes, the majority of FhaB* associated with the membrane vesicles. Even if the nonfloating FhaB*, which accumulated predominantly in fractions 4 and 5 both in the absence or presence of membranes, were to prevail in the form of aggregates, it could be digested by PK as demonstrated for the proteoliposome-free controls in Fig. 1, A and C.

The formation of a β-helix by TpsA proteins, such as FhaB*, is a potential driving force of secretion, which is totally independent of nucleotide hydrolysis. Demonstration of some degree of folding of FhaB* inside the OMP proteoliposomes would be another strong indication for the occurrence of a true translocation event. We therefore performed the experiment depicted in Fig. 1F. FhaB* was allowed to translocate into FhaC-containing OMP proteoliposomes and was then exposed to PK by disintegrating the vesicles by Triton X-100. At 10 µg/ml PK, FhaB* was completely digested (Fig. 1F, lane 6), similar to what had been obtained with 500 µg/ml protease (Fig. 1A, lane 7). Reducing the PK concentration to 2.5 µg/ml failed to digest any FhaB*, even material that had not been incubated with proteoliposomes (Fig. 1F, lane 1). However, at 5 µg/ml PK, vesicle-free FhaB* was digested (lane 3), whereas FhaB* incubated with the OMP proteoliposomes remained resistant even though vesicles were disrupted with Triton X-100 (Fig. 1F, lane 4). An obvious explanation for this result is a partially folded conformation of FhaB* adopted after its translocation into the proteoliposomes. Collectively, the results summarized in Fig. 1 demonstrate that the TpsA protein FhaB* when secreted from spheroplasts is translocated into OMP proteoliposomes, provided that they contain an active form of the TpsB protein FhaC.

To further investigate, if FhaC was not only necessary but also sufficient for transporting FhaB* into the proteoliposomes, we purified FhaC from solubilized outer membrane proteins by means of cation exchange and metal affinity chromatography. The latter was possible because the FhaC construct used here contains an N-terminal His tag. On Coomassie Blue-stained SDS gels, FhaC thus obtained was essentially pure (Fig. 2A, lane 4). As illustrated in Fig. 2, B–D, proteoliposomes reconstituted with this pure FhaC clearly showed translocation activity toward FhaB*. The translocation efficiency of the pure FhaC proteoliposomes was dependent on the amount of FhaC used for reconstitution (Fig. 2B) and on the amount of FhaC proteoliposomes added per single reaction (Fig. 2C). By optimizing the FhaC/phospholipid ratio during the reconstitution process, up to 33% of FhaB* was found translocated into FhaC proteoliposomes (Fig. 2D). Thus, proteoliposomes containing only FhaC were at least as active as if they contained in addition to FhaC the whole spectrum of E. coli outer membrane proteins. Because β-barrel proteins, such as FhaC, seem to interact with the outer membrane-specific phospholipid lipopolysaccharide (LPS), we also included a commercial LPS preparation at various amounts during the reconstitution procedure of FhaC proteoliposomes. A stimulating effect was, however, not observed. Rather at higher concentrations of LPS, translocation of FhaB* into FhaC proteoliposomes was inhibited presumably due to disturbing effects of LPS on the lipid bilayer (data not shown).

Because recent data suggest that the β-barrel assembly machinery of the E. coli outer membrane is involved in the two-partner secretion-related autotransporter pathway (32–34), we considered the possibility that the translocation activity of FhaC proteoliposomes might be influenced by contaminating traces of the Bam complex. This was examined by Western blot analysis of the BamA content as shown in Fig. 2E. As expected, BamA as an outer membrane constituent was present in substantial amounts in the crude OMP proteoliposomes (Fig. 2E, lane 2) that had been used for the experiments shown in Fig. 1. In contrast, pure FhaC proteoliposomes hardly contained any BamA (Fig. 2E, lane 3). Despite lacking BamA, FhaC proteoliposomes did not show a lower translocation activity toward FhaB* than OMP proteoliposomes. Therefore, their translocation activity can only be ascribed to FhaC.

In the transport experiments described thus far, FhaC-containing vesicles had always been present during secretion of FhaB* from the spheroplasts. It was therefore theoretically possible that outer membrane proteins that were still associated with the surface of the spheroplasts might influence the transport of FhaB* into the OMP proteoliposomes. To prevent such a situation, we performed a post-secretional assay as shown in Fig. 3. After a 2-min pulse with [35S]methionine/cysteine, spheroplasts were removed by centrifugation, and the secreted FhaB* present in the supernatant was incubated with OMP proteoliposomes. If the proteoliposomes contained FhaC (Fig. 3, lanes 3 and 4), a substantial amount of released FhaB* (12.7% as a means of five experiments) proved to be protease-protected. Notably, translocation was also obtained when proteoliposomes had been reconstituted...
Reconstitution of Two-partner Secretion

from purified FhaC only (Fig. 3, lanes 7 and 8). Although the translocation efficiency in this post-secretional mode was lower than under co-secretional conditions (Fig. 3, lanes 1 and 2), these results strongly confirm that two-partner secretion does not seem to require any other outer membrane protein besides the TpsB transporter.

**DISCUSSION**

We have reconstituted in vitro the transport of a two-partner secretion passenger protein (TpsA) across the outer membrane of Gram-negative bacteria. This is the first time that secretion of a bacterial protein has been reproduced in vitro using outer membrane-derived membrane vesicles. The only membrane
Reconstitution of Two-partner Secretion

protein required for secretion of the TpsA passenger protein was the cognate TpsB transporter. In addition, our experimental setup revealed that secretion of a TpsA protein can be uncoupled from its translocation across the inner membrane, as was previously inferred from in vivo experiments (35). We could also demonstrate that a TpsA protein acquires a folded conformation after its translocation into TpsB-containing proteoliposomes, which might actually provide the driving force of the translocation process.

The novel experimental system now provides the clearest evidence that the TpsB protein FhaC functions as an autonomous protein-conducting channel, an assumption that thus far was largely based on circumstantial evidence (24). Thus FhaC was demonstrated to be a necessary component for secretion of FHA from B. pertussis cells (36) and from the heterologous host E. coli (23). Consistent with being a transporter, FhaC was shown to form ion-permeable channels in artificial membranes (37). Finally, x-ray structural analysis of FhaC revealed that it folds into a 16-stranded β-barrel in the outer membrane, which in the crystal structure was, however, largely occluded by an N-terminal α-helix and a flexible extracellular loop (18).

TpsB proteins such as FhaC are members of the Omp85 protein family, including Omp85 and BamA (YaeT) in the outer membrane of Gram-negative bacteria, Sam50 in the outer mitochondrial membrane, and Toc75 in the outer envelope of plant chloroplasts. The common structural denominator of Omp85 homologues are the occurrence of N-terminal POTRA domains followed by a membrane-embedded β-barrel domain (8, 38). Omp85 family members are involved in protein transport as well as in the membrane integration of β-barrel proteins. Thus, BamA together with four outer membrane lipoproteins (BamB–E) forms the β-barrel assembly machinery complex that is required for the integration and assembly of β-barrel proteins into bacterial outer membranes (11–13). Likewise, the SAM (sorting and assembly machinery) complex of mitochondria allows integration of newly imported proteins from the intermembrane space into the outer membrane of mitochondria (14–16). In contrast, FhaC is a member of the Omp85 family that facilitates only the transmembrane translocation of its cognate substrate protein. Similarly, Toc75-III is the channel subunit of the protein translocase of the chloroplast outer envelope (10). Thus, some of the Omp85 homologues function as true protein-conducting channels, whereas others help β-barrel proteins to insert into lipid bilayers. Recent findings suggest, however, that BamA, in addition to being involved in the membrane integration of β-barrel proteins, also functions as a translocation pore. Thus, a translocation intermediate of an autotransporter passenger domain was found in close proximity to BamA (33). A plausible evaluation of these results would be that the basic design of Omp85 homologues allows them to serve as protein conduits across membranes, whereas the β-barrel insertase function of some of the Omp85 family members necessitates further molecular equipment such as more POTRA domains and/or the cooperation with additional subunits such as BamB–E (17).

Traditionally two-partner secretion and the autotransporter pathway have been classified as two branches of the type V secretion pathway of Gram-negative bacteria, with the underlying idea that autotransporters would essentially represent fused versions of a pair of otherwise individual TpsA and TpsB proteins. However, the β-domains of autotransporters, which form conserved 12-stranded β-barrels in the outer membrane, have long been questioned to function as the sole translocators for their proximal passenger domains (39–42). In fact, a direct involvement of BamA in the secretion of autotransporters has become highly likely, because recent analyses of secretion intermediates stalled in the outer membrane disclosed an immediate vicinity of BamA to translocating autotransporters (33, 34). On the contrary, our results largely rule out any essential participation of BamA and other outer membrane proteins in the secretion of FhaB*, demonstrating that in contrast to the β-domains of autotransporters, TpsB proteins function as the sole translocating device in the outer membrane.

Our experimental system will enable us to explore the involvement of soluble auxiliary factors, such as chaperones or targeting factors in the translocation of FhaB* across FhaC. The periplasmic chaperones SurA and Skp have both been shown to directly interact with, and contribute to, the efficient secretion of autotransporters (33, 34, 43, 44), and SurA was found to physically and functionally interact with BamA (17, 45). Similarly, the DegP orthologue of B. pertussis binds to non-native forms of FHA in vitro and seems to contribute to secretion efficiency in vivo (46, 47), but the mechanisms by which it might assist secretion remain to be deciphered. In our in vitro system, E. coli periplasmic chaperones associated with the spheroplasts might have replaced missing B. pertussis chaperones, because bona fide soluble periplasmic chaperones of E. coli such as Skp and DegP have the propensity to associate with the inner membrane (48–50). The involvement of periplasmic chaperones in the transfer of FhaB* from the inner membrane to FhaC is strongly suggested by our preliminary experiments indicating a fast loss of the translocation competence of FhaB* in post-secretional assays. Moreover, because the natural FhaB precursor is considerably longer than FhaB*, a requirement for periplasmic chaperones to orchestrate release from the inner membrane to targeting to the outer membrane becomes even more obvious. Besides studying the involvement of chaperones, our novel experimental system now opens the possibility of analyzing in detail the mechanisms of targeting, translocation, and folding during two-partner secretion and possibly also during related clinically relevant protein secretion pathways.

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